Supplementary methods and figures

DNA constructs.

The human wild type α_5 and β_1 integrins were in pcDNA3.1/Myc-His (-) A and pEF vectors, respectively (1). The α_5 with C-terminal EGFP tag (α_5-EGFP) was a gift from Rick Horwitz (Addgene plasmid #15238) (2). The pQCXIP-GFP1-10 (Addgene plasmid #68715) and pQCIP-BSR-GFP11 (Addgene plasmid #68716) were a gift from Yutaka Hata (3). The α_5-FKPB12 construct was generated by replacing the EGFP with FKPB12 in α_5-EGFP plasmid. The β_1 with C-terminal GFPspark tag (β_1-GFP) was from Sino Biological. The α_5 CT truncation (α_5-CTTr, at R987) was as described before (1). The β_1 S132A-S134A (β_1-SSAA) mutations were generated by QuikChange site-directed mutagenesis. Integrin α_5 CRISPR/Cas9 KO plasmid was from Santa Cruz Biotechnology. The human full-length ACE2 with N-terminal Flag-tag was from Sino Biological. The full-length SARS-CoV-2 spike, pcDNA3.1-SARS2-Spike, and SARS-CoV spike, pcDNA3.1-SARS-Spike, both of which contain a C-terminal C9 tag, were a gift from Fang Li (Addgene plasmids #145032 and #145031) (4). The pCMV/R vector containing SARS2-S gene from Omicron variant (B.1.1.529) was from BEI resources (NR-56470). The SARS2-S RGD to RGA mutation was generated by QuikChange site-directed mutagenesis. The SARS-CoV-2 and SARS-CoV full-length spike proteins were cloned with N-terminal Flag tag into a modified pIRES2-DsRed vector with a C-terminal PC-tag. The S1 and S2 subunits of the SARS-CoV-2 spike ectodomain were cloned with C-terminal human IgG1 Fc tag into a modified pIRES2-EGFP vector.

Cell lines.

HEK293T-ACE2 stable cells were generated by stable transfection of N-Flag tagged human full-length ACE2 into HEK293T cells. Single-cell clones with high ACE2 surface expression were selected by fluorescence activated single-cell sorting. The Vero E6 cells expressing high endogenous ACE2 were from BEI Resources (NR-53726). The primary human peripheral blood mononuclear cells (PBMC) and neutrophils were obtained from ReachBio Research Labs or Cellero. The HEK293 and Vero E6 cells were grown in complete DMEM (Corning) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich). Primary human umbilical vein endothelial cells (HUVEC) were provided by the Blood Research Institute Core facility. The immortalized HUVEC cells (HUVEC) were generated by transducing HUVEC cells with recombinant retrovirus LXSN16 E6/E7 as previously described (5). HUVEC cells were grown in 0.1% gelatin-precoated T75 flasks or 100mm tissue culture dishes, in DMEM medium completed with 15% FBS, 0.05 mg/ml endothelial cell growth supplement (ECGS, BD Biosciences), 6.452 U/ml of Heparin (Sigma), 5 μg/ml gentamicin sulfate (Cellgro). The α_5 expression was knocked out by transfection of CRISPR/Cas9 α_5-KO plasmid into HEK293T, HEK293T-ACE2 and Vero E6 cells as described before (1, 6). For α_5 knockout in HUVEC cells, 2 μg plasmid in 100 μl of HUVEC growth medium was mixed with 4×10^6 HUVEC cells. The transfection was performed using Amaxa™ Cell Line Nucleofector™ Kit C (Lonza, US) and Nucleofector™ 2b Device (Lonza) according to the manufacture's protocol. The α_5 negative cells were sorted using a BD FACSMelody™ Cell Sorter (BD Biosciences). The cell surface

expression of α_5 was detected by flow cytometry with mAb VC5 (BD Biosciences) as described (7). The total α_5 in cell lysate was detected by immunoblot with mAb A-11 (Santa Cruz Biotechnology).

Recombinant proteins.

The following purified proteins were obtained through BEI Resources, NIAID, NIH: the SARS-CoV-2 spike (SARS2-S, Wuhan-Hu-1) ectodomain stabilized by substitution at the S1/S2 furin cleavage site (RRAR to GSAS, residues 682 to 685) and stabilizing mutations (K986P and V987P), expressed in HEK293 cells with a C-terminal T4 foldon trimerization domain and octa-histidine tag, NR-53257; the same SARS2-S ectodomain construct as NR-53257 with an additional C-terminal Twin-Strep tag, expressed in CHO pYW cells, NR-53937; the SARS2-S RBD domain with a C-terminal hexa-histidine tag, expressed in HEK293T cells, NR-52946; The SARS2-S (Stabilized) from SARS-CoV-2, BA.2 Lineage (Omicron variant) with C-terminal His and Avi tags, recombinant from HEK293 cells, NR-56517; The SARS2-S (Stabilized) from SARS-CoV-2, Delta variant with C-terminal His and Avi tags, recombinant from HEK293 Cells, NR-55614. The CHO cells produced SARS-CoV spike (SARS-S) ectodomain stabilized with two proline mutations (K968P and V969P) and a C-terminal GCN4-IZ trimerization domain plus a hexa-histidine tag was obtained from R&D Systems. The human ACE2 ectodomain with a human IgG1 Fc tag (ACE2-Fc) was obtained from GenScript. The S1-Fc and S2-Fc of SARS2-S and S2-Fc of SARS1-S were expressed in HEK293T cells and used as cell culture supernatant. The purified SARS2-S S1-Fc and S2-Fc from HEK293 cells were also obtained from Sino Biological. The purified human $\alpha_5\beta_1$ and $\alpha_8\beta_1$ ectodomains with C-terminal ACID-BASE coiled-coil and Flag tag were obtained from Sino Biological. Human $\alpha_5\beta_1$ ectodomain with C-terminal ACID-BASE coiled-coil, Strep-Tag II, and His-tag was purified from HEK293 cells as described before (8).

Virus infection assay.

The SARS2-S-Pseudotyped Lentiviral Kit was from BEI Resources (NR-52948). The SARS-CoV-2 S-pseudotyped lentiviral particles were generated using the SARS-CoV-2, Wuhan-Hu-1 Spike-Pseudotyped Lentiviral kit following the published protocol (9). In brief, HEK-293T-α_5-KO cells were seeded in a 6-well plate one day before transfection to reach 80% confluence. One well of cells were co-transfected using TransIT-VirusGEN Transfection Reagent (Mirus) with 1 µg lentiviral backbone plasmid pHAGE-CMV-Luc2-IRES-ZsGreen-W, 0.22 µg each of lentiviral helper plasmids HDM-Hgpm2, pHDM-tat1b, and pRCCMV-Rev1b, and 0.34 µg plasmid expressing viral entry protein: vesicular stomatitis virus G (VSV-G), SARS-CoV S, or SARS-CoV-2 S. The cell culture supernatant containing pseudoviruses were harvested 60 hours after transfection and centrifuged at 500g for 5 min. The tilters of pseudoviruses were measured using HEK293T-ACE2 cells by the luciferase assay (9). The supernatants were aliquoted in small volumes and stored at -80°C until use. For the pseudovirus entry assay, HEK-293T-ACE2 or HEK-293T-ACE2-α_5-KO cells were seeded at 1.25×10^5 cells/well into 48-well plates that were pre-coated with 5 µg/ml poly-lysine. In some experiments, HEK-293T-ACE2-α_5-KO cells were transfected with human α_5 plasmid for 48 hours before seeding. At 18 hours post-seeding,

cells were infected with pseudotyped lentiviral particles supplemented with 5 μg/ml polybrene. 48 hours after infection, cells were harvested for luciferase activity measurement using Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's instruction. Briefly, 30 μl of suspension cells at 5 x 10^6/ml were incubated with 30 μl luciferase reagent for 4 min at room temperature in the dark. Luciferase activity was measured using Enspire Multimode Plate Reader (Perkin Elmer). All experiments were performed at least three times and expressed as mean ± standard deviation (SD).

The recombinant Vesicular Stomatitis Virus (rVSV) expressing SARS2-S and EGFP was from BEI resources (NR-55284) (10) or provided by Sean Whelan (11, 12). The rVSV expressing VSV-G and EGFP was a gift from Kartik Chandran. For the rVSV-S and rVSV-G infection assay, Vero E6 and Vero E6 α5-KO cells were seeded at 2.4 x 10^5 cells/well into 48-well plate. Subsequently, cells were incubated with rVSV-S (3.56 x 10^5 TCID50) or rVSV-G for 6 hours at 37°C. The infected cells were imaged using EVOS M7000 imaging system with Plan Fluor 4X objective lens (numerical aperture of 0.13). The infection was quantified by measuring the number of EGFP-positive cells, mean EGFP area, or total EGFP-positive areas using CellProfiler software.

For virus inhibition assay, HEK-293T-ACE2 or HEK-293T-ACE2-α5-KO cells were seeded at 6 × 10^5 cells/well into 24-well plates the day before infection. Cells were incubated with 0.1 mM ATN-161 (Selleckchem) for 1 hour at 37°C. Subsequently, rVSV-S (1.78 x 10^5 TCID50) was used to infect the cells. As a control, rVSV-S (1.78 x 10^5 TCID50) was incubated with pooled human sera from COVID-19 vaccinated donors (NRH-21765, BEI Resources) for 1 hour at 37°C. The sera/virus mix was then added to HEK-293T-ACE2 or HEK-293T-ACE2-α5-KO cells. 6 hours post-infection, cells were harvested for infection measurement using flow cytometry.

Cell-cell fusion assay.

For cell-cell fusion induced by virus infection, the infected cells were imaged using EVOS fluorescence microscope. The cell-cell fusion was quantified by measuring the mean or total EGFP areas using CellProfiler. For cell-cell fusion assay based on the split GFP system, HEK-293T-α_5-KO cells were transfected with SARS2-S plus GFP11 with or without $\alpha_5\beta_1$ or transfected with ACE2 plus GFP1-10 for 48 hours. The SARS2-S cells and ACE2 cells were detached by repeatly pipetting without trypsinization. Equal number of cells were then co-cultured for 6 and 24 hours before imaging with EVOS fluorescence microscope. Alternatively, the detached SARS2-S/$\alpha_5\beta_1$ cells were pre-treated with 1 mM ATN-161 or 200 nM MK-0429 (MedChemExpress) for 1 hour before co-culturing with ACE2 cells in the presence of inhibitor. Three random images were taken with Plan Fluor 4X objective lens (numerical aperture of 0.13), equipped with Sony 1cx285AQ color CCD camera. The cell-cell fusion was quantified by measuring the mean or total EGFP areas using CellProfiler.

Protein-protein interaction assay.

The interaction between the S proteins and integrin $\alpha_5\beta_1$ was analyzed by ELISA and pull-down assays. For ELISA, 96-well ELISA plate was coated with 50 μl/well of purified SARS2-S or SARS1-S ectodomain, SARS2-S RBD, or BSA at an equal molar concentration in PBS at 4ºC overnight. The coating concentration of SARS2-S was 1μg/ml for the ACE2 binding assay. The plate was blocked with 200 μl/well of 1% (w/v) BSA in TBS-Ca/Mg (Tris-buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 37°C for 1 hour. Each well was then washed three times with 200 μl of TBS plus 1% BSA. 50 μl of purified $\alpha_5\beta_1$ ectodomain or ACE2-Fc at different concentrations were added into each well and incubated for 1 hour at room temperature. After washing three times with TBS-Ca/Mg plus 0.05% (v/v) Tween 20, 50 μl of anti-α_5 mAb VC5 (BD Biosciences) at 5 μg/ml was added into each well and incubated for 1 hour at room temperature. After repeating the washing step, 50 μl of 1:5000 diluted goat anti-mouse IgG-HRP (Jackson Laboratory) was added into each well and incubated for 1 hour at room temperature. Each well was then washed 4 times and detected by adding 100 μl of 1-Step™ Ultra TMB-ELISA Substrate Solution (ThermoFisher Scientific) for 30 min at room temperature. The reaction was stopped by adding 100 μl of 2M sulfuric acid, and OD_{450} was measured using Infinite M200 PRO Multimode Microplate Reader (Tecan). At least three independent experiments were performed for each assay.

Either purified proteins, cell culture supernatants, or cell lysates were used for the pull-down assay. For the purified proteins, 1 μg of Flag-tagged integrin ectodomain was mixed with 60 μl slurry of protein A beads in the absence or presence of 0.5 μg SARS2 S1-Fc or SARS2 S2-Fc in 500 μl PBS buffer. Alternatively, the supernatant of total cell lysates of HEK293T-ACE2 and the cell culture supernatant of HEK293T cell transfected with SARS2 S2-Fc or SARS1 S2-Fc were used. In another pull-down assay using purified proteins, 1 μg each of Flag-tagged $\alpha_5\beta_1$ ectodomain and ACE2-Fc were mixed with protein A beads in the absence or presence of SARS2-S ectodomain. For the pull-down assay of full-length proteins, HEK293T-α_5-KO cells were transfected with C9-tagged or PC-tagged full-length S constructs plus EGFP or α_5-EGFP/β_1 or α_5/β_1-GFP for 36-48 hours. The cells were lysed by PBS plus 1% Triton X-100 and Mammalian ProteaseArrest™ protease inhibitor cocktail (GBiosciences) and cleared by centrifugation at 15,000 rpm for 15 minutes. The supernatants were mixed with GFP-Trap Magnetic Agarose (Chromotek). For all the pull-down assays, the mixtures were incubated overnight on a roller at 4°C. The beads were washed at least three times with PBS buffer or PBS plus 1% Triton X-100, drained by gel-loading pipette tips, and eluted by 1 x SDS-PAGE loading buffer. The eluted samples were subjected to western blot and detected by anti-Flag (FG4R, Invitrogen), anti-human IgG Fc (Jackson ImmunoResearch), anti-His (27E8, Cell signaling), anti-C9 (1D4, Novus), anti-PC (ICL, Inc), or anti-EGFP (OriGene Technologies).

For protein analysis using dynamic light scattering (DLS), $\alpha_5\beta_1$ ectodomain was further purified with an analytical microscale gel filtration column Suprose™ 6 Increase 3.2/300. Protein in each fraction was diluted to 0.2 mg/ml and filtered through a 0.1 μm inorganic membrane filter (Whatman™, GE Healthcare Life Sciences). 2 μl of filtered $\alpha_5\beta_1$ from each fraction was loaded into a 2 μl quartz cell (ZMV1002, Hellma Analytics), which was then inserted into the Zetasizer μV instrument (Marlvern) for DLS analysis. The Z-

Average (d.nm) in the Intensity PSD (M) or the Volume PSD (M) was used for diameter determination.

NF-κB activation, cytokine release, and GSDMD cleavage.

The S-induced NF-κB activation, IL-6 release, and GSDMD cleavage in iHUVEC cells were performed with a cell-adhesion-based assay. For NF-κB activation assay, 6-well plates were coated with 1 ml/well of 10 μg/ml purified S protein constructs or poly-K in PBS at 4ºC overnight, and then blocked with 1% BSA at 37ºC for 1 hour. 2×10^6 HUVEC cells suspended in DMEM without FBS were seeded into each well and incubated for 2 hours in a 37°C incubator with 5% CO_2. The adhered cells were detached, spun down, and solubilized by adding 50 μl of SDS-PAGE loading buffer and boiled for 20 minutes. The samples were analyzed using the Jess automated western blotting system with 12-230kDa Separation Module following the manufacturer's protocol (ProteinSimple). Anti-NF-κB(p65) (Santa Cruz Biotechnology) and anti-phospho-NF-κB(p65) (Ser536) (Cell Signaling) were used for protein detection. The data were analyzed using AlphaView software (ProteinSimple) and presented by phospho-NF-κB as a percentage of total NF-κB. The total and the cleaved N-terminal fragment of GSDMD in the total cell lysates were detected by anti-GSDMD (Cell Signaling) using standard western blot. The cleavage of GSDMD was calculated by the intensity of N-terminal fragment as a percentage of total GSDMD intensity (full-length plus N-terminal fragment).

For the IL-6 release assay, 48-well plates were coated with 120 μl/well of 10 μg/ml purified S protein constructs, poly-K, or LPS in PBS at 4ºC overnight, and then blocked with 1% BSA at 37ºC for 1 hour. 200 μl of HUVEC cells at 2×10^6 /ml were seeded on each well and incubated in a 37 °C incubator with 5% CO_2 for 2 hours. Triplicate wells were used for each condition. For rolipram and cAMP inhibition assay, the cells were pre-treated with different concentrations of rolipram or cAMP for 30 minutes before seeding to the coated plate. As a control, the cells were also treated with DMSO at the concentration (1%) corresponding to that in the highest tested concentration of rolipram. The supernatants were collected and cleared by centrifugation at 13,000 rpm for 2 minutes. The IL-6 concentration in the supernatant was measured using human IL-6 Quantikine ELISA kit according to the manufacture's protocol (R&D Systems). The data of rolipram assay were presented as a percentage of inhibition calculated based on the DMSO control.

The S-induced NF-κB activation, IL-6 release, and GSDMD cleavage in HULEC-5a cells were performed using suspension cells. For NF-κB activation assay, suspended 1×10^6 HULEC-5a cells in DMEM without FBS were incubated with 10 μg/ml SARS2-S, 10 μg/ml RBD, or 5 μg/ml LPS in the presence or absence of 1 mM D-cAMP for 2 h. The cells were spun down and solubilized by adding 50 μl of SDS-PAGE loading buffer and boiled for 20 minutes. The samples were analyzed using the Jess automated western blotting system with 12-230kDa Separation Module following the manufacturer's protocol (ProteinSimple). Anti-NF-κB(p65) (Santa Cruz Biotechnology) and anti-phospho-NF-κB(p65) (Ser536) (Cell Signaling) were used for protein detection. The data were analyzed using AlphaView software (ProteinSimple) and presented by phospho-NF-κB

as a percentage of total NF-κB. The total and the cleaved N-terminal fragment of GSDMD in the total cell lysates were detected by anti-GSDMD (Cell Signaling) using standard western blot. The cleavage of GSDMD was calculated by the intensity of N-terminal fragment as a percentage of total GSDMD intensity (full-length plus N-terminal fragment). The supernatant was used for IL-6 and IL-1β release analysis. Triplicate wells were used for each condition. The IL-6 concentration in the supernatant was measured using human IL-6 and IL-1β Quantikine ELISA kit according to the manufacture's protocol (R&D Systems).

Statistical analysis.

Statistical analysis was carried out on at least three individual datasets and analyzed with GraphPad Prism software. Unpaired two-tailed t test or One-Way ANOVA Tukey's multiple comparisons test was performed between control and treated experimental groups. P-values ≤ 0.05 were considered significant. For dose–response experiments, data were normalized and analyzed using nonlinear curve fitting for the log (inhibitor) versus response (three parameters) curves.

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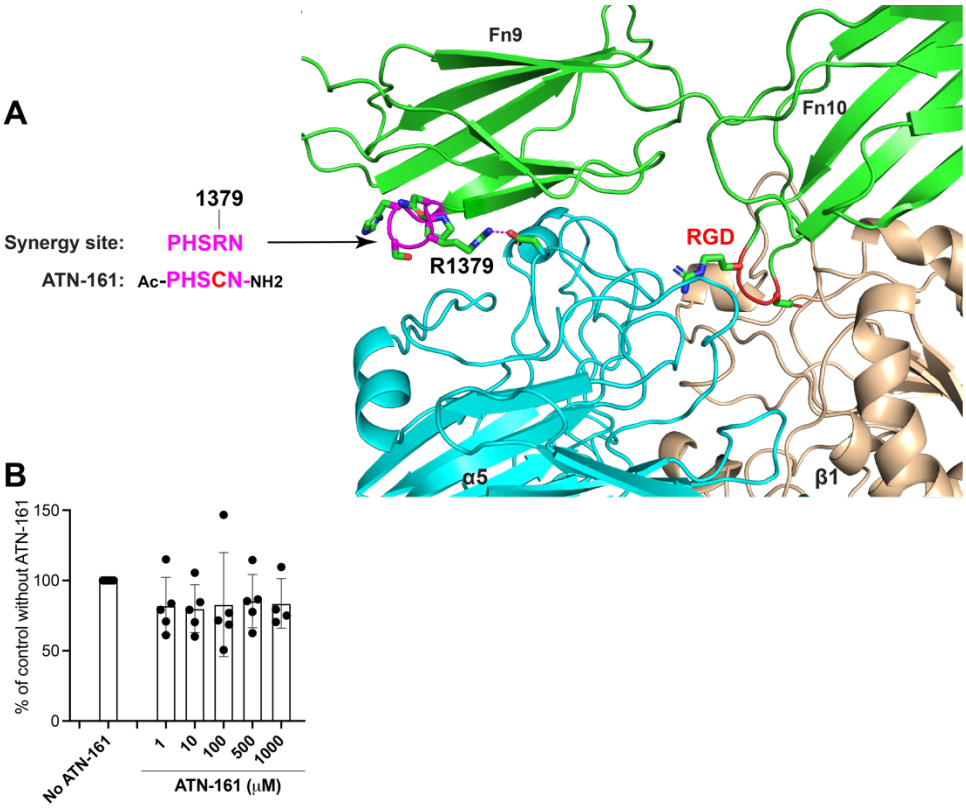

Fig. S1. The design of ATN-161 and the interaction of the RGD and synergy sites of fibronectin with $\alpha_5\beta_1$. (**A**) Cryo-EM structure of $\alpha_5\beta_1$ in complex with fibronectin domains 7-10 (PDB 7NWL) shows the interaction of the Fn10 RGD site with both α_5 and β_1 and the interaction of Fn9 PHSRN synergy site with α_5 only. The peptide inhibitor ATN-161 was designed based on the PHSRN synergy site with an Arg to Cys substitution at position 1379. However, the R1379 is critical for the PHSRN site to interact with α_5 by forming a salt bridge, which is the only polar contact between PHSRN and α_5. The Arg mutation in ATN-161 may decrease its binding with α_5 if it binds to the synergy binding site of α_5. The structural basis of how ATN-161 blocks $\alpha_5\beta_1$ function requires further investigation. (**B**) ATN-161 does not inhibit the interaction between SARS2-S and $\alpha_5\beta_1$. The purified $\alpha_5\beta_1$ ectodomain at 25 μg/ml was incubated with ATN-161 at different concentrations for 1 h before adding to the ELISA plate coated with 15 μg/ml purified SARS2-S protein. The binding of $\alpha_5\beta_1$ was detected by the anti-α_5 mAb VC5 and presented as a percentage of control without ATN-161. Data are mean ± SD from four independent repeats.

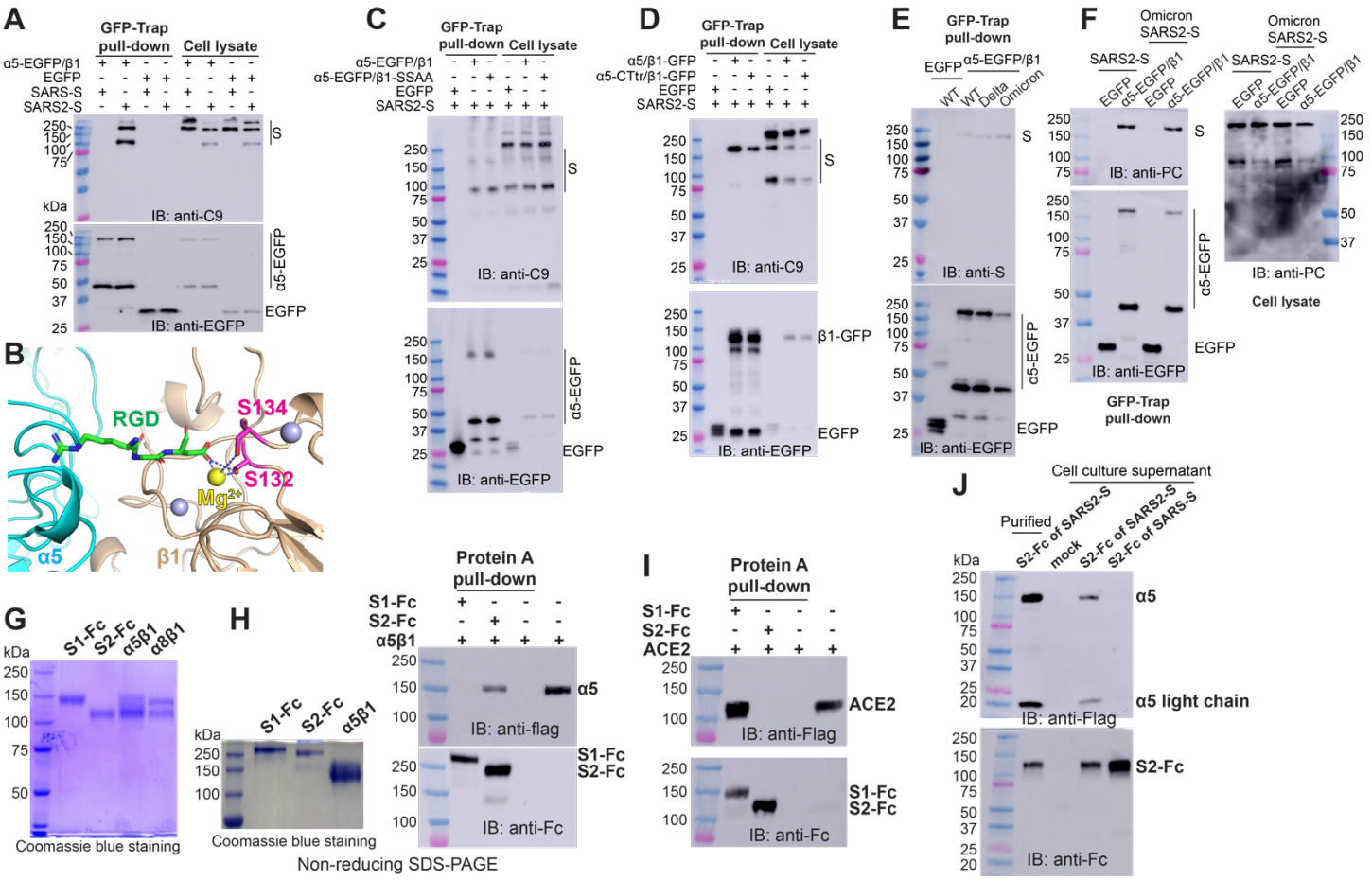

Fig. S2. **Integrin $\alpha_5\beta_1$ interacts with SARS2-S protein independent of the RGD motif.** (**A**) Pull-down assay of full-length $\alpha_5\beta_1$ and S proteins. HEK293T-α_5-KO cells were transfected with α_5-EGFP/β_1 or EGFP plus C-terminal C9-tagged SARS-S or SARS2-S. The cell lysates were immunoprecipitated with GFP-Trap beads. The precipitated samples were immunoblotted with anti-C9 and reblotted with anti-EGFP under reducing condition. The SARS2-S was cleaved into S1 and S2 subunits. The α_5 light chain tagged with EGFP was also detected. (**B**) Crystal structure of $\alpha_5\beta_1$ bound with RGD peptide (PDB 4WK0). Metal ions are shown as spheres. The conserved S132 and S134 of β_1 subunit coordinate with RGD and Mg^{2+}. Mutating the S132S134 to Ala (β_1-SSAA) abolishes Mg^{2+} and RGD binding. (**C**) HEK293T-α_5-KO cells were transfected with α_5-EGFP/β_1 α_5-EGFP/β_1-SSAA, or EGFP plus C-terminal C9-tagged SARS-S or SARS2-S. The cell lysates were immunoprecipitated with GFP-Trap beads. (**D**) HEK293T-α_5-KO cells were transfected with α_5/β_1-GFP, α_5-CTtr/β_1-GFP, or EGFP plus C-terminal C9-tagged SARS1-S or SARS2-S. The cell lysates were immunoprecipitated with GFP-Trap beads. (**E**) HEK293T-α_5-KO cells transfected with α_5-EGFP/β_1 or EGFP were incubated with purified S protein of WT, Delta, or Omicron variant. The cell lysates were immunoprecipitated with GFP-Trap beads. (**F**) HEK293T-α_5-KO cells were transfected with full-length PC-tagged SARS2-S or Omicron SARS2-S plus EGFP or α_5-EGFP/β_1 for 36 hours. Total cell lysates were precipitated with GFP-Trap beads. The S proteins were detected by immunoblot with anti-PC. (**G**) SDS-PAGE

of purified S1-Fc and S2-Fc of SARS2-S and Flag-tagged integrin ectodomains. (**H**) SDS-PAGE of purified S1-Fc and S2-Fc under non-reducing condition and protein A beads pull-down assay. The purified $\alpha_5\beta_1$ was incubated with S1-Fc or S2-Fc and precipitated by protein A beads. The precipitated samples were immunoblotted with anti-Flag and anti-Fc under non-reducing condition. (**I**) The purified S1-Fc or S2-Fc were mixed with cell lysates containing Flag-tagged full-length ACE2 and precipitated by protein A beads. (**J**) The mixture of purified Flag-tagged $\alpha_5\beta_1$ ectodomain and purified SARS2-S S2-Fc or the cell culture supernatant of HEK293T cells transfected with the DNA constructs of SARS2-S S2-Fc or SARS-S S2-Fc were immunoprecipitated with protein A beads. The samples were first immunoblotted with anti-Flag and then re-blotted with anti-Fc. The mock control was the cell culture supernatant of HEK293T transfected with the empty vector.

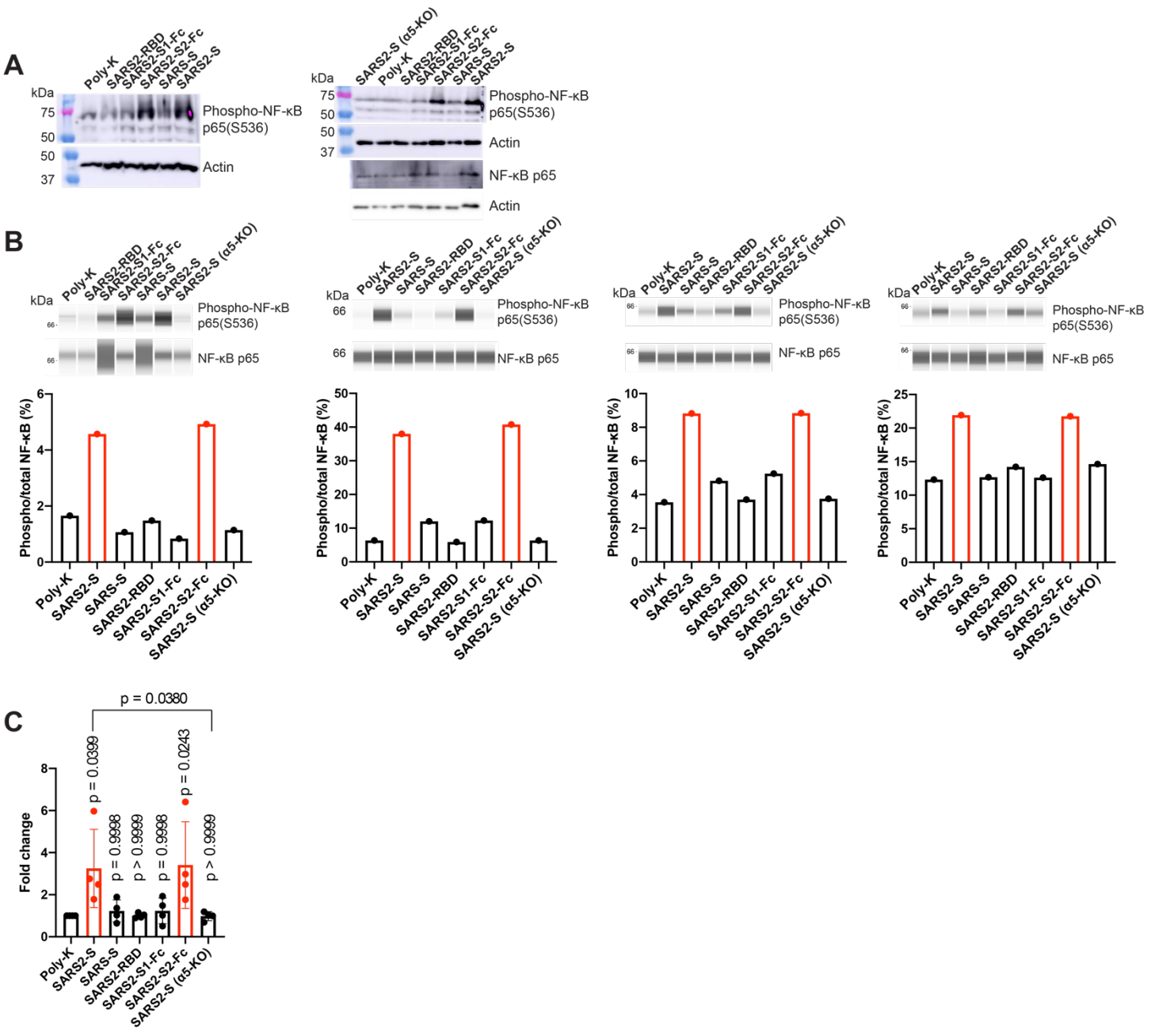

Figure S3. SARS-CoV-2 S protein induces NK-κB activation and TNT formation via α_5 integrin in HUVEC cells. (**A, B**) NF-κB activation in HUVEC cells attached to immobilized S proteins. HUVEC or HUVEC-α_5-KO cells were seeded into a plate coated with 10 μg/ml indicated proteins for 2 h. Total cells were collected for Western blotting by traditional method (A) or automatic method by Jess (ProteinSimple) (B). Results of different repeats are shown. The data quantitation was shown for each repeat of automatic Western blot in B. (**C**) The quantitation of combined data of four individual repeats in panel B. The data are presented as fold changes relative to the control condition of poly-K. One-Way ANOVA Tukey's multiple comparisons test between poly-

K control and each experiment group or as indicated. Data are mean ± SD from four independent repeats shown in panel B.