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SARS-CoV-2 infection induces expression and secretion of lipocalin-2 and regulates iron in human lung cancer xenograft model

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Supplemental Table 1. The proteins whose expression were significantly changed after SARS-CoV-2 infection.

Protein	Normalized dot density		Ratio	Function	Ref
	Mock	SARS-CoV-2			
EMAP-II	1.257	24.259	19.3	EMAP-II is a cytokine derived from the cleavage of AIMP1. It plays a role in regulating inflammatory responses, angiogenesis, and exhibits anti-tumor effects.	(1)
HCR /CRAM-A/B	1	10.142	10.142	The HCR/CRAM-A/B is a type of chemokine receptor that triggers chemotaxis when it encounters chemokines such as CCL2, CCL5, CCL7, and CCL8.	(2)
IL-7	1	12.164	12.164	IL-7 is a cytokine that plays a pivotal role in the growth and maintenance of immune cells, encompassing T and B lymphocytes, and natural killer (NK) cells.	(3)
Lipocalin-2	1	10.364	10.364	Lipocalin-2 contributes to innate immunity against bacterial infection by sequestrating iron.	(4)
SDF-1/CXCL12	1	11.324	11.324	SDF-1/CXCL12 is a chemokine that plays a role in recruiting endothelial progenitor cells and facilitating the accumulation of immune cells.	(5, 6)

Materials and Methods

Cell lines and viruses

Vero E6 cells (Catalogue no. 21587) derived from African green monkey kidney cells, Calu-3 cells (Catalogue no. 30055) derived from human airway epithelial cells, A549 cells derived from adenocarcinomic human alveolar basal epithelial (Catalogue no. 10185), Caco-2 cells derived from human colorectal adenocarcinoma (Catalogue no. 30037.1), and Huh-7 cells derived from human hepatocarcinoma (Catalogue no. 60104) were purchased from the Korean Cell Line Bank (Seoul, Korea). MRC-5 cells from human lung fibroblasts (Catalogue no. CCL-171) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells except the MRC-5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 25 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin in 95% atmospheric air and 5% CO_2 at 37˚C. MRC-5 cells were maintained in Eagle's Minimum Essential Medium (EMEM, ATCC) containing 10% FBS. The SARS-CoV-2 parental strain (BetaCoV/Korea/KCD03/2020, NCCP43326), Alpha variant (hCoV-19/Korea/KDCA51463/2021, lineage B.1.1.7, NCCP43381), Beta variant (hCoV-19/Korea/KDCA55905/2021, lineage B.1.351, NCCP43382), and Delta variant (hCoV-19/Korea/KDCA119861/2021, lineage B.1.617.2, NCCP43390) were obtained from the National Culture Collection for Pathogens of Korea (Osong, Korea). Human coronavirus 43 (HCoV-OC43, KBPV-VR-8) was from the Korea Bank for Pathogenic Viruses (College of Medicine, Korea University, Seoul, Korea).

Virus amplification

SARS-CoV-2 was amplified in Vero E6 cells as previously described (7, 8). Vero E6 cells (2×10^5 cells/well) were cultured on six-well plates (Corning, NY, USA) in DMEM containing 10%

FBS at 37°C in a CO_2 incubator for 18 h. After the cells were washed with phosphate-buffered saline (PBS), the cells were infected with SARS-CoV-2 in PBS at a multiplicity of infection (MOI) of 0.01 for 1 h at 37°C with shaking every 20 min. After adsorption, the medium was changed with DMEM containing 2% FBS. After 72 hours of cultivation, the culture supernatants were prepared by centrifugation. Virus titers were quantified by plaque assay as previously described (9, 10). Vero E6 cells (7×10^5 cells/well) were cultured in 6-well plates for 18 h. The cells were washed with PBS and infected with 10-fold serial dilutions of SARS-CoV-2 for 1 h at 37°C. After adsorption, the wells were refilled with 3 mL DMEM/F12 medium (Thermo Fisher Scientific) containing 0.6% bacteriological agar. After 72 hours of cultivation, the cells were stained with 0.1% crystal violet for 1 h and then evaluated for plaque formation. Virus stocks (1×10^7 pfu/mL) were stored at -70°C. SARS-CoV-2 was amplified in biosafety level 3 (BSL-3) conditions at the Research Institute of Medical-Bio Convergence of Hallym University. All SARS-CoV-2-related procedures received approval from the Institutional Biosafety Committee (IBC) of Hallym University (Hallym2020-12, Hallym2021-04). HCoV-OC43 was amplified in Vero cells as described previously (11). HCoV-OC43 was amplified, and cell infection was carried out under biosafety level 2 (BSL-2) conditions.

Human antibody array

The RayBio® L-Series Human Antibody Array 507 Membrane Kit (RayBiotech, Catalogue no. AAH-BLM-1-2, Peachtree Corners, GA, USA) was utilized to identify alterations in protein expression due to SARS-CoV-2 infection, following the manufacturer's instructions. Calu-3 cells were infected with 0.1 MOI of parental SARS-CoV-2. After a 48-h cultivation, the cells were lysed in cell lysis buffer (10 mM HEPES, 150 mM NaCl, 5 mM EDTA, 100 mM NaF, 2 mM Na_3VO_4, protease inhibitor cocktail, and 1% NP-40), and then, the cell lysates were obtained by centrifugation. The cell lysates of uninfected and SARS-CoV-2-infected Calu-3

cells were dialyzed with PBS overnight at 4℃ and biotinylated following the manufacturer's instructions. The biotinylated cell lysates were incubated with the Human Antibody Array 507 Membrane for 2 h at room temperature. The membranes were treated with HRP-conjugated streptavidin and imaged using a chemiluminescence imaging system (iBright CL1000, Thermo Fisher Scientific). The imaged blot density was calculated with ImageJ (NIH, Bethesda, MD, USA) and analysis tool software (RayBiotech). The dot blot density was adjusted by subtracting the background and then normalized using the positive control from an uninfected dot blot (reference array). The normalization was calculated according to the following formula:

$$X(Ny) = X(y) \times \frac{P1}{P(y)}$$

X(Ny): Normalized value, *X(y)*: the signal spot density of SARS-CoV-2 array blot, *P1*: the positive control spot density of reference array blot, *P(y)*: the positive control spot density of SARS-CoV-2 array blot. Based on the normalized spot density, we calculated the ratio of change in protein expression. Proteins were chosen for further analysis if their expression level altered by a factor of more than 10.

Western blot analysis

Calu-3 cells, both uninfected and infected with SARS-CoV-2, were subjected to lysis using cell lysis buffer. The lysates were then obtained by centrifuging at 14,000 rpm for 20 minutes at 4°C. These lysates were electrophoresed on an 8–16% Bis-Tris gradient polyacrylamide gel (Komabiotech, Seoul, Korea), and the proteins were electro-transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% dry milk in PBS-Tween-20 (PBS-T; 140 mM NaCl, 2.7 mM KCl, 10 mM, Na2HPO4, 2mM KH_2PO_4, and 0.05% Tween-20) to prevent non-specific binding. Then, the membranes were then probed with the

anti-human Lipocalin-2/NGAL rabbit polyclonal antibody (Catalogue no. ab63929, Abcam, Cambridge, UK) and anti-GAPDH mouse monoclonal antibody (Catalogue no. sc-32233, Santa Cruz Biotechnology, Dallas, TX, USA). Signals were detected by horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary-antibodies (Thermo Fisher Scientific) using an ECL solution (ATTO, Tokyo, Japan).

RT-PCR and quantitative real-time PCR (qRT-PCR)

Total RNA of the SARS-CoV-2-infected cells was prepared and reverse-transcribed using M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) at 42ºC for 1 h. Quantitative real-time PCR (qRT-PCR) was performed with the Rotor-Gene SYBR® Green PCR Kit (Qiagen, Hilden, Germany). The PCR reaction was performed as follows: 10 min at 95°C for 1 cycle and 20 sec at 95°C and 40 sec at 60°C for 40 cycles. The following primer sequences were used: lipocalin-2, 5'- GGC CTC CCT GAA AAC CAC AT-3' (sense) and 5'-CCT CAA TGG TGT TCG GGC T-3' (antisense); CCL2, 5'-CTT CAT TCC CCA AGG GCT CG-3' (sense) and 5'-GTC TTC GGA GTT TGG GTT TGC-3' (antisense); IL-15, 5'-GTG CAG GGC TTC CTA AAA CA-3' (sense) and 5'-TAC TTG CAT CTC CGG ACT CA-3' (antisense); IL-6, 5'-AGA CAG CCA CTC ACC TCT TC-3' (sense) and 5'-TTT CAC CAG GCA AGT CTC CT-3' (antisense); glyceraldehyde phosphate dehydrogenase (GAPDH), 5'-AGC CAC ATC GCT CAG ACA C-3' (sense) and 5'-GCC CAA TAC GAC CAA ATC C-3' (antisense). The relative expression of each gene was determined with the $\Delta\Delta C_T$ technique, using GAPDH as the reference standard. After virus infection, the relative expression (expressed as fold change) of the genes was calculated by normalizing to the values in the uninfected cells, which were set as 1.

Mouse

Four-week-old female NOD/ShiLtJ-Rag2^{em1AMC}Il2rg^{em1AMC} (NRGA) mice were purchased from JA BIO, Inc. (Suwon, Korea). The immunocompromised NRGA mice were used for lung cancer xenograft transplantation as previously described (12). The mice were kept in a regulated environment (20–25℃, 40–45% humidity, 12-hour light/dark cycle, with unrestricted access to food and water) at Hallym University's Experimental Animal Center, ensuring they were free from specific pathogens. If the mice showed a 30% loss in adult body weight, reached a tumor size of 1,000 mm^3, or displayed signs of discomfort, pain, or distress, such as a hunched stance, rough coat, decreased food intake, wasting, lethargy, walking difficulties, or breathing issues, a humane protocol was in place. In such cases, the mice would be anesthetized with a 0.2 mL intraperitoneal injection of avertin (from Sigma-Aldrich) and then humanely euthanized via cervical dislocation, following the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of Hallym University. All animal-related procedures received approval under the code Hallym2020-26. Any experiments associated with SARS-CoV-2 infection were conducted under animal biosafety level 3 (ABSL-3) conditions at the Research Institute of Medical-Bio Convergence of Hallym University, adhering to the guidelines provided by Hallym University's Institutional Biosafety Committee (IBC) (Hallym2020-12, Hallym2021-04).

Mouse xenograft model and SARS-CopV-2 infection experiments

Calu-3 cells (2×10^6 cells/mouse) prepared in 50% Matrigel (Corning, PBS/Matrigel, 1:1 *v*/*v*) were subcutaneously injected into the right flank of four-week-old female NRGA mice. The mice (n = 3/group) were intratumorally inoculated with 1×10^6 pfu of parental SARS-CoV-2 or SARS-CoV-2 Delta at tumor volumes that reached an average of 100 mm^3. The mice were observed daily to monitor body weight, clinical signs, and survival. Tumor sizes were measured

with calipers, and the tumor volumes were then estimated as width^2 × length/2 as described previously (13). The mice were sacrificed at 3-, 5-, 10-, or 15-days post-infection, and then, the tumors were surgically removed and weighed. Tumor tissues were lysed with cell lysis buffer and centrifuged at 14,000 rpm at 4ºC for 20 min. The supernatants were harvested, and the Lipocalin-2 concentration was determined by ELISA. Blood was collected, and the mice were sacrificed 15 days post-infection. The tumors were surgically removed, homogenized with Tissue Lyser II (Qiagen, Germantown, MD, USA) using stainless beads, and centrifuged at 14,000 rpm and 4ºC for 20 min. The supernatants were taken, and virus titers were measured by the plaque formation assay. Lipocalin-2 and iron concentrations were determined by ELISA and iron colorimetric assay, respectively.

Measurement of Lipocalin-2 by ELISA

The levels of Lipocalin-2 in sera, tumor tissue lysates and supernatants of tumor tissue homogenates were measured by ELISA using the Human Lipocalin-2/NGAL immunoassay kit (Catalogue no. DLCN20, R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's instructions. Briefly, tissue lysates and sera were added to a 96-well polystyrene microplate coated with an anti- human Lipocalin-2 monoclonal antibody and incubated for 2 h at 4 ºC. To take the standard curve for calculating the Lipocalin-2 amounts in the samples, serially diluted recombinant human Lipocalin-2 was added to the wells. After washing, the plates were incubated with an anti-human Lipocalin-2 monoclonal antibody conjugated to horseradish peroxidase for 2 h at 4 ºC. After washing, the HRP activity was developed with stabilized chromogen (tetramethylbenzidine). The concentration of Lipocalin-2 in each well was ascertained by gauging the absorbance at 450 nm using a Multiskan GO ELISA reader (Thermo Fisher Scientific).

Iron colorimetric assay

The level of total iron in the sera and supernatants of tumor tissue homogenates were measured by ELISA using the Iron Colorimetric assay kit (Catalogue no. #K390-100, BioVision, Milpitas, CA, USA) following the manufacturer's protocol. Briefly, sera, supernatants of tumor tissue homogenates and a serially diluted iron standard were added to each well of a 96-well microplate, followed by Iron Assay buffer and Iron Reducer to the sample wells, and incubated for 30 min at 25 ºC. After incubation, an iron probe was mixed into each well containing the iron standard and samples and reacted for 60 min at 25 ºC. The total iron concentration was measured with an absorbance of 593 nm using a Multiskan GO ELISA reader.

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