AXL Promotes SARS-CoV-2 Infection of Pulmonary and Bronchial Epithelial Cells

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Research Article

Keywords: SARS-CoV-2, AXL, spike glycoprotein, host receptor, pulmonary and bronchial epithelial cells

Posted Date: June 18th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-35387/v1>

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Abstract

The current coronavirus disease 2019 (COVID-19) pandemic presents a global public health challenge. The viral pathogen responsible, Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), binds to a host receptor ACE2 through its spike (S) glycoprotein, which mediates membrane fusion and virus entry. Although the role of ACE2 as a receptor for SARS-CoV-2 is clear, studies have shown that ACE2 expression across different human tissues is extremely low, especially in pulmonary and bronchial cells. Thus, other host receptors and/or co-receptors that promote the entry of SARS-CoV-2 into cells of the respiratory system might exist. In this study, we have identified tyrosine-protein kinase receptor UFO (AXL), specifically interacts with SARS-CoV-2 S on the host cell membrane. When overexpressed in cells that do not highly express either AXL or ACE2, AXL promotes virus entry as efficiently as ACE2. Strikingly, deleting AXL, but not ACE2, significantly reduces infection of pulmonary cells by the SARS-CoV-2 virus pseudotype. Soluble human recombinant AXL, but not ACE2, blocks SARS-CoV-2 virus pseudotype infection in pulmonary cells. Taken together, our findings suggest AXL may play an important role in promoting SARS-CoV-2 infection of the human respiratory system and is a potential target in future clinical intervention strategies.

Main Text

COVID-19 has caused a global pandemic since December 2019 and presents a global public health threat. The viral pathogen responsible, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a highly contagious enveloped, positive-strand RNA virus ¹ that causes upper respiratory diseases, fever and severe pneumonia in humans ^{1,2}. SARS-CoV-2 belongs to the β coronavirus genus. Other family members include severe acute respiratory syndrome coronavirus (SARS-CoV) and middle east respiratory syndrome coronavirus (MERS-CoV), which caused outbreaks in 2003 and 2012, respectively, though on a much smaller scale ^{3,4}. SARS-CoV-2 preferentially infects cells of the respiratory tract ⁵ but has also been detected in almost all human organs, including the lungs, pharynx, heart, liver, brain, kidneys and digestive system ⁶⁻⁸.

Coronavirus binds to host receptors through its spike (S) glycoprotein, which mediates membrane fusion and virus entry ⁹. The S protein is cleaved into the N-terminal S1 subunit and C-terminal S2 subunit by host proteases Transmembrane protease serine 2 (TMPRSS2) and FURIN (**Fig. 1a**) ¹⁰. SARS-CoV-2 shares 79.5% genetic identity with SARS-CoV, and studies have shown that ACE2, a cellular receptor for SARS-CoV ¹¹, also binds SARS-CoV-2 S and serves as the entry point for SARS-CoV-2 (**Fig. 1a**) ^{1,12}. The complex structures between ACE2 and the receptor binding domain (RBD) of SARS-CoV-2 S have recently been resolved ^{13,14}. Although the role of ACE2 as the receptor for SARS-CoV-2 is quite clear, the efficacy of drugs targeting ACE2 is still debatable. Current efforts regarding therapeutic interventions are focused on developing monoclonal antibodies targeting the SARS-CoV-2 S protein ¹⁵⁻¹⁷. However, no SARS-CoV monoclonal antibodies isolated to date are able to neutralize SARS-CoV-2 ^{18,19}, highlighting the importance of understanding the infection machinery in detail.

Although SARS-CoV-2 infection largely manifests as respiratory system symptoms, single-cell sequencing data indicate that overall ACE2 expression across different human tissues is low, especially in pulmonary and bronchial cells^{20,21}. In addition, a recently published single-cell mRNA sequencing dataset of 232,905 single cells from major adult organs²² revealed that ACE2 is specifically expressed in the kidney but rarely in organs such as the lung and trachea. Thus, other important host receptors and/or co-receptors that facilitate the entry of SARS-CoV-2 into cells of the respiratory system might exist.

Using TAP-MS to analyse protein complexes interacting with SARS-CoV-2 S in pulmonary and bronchial cells, we identified 22 candidates that may facilitate SARS-CoV-2 entry. The candidates were further enriched via molecular simulations and then tested through a series of biochemical and cell biology assays. We found that AXL specifically interacts with SARS-CoV-2 S on the host cell membrane and facilitates virus entry. Overexpressing AXL in HEK293T cells promotes virus entry as efficiently as ACE2. Downregulating AXL, but not ACE2, significantly reduces infection of pulmonary cells by the SARS-CoV-2 virus pseudotype. Soluble human recombinant AXL, but not ACE2, blocks SARS-CoV-2 virus pseudotype infection in pulmonary cells. Taken together, our results suggest that AXL promotes SARS-CoV-2 entry into human pulmonary and bronchial epithelial cells.

Identification of SARS-CoV-2 S candidate receptors in A549, H1299 and BEAS-2B cells

We analysed the expression level of ACE2 using a recently published single-cell mRNA sequencing dataset of 232,905 single cells from major adult human organs (all from non-SARS-CoV-2-infected samples)²² and found that ACE2 is specifically expressed in the jejunum (426/3075), duodenum (95/2305) and kidney (681/20053) but rarely in organs such as the lung (16/17628) and trachea (20/9521) (**Figs. 1b-g, Extended Data Fig. 1, Supplementary Table 1**). To identify additional candidate receptors and/or co-receptors for SARS-CoV-2, we first assessed which of the following cell lines are vulnerable to infection using the SARS-CoV-2 virus pseudotype: the human kidney-origin cell line HEK293T; the lung-origin cell lines A549, NCI-H1299 (H1299), NCI-H460, NCI-H292, and CALU-3; the bronchus-origin cell line BEAS-2B; the stomach-origin cell line HGC-27; the liver-origin cell lines HepG2 and HL7702; the breast-origin cell line MDA-MB-231; and the neuron-origin cell line SH-SY5Y (**Fig. 1h**). The SARS-CoV-2 virus pseudotype successfully infected lung alveolar epithelial-like A549 and H1299 cells, immortalized normal bronchus BEAS-2B cells, immortalized liver carcinoma HepG2 cells and the normal liver cell line HL7702 (**Fig. 1h**). We used A549, H1299 and BEAS-2B cells for ensuing studies to identify putative additional receptors or co-receptors responsible for SARS-CoV-2 infection of pulmonary and bronchial cells.

To identify host proteins responsible for SARS-CoV-2 infection of pulmonary and bronchial cells, we explored those interacting with SARS-CoV-2 S in A549, H1299 and BEAS-2B cells. To this end, we overexpressed FLAG-tagged full-length SARS-CoV-2 S, SARS-CoV-2 S RBD, or SARS-CoV-2 S S1+S2 in these cells or added recombinant full-length FLAG-tagged SARS-CoV-2 S or SARS-CoV-2 S RBD to the cells. Only cells overexpressing full-length SARS-CoV-2 S showed strong membrane localization of the S protein (**Extended Data Fig. 2a**). Moreover, the full-length SARS-CoV-2 S was successfully cleaved in

A549, H1299 and BEAS-2B cells, generating fragments with sizes similar to S1-cleaved SARS-CoV-2 S (**Extended Data Fig. 2b**).

Thus, we established A549, H1299 and BEAS-2B cells stably expressing SARS-CoV-2 S or control influenza A virus (A/Guangzhou/39715/2014 (H5N6)) haemagglutinin (HA), which were both fused to N-terminal SFB triple tags (S tag-2x Flag tag-SBP tag) (**Fig. 2**). Twelve clones of each bait in each cell line were examined in follow-up experiments, and bait protein expression and localization were confirmed using Western blotting and immunostaining, respectively. We chose two clones of each bait in each cell line with membrane/cytosol localization and moderate expression as biological repeats for TAP-MS analysis and isolated, combined, and affinity-purified membrane and soluble fractions using TAP. Proteins associated with the bait in isolated complexes were identified by MS, and human, SARS-CoV-2 and H5N6 databases were searched for these proteins (**Fig. 2, Supplementary Tables 2, 3**).

TAP-MS recovered a reasonable number of peptides, with high coverage for the bait proteins (**Extended Data Fig. 2c, Supplementary Table 2**) and reasonable data reproducibility (**Extended Data Fig. 2d**). In total, we identified 59,895 peptides of 9,479 proteins, representing 3,725 unique preys (**Supplementary Table 3**), 524 of which are membrane proteins or are able to translocate to the membrane. Interestingly, we did not identify ACE2 in any of our experiments, indicating ACE2 may not be the major host receptor of SARS-CoV-2 in these cells due to its low expression (**Extended Data Fig. 2e**). Nonetheless, we did consistently identify ACE2 in TAP-MS for SARS-CoV-2 S in HEK293T cells, which express very low levels of ACE2¹, indicating that our approach is able to recover host receptors for SARS-CoV-2 S (**Extended Data Fig. 2e, Supplementary Table 3**).

To distinguish *bona fide* SARS-CoV-2 S-interacting proteins from the large number of non-specific interactors frequently obtained in AP-MS results²³, we carried out H5N6 HA TAP-MS experiments under identical experimental conditions as controls and assigned a quality-associated probabilistic score to each binary interaction using the MUSE algorithm^{24,25} to remove non-specific interacting proteins. Twenty-two high-confidence candidate receptors/co-receptors that were present in protein complexes identified from at least two cell lines were chosen.

Computational screening of the top candidate receptors for SARS-CoV-2 S

To find host receptor(s) with the highest binding affinity for SARS-CoV-2 S, TAP-MS results were further enriched using a hierarchical computational protocol combining protein-protein docking, molecular modelling, MD simulations, and MM/PBSA calculations (**Fig. 2**). For each candidate receptor, we docked its extracellular domain with the full-length SARS-CoV-2 S and performed multiple rounds of conformational optimization using HADDOCK²⁶. Top-ranking poses were filtered by building full-length proteins and excluding complex conformations with potential steric clashes. Moreover, the stability of these docking poses was further assessed with explicit solvent atomistic simulations. Eleven-nanosecond MD simulations were performed for each protein-protein docking conformation; to estimate the most likely binding conformation and the corresponding binding affinity, MM/PBSA calculations were

conducted using the last 1-ns MD trajectories. Although MM/PBSA is not able to provide quantitatively accurate binding affinities, we consider the computation to be an approach for prioritizing candidate receptors for viral entry experiments. The top three candidate receptors with the most favourable affinity scores, namely, AXL, epidermal growth factor receptor (EGFR), and low-density lipoprotein receptor (LDLR) (**Fig. 2**), were subjected to further biochemical experiments.

SARS-CoV-2 S interacts with host AXL

We first validated the observed interactions using FLAG-tagged SARS-CoV-2 S and MYC-tagged receptor proteins. All three top candidates exhibited a binding affinity similar to that of ACE2 by SARS-CoV-2 S *in vivo* (**Fig. 3a**). AXL and SARS-CoV-2 S were mainly co-localized to the cell membrane, as is ACE2, whereas LDLR and EGFR were not (**Fig. 3b**). In addition, recombinant His-tagged AXL was able to bind FLAG-tagged SARS-CoV-2 S purified from HEK293T cells *in vitro* (**Fig. 3c**). Furthermore, recombinant mFc-tagged SARS-CoV-2 S pulled down AXL in H1299 cells, indicating that extracellular S is also capable of binding to AXL on the cell surface (**Supplementary Table 4**). To look into the interaction between AXL and SARS-CoV-2 S, the interface was analysed and an extensive hydrophilic network was found along the interface. In contrast to ACE2 receptor, AXL interacted with spike NTD domain, rather than RBD domain (**Extended Data Fig. 3**).

Next, we measured the protein and mRNA levels of these proteins in HEK293T, A549, H1299 and BEAS-2B cells. The level of ACE2 expression was low in all the cell lines tested; LDLR and EGFR expression was detected in all cell lines, and AXL was highly expressed in A549, H1299 and BEAS-2B cells (**Figs. 3d, e**). AXL belongs to the phosphatidylserine receptor family, many of which may serve as factors or co-factors for viral entry²⁷. Regardless, none of the other receptors in this family were specifically recovered by SARS-CoV-2 S TAP-MS or AP-MS in A549, H1299 or BEAS-2B cells (**Supplementary Tables 2-4**), indicating that this interaction between AXL and SARS-CoV-2 S is highly specific.

AXL is a receptor tyrosine kinase that transduces signals from the extracellular matrix into the cytoplasm²⁸ and regulates many physiological processes, including cell survival, proliferation, differentiation and immunity²⁹⁻³². AXL forms a stable complex with its ligand growth arrest-specific protein 6 (GAS6) via interaction interfaces involving its Ig-like domains³³. In fact, AXL has been reported to act as a cell surface receptor for Ebola virus³⁴ and plays important roles in mediating virus–host interactions²⁷. Analysis of the human cell landscape at the single-cell level revealed that AXL is expressed in most human tissues tested (**Extended Data Fig. 1c, Supplementary Table 1**). For example, many more cells in the lung and trachea expressed AXL (1232/17628 and 456/9521, respectively) than expressed ACE2 (16/17628 and 20/9521, respectively) (**Figs. 3f-i, Supplementary Table 1**). Thus, AXL might play important roles in facilitating SARS-CoV-2 entry into pulmonary and bronchial epithelial cells.

AXL facilitates SARS-CoV-2 entry into human cells

To determine whether SARS-CoV-2 uses AXL to facilitate entry into human cells, we conducted virus infectivity analyses using the SARS-CoV-2 virus pseudotype system in HEK293T cells, which express very low levels of ACE2 and AXL (**Figs. 3d, e**) and cannot be infected by the SARS-CoV-2 virus pseudotype (**Fig. 1h**). We overexpressed ACE2, AXL, LDLR, EGFR or the empty vector in HEK293T cells and infected the cells with the SARS-CoV-2 virus pseudotype. Viral infection of HEK293T cells was significantly enhanced in those overexpressing ACE2, reproducing previous results ^{1,12} and confirming that ACE2 is a SARS-CoV-2 receptor. Similar enhancement was observed for viral infection of HEK293T cells overexpressing AXL but not cells expressing LDLR or EGFR (**Figs. 4a, b**). Moreover, SARS-CoV-2 virus pseudotype particles were only observed in cells overexpressing AXL (**Fig. 4c**). We also overexpressed tyrosine-protein kinase Mer (MER) or fibroblast growth factor receptor (FGFR), which belong to the same TAM/TAM-like receptor families of receptor tyrosine kinases. However, MER and FGFR both failed to promote viral entry into cells, also indicating that the function of AXL in facilitating viral entry is highly specific (**Figs. 4d-f**). To identify the region of AXL that binds the SARS-CoV-2 S protein, we generated several truncation mutants for AXL and found its extracellular N-terminal domain, but not the kinase domain, to be responsible for the interaction (**Fig. 4g**) and viral entry into host cells (**Figs. 4h, i**). In addition, our results show that SARS-CoV-2 pseudoviral particles colocalized with endocytosis and vesicle trafficking markers including Early endosome antigen 1 (EEA1), DCC-interacting protein 13- α (APPL1), Clathrin heavy chain 1 (CLTC) and Syntaxin-6 (STX6), indicating AXL-facilitated viral entry utilizes the host endocytosis system (**Fig. 4j**). Taken together, these results indicate that AXL facilitates SARS-CoV-2 entry as potently as ACE2.

AXL is required for SARS-CoV-2 entry into human pulmonary epithelial cells

To evaluate the significance of AXL in SARS-CoV-2 infection of pulmonary epithelial cells, we knocked down ACE2, AXL, LDLR, and EGFR (**Extended Data Fig. 4**) in H1299 and A549 cells by transfecting corresponding siRNAs and then infected the cells with the SARS-CoV-2 virus pseudotype (**Figs. 5a, b**). Downregulation of AXL, but not ACE2, LDLR or EGFR, drastically reduced SARS-CoV-2 virus pseudotype infection of H1299 and A549 cells at 24 h post-infection (**Figs. 5a-d**), indicating that AXL is required for SARS-CoV-2 entry into these cells. Next, we established H1299 AXL-KO or ACE2-KO cells using the CRISPR-Cas9 system (**Fig. 5e**). Knocking out AXL but not ACE2 completely prevented viral infection in H1299 cells (**Figs. 5f, g**), indicating that AXL is required for SARS-CoV-2 entry into pulmonary and bronchial epithelial cells.

Soluble human recombinant ACE2 has reduced SARS-CoV-2 recovery from Vero cells which expresses high level ACE2 ³⁵. To further confirm AXL's indispensable role in SARS-CoV-2 infections of pulmonary and bronchial cells, and determine the potential impact on clinical practice, we added soluble human recombinant ACE2 or AXL into HEK293T cells overexpressing ACE2 or AXL, and then infected the cells with the SARS-CoV-2 virus pseudotype (**Figs. 6a-d**). Soluble human ACE2 and AXL blocks SARS-CoV-2 virus pseudotype infection in cells overexpressing ACE2 and AXL, respectively. However, they failed to block viral infection in cells overexpressing each other (**Figs. 6a-d**), indicating AXL's function in mediating viral entry is very likely to be independent of ACE2. Soluble human AXL but not ACE2 blocks SARS-CoV-2

virus pseudotype infection in H1299 cells (**Figs. 6e-g**), confirming that AXL is required for SARS-CoV-2 entry into pulmonary epithelial cells.

Taken together, our results not only confirm that the human tyrosine-protein kinase receptor AXL specifically interacts with the SARS-CoV-2 S glycoprotein but also clarify its indispensable role in facilitating SARS-CoV-2 entry into human pulmonary epithelial cells, and the cellular significance deserves further investigation (**Fig. 6h**).

Discussion

COVID-19 primarily causes respiratory system illness; however, an increasing number of case reports of SARS-CoV-2 viral infection have shown that it can also affect almost all of the body's primary organs, including the lungs, pharynx, heart, liver, brain, and kidneys⁷. SARS-CoV-2 viral particles were originally visualized in ultrathin sections of human airway epithelial cells¹. Later, SARS-CoV-2 RNA was detected in oesophagus, stomach, duodenum, and rectum specimens⁸ as well as in the kidney⁷. These results are consistent with single-cell sequencing data (**Supplementary Table 1**) showing that ACE2 is highly expressed in the kidney and digestive system. However, in other tissues, including the heart (0.3%), liver (0.1%), brain (0%), lung (0.1%) and trachea (0.2%), ACE2 expression was under 1% (**Supplementary Table 1**). Accordingly, we speculated on the existence of additional proteins that are responsible for viral entry in these tissues.

In our current study, we found that SARS-CoV-2 is able to utilize either ACE2 or AXL for entry into human cells. AXL is widely expressed in almost all human organs, and particularly in human pulmonary and bronchial epithelial tissue and cells, its expression is much higher than that of ACE2 (**Figs. 1b-g, 3g-j, Extended Data Fig. 1, Supplementary Table 1**). Given that AXL does not co-express with ACE2 or TMPRSS2 in the human lung or trachea (**Extended Data Fig. 5, Supplementary Table 1**), and the fact they could not block viral infection when the other protein is overexpressed (**Figs. 6a-d**), AXL's function in mediating SARS-CoV-2 infection is very likely to be independent of ACE2. Nevertheless, further investigation is needed to determine whether AXL and ACE2 utilize the same co-factors or are involved in similar infection processes.

Since the outbreak of COVID-19, extensive effort has been devoted to developing drugs that target the human cell viral receptor ACE2. Unfortunately, the outcomes were not satisfying. Considering the low expression level of ACE2 in human pulmonary and bronchial epithelial cells, downregulation of ACE2 elicited a minor effect in reducing SARS-CoV-2 virus pseudotype infection of H1299 and A549 cells (**Figs. 5a-d**), indicating that ACE2 might not be a good drug target for the lungs and bronchi.

As AXL is overexpressed in numerous cancer types, it has already been pursued as a drug target. However, we note that most reported AXL inhibitors target its kinase domain and would thus not have efficacy against SARS-CoV-2 infection because the virus interacts with the extracellular Ig-like domains of AXL (**Figs. 4 g-i**). Alternative approaches include targeting the AXL extracellular Ig-like domains and the

region of the SARS-CoV-2 S protein that interacts with AXL. Clinical grade human recombinant soluble AXL may be used to block viral infection (**Figs. 6 c-g**). Based on our data, AXL is likely to bind the N-terminal region of SARS-CoV-2 S, unlike the binding of ACE2 to the RBD (**Extended Data Fig. 3**). Interestingly, a recent study identified a potent neutralizing human antibody that binds to the N-terminal domain of SARS-CoV-2 S ³⁶. Although this antibody does not bind to the RBD or block its interaction with ACE2, its neutralizing activity was much higher than that of antibodies targeting the RBD ³⁶. This report highlights the importance of the N-terminal domain of SARS-CoV-2 S during viral infection and may support the important role of AXL during infection of the human pulmonary and bronchial systems.

Taken together, our findings demonstrate AXL's indispensable role in facilitating SARS-CoV-2 infection. We anticipate that upon further validation and mechanistic understanding, our findings will contribute to the development of therapeutic solutions to COVID-19.

Methods

Cell culture, plasmid construction, transfection

HEK293T, NCI-H1299, A549, BEAS-2B, NCI-H292, CALU-3, HepG2, HGC-27, MDA-MB-231 and SH-SY5Y cells were purchased from American Type Culture Collection (ATCC, USA). NCI-H460 and HL7702 cells were purchased from the Cell Bank of the Chinese Academic of Science. HEK293T, HepG2 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium, NCI-H1299, NCI-H292, NCI-H460, HL7702 and HGC-27 cells in RPMI-1640 medium, A549 cells in McCoy's 5A medium (modified with tricine) (Sigma-Aldrich, UK), BEAS-2B cells in Bronchial Epithelial Cell Growth Basal Medium (Lonza, Switzerland), CALU-3 cells in Eagle's Minimum Essential Medium (ATCC, USA), and SH-SY5Y cells in Eagle's Minimum Essential and F12 Medium (50:50) (ATCC, USA). All culture media contained 10% foetal bovine serum (Gibco, Australia) and were supplemented with 1% penicillin and streptomycin (Sigma-Aldrich, UK).

pDEST-SFB-SARS-CoV-2-S and pCMV-Myc-ACE2, AXL, EGFR and LDLR plasmids were synthesized directly. The influenza A virus haemagglutinin plasmid, a generous gift from Dr Qiyun Zhu (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences), was cloned into the pDEST-SFB vector. Deleted and truncated SARS-CoV-2-S and AXL constructs were generated by PCR-based amplification using the wild-type construct as the template. All constructs were confirmed by sequencing. Plasmids were transiently transfected into the indicated cells using Jetprime DNA transfection reagents (Polyplus, France) according to the manufacturer's instructions.

siRNAs against ACE2 (si-ACE2-1): 5'-GCGAGUGGCUAAUUUGAAAtt-3', (si-ACE2-2): 5'-GCACUUUGUCAAGCAGCUAtt-3', AXL (si-AXL-1): 5'-GGGUGGAGGUUAUCCUGAAAtt-3', (si-AXL-2): 5'-CCUGUGGUCAUCUUACCUUtt-3', EGFR (si-EGFR-1): 5'-GUCCGCAAGUAAGAAGUtt-3', (si-EGFR-2): 5'-GCAACAUGUCGAUGGACUtt-3', LDLR (si-LDLR-1): 5'-CGGCUUAAGAACAUCAACAtt-3', (si-LDLR-2): 5'-GGGUCUCCUUCUAUGGAAtt-3' were synthesized. Plasmid and siRNA transfection were performed as

follows. Cells were cultured in six-well plates, and 2 µl of plasmid (1 µg/µl) or siRNA (50 nM) was mixed with 200 µl Jetprime buffer for 10 s. Five microlitres of Jetprime transfection reagent (Polyplus, France) was added and incubated for 10 min. The mixture was then added to one well of six-well plates and incubated 24 h.

sgRNAs against ACE2 (sg-ACE2): 5'-GAAAGCTGGAGATCTGAGGT-3', AXL (sg-AXL-1): 5'-GCGAAGCCCATAACGCCAAGG-3', (sg-AXL-2): 5'-GGTTCAGGGAGAGCCCCCG-3' were synthesized, and cloned into pLenti-V2 vector. Lenti-virus were packaged in HEK293T cells, condensed by ultracentrifugation and infected A549 cells. Cells were selected with puromycin (2 µg/ml) for two days and subcloned to form single colonies. Knockout cell clones were screened by Western blotting to verify the loss of ACE2 or AXL expression.

Tandem affinity purification

H1299, A549, BEAS-2B and HEK293T cells stably expressing an N-terminal SFB-fused SARS-CoV-2 S protein or control influenza A virus haemagglutinin were selected via culture in medium containing 2 µg/ml puromycin. Protein expression was confirmed by immunostaining and Western blotting as described previously³⁷. For TAP, the membrane and soluble proteins of 1×10^8 cells were extracted using a membrane/soluble protein isolation kit (Beyotime, China) with protease inhibitors at 4°C. The lysates were combined and incubated with streptavidin-conjugated beads (Thermo Fisher Scientific, USA) for 2 h at 4°C. The beads were washed three times with 1 × NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), and the bound proteins were eluted with NETN buffer containing 2 mg/ml biotin (Sigma-Aldrich, USA) for 2 h at 4°C. The eluates were incubated with S-protein beads (Millipore, USA) for 1 h; the beads were washed three times with NETN buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Each pull-down sample was electrophoresed. The whole band was excised as one sample and subjected to in-gel trypsin digestion and LC-MS.

Liquid chromatography and mass spectrometry

LC and MS were performed as described previously^{24,25}. Briefly, the gel bands excised as described above were cut into approximately 1-mm³ pieces, which were then subjected to in-gel trypsin digestion³⁸ and dried. The samples were reconstituted in 5 ml of high-performance liquid chromatography solvent A (2.5% acetonitrile and 0.1% formic acid). A nanoscale reverse-phase high-performance liquid chromatography capillary column was created by packing 5-mm C18 spherical silica beads into a fused silica capillary (100 mm inner diameter × ~20 cm length) using a flame-drawn tip. After the column was equilibrated, each sample was loaded using an autosampler. A gradient was formed, and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile and 0.1% formic acid); the peptides were subjected to MS as they eluted.

The MS conditions were as follows. For Orbitrap Fusion Lumos, the source was operated at 1.9 kV, with no sheath gas flow and with the ion transfer tube at 350°C. The mass spectrometer was programmed to acquire in a data-dependent mode. The survey scan was from m/z 350 to 1500, with a resolution of

60,000 at m/z 200. The 20 most intense peaks with charge state 2 and greater were acquired with collision-induced dissociation with a normalized collision energy of 30% and one microscan; the intensity threshold was set at 1000. MS2 spectra were acquired with 15,000 resolution. The peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide.

Mass spectrometry data analysis

MS peptide sequences and, hence, protein identity were determined by matching fragmentation patterns in protein databases using the Mascot software program (Matrix Science, USA). Enzyme specificity was set to partially tryptic with two missed cleavages. Modifications of the peptides included carboxyamidomethylation (cysteines, variable), oxidation (methionine, variable), phosphorylation (S, T, Y, H, variable) and acetylation (N-term, K, variable). Mass tolerance was set to 20 ppm for precursor ions and fragment ions. UniProt was the database searched (*Homo sapiens*, *SARS-CoV-2* and *Influenza A virus* (A/Guangzhou/39715/2014(H5N6))). Spectral matches were filtered for a false-discovery rate of less than 1% at the peptide level using the target-decoy method³⁹, and protein inference was considered following the general rules⁴⁰, with manual annotation applied when necessary. This same principle was used for protein isoforms when they were present; in general, the longest isoform is reported. Interaction filtration was further performed using the MUSE algorithm as described previously^{24,25} to assign quality scores for the identified PPIs. TAP-MS performed under identical experimental conditions for influenza A virus haemagglutinin from H1299 cells was used as a true negative control for the MUSE analysis.

Enrichment of the overall and individual groups of proteins in canonical signalling pathways, functional categories and diseases was assessed. P values were estimated using Knowledge Base included with the Ingenuity Pathway Analysis software program (Ingenuity Systems, USA). Only statistically significant correlations ($P < 0.01$) are shown. The $-\log(P \text{ value})$ for each function and related HCIPs is listed.

Analysis of protein expression levels in human tissue cells

Analysis of ACE2 and AXL expression in the cells of human tissues was performed using a recently published single-cell mRNA sequencing dataset consisting of 232,905 single cells from all major adult organs after the batch gene background removed and cells with detection less than 500 transcripts filtered out. For digital gene expression data matrices were $\ln(\text{CPM}/100+1)$ transformed and downstream procedures for filtering and dimensional reduction were performed using Seurat v3.1.0⁴¹. All the genes were used for initial principle component analysis, and the top 10 principal components were used for nonlinear dimensional reduction (t-SNE) analysis.

Computational modelling of protein-protein complexes

Protein-protein docking calculations were performed using HADDOCK²⁶. For each receptor, 600,000 docking poses were randomly generated and rigid-body minimized in the first stage, after which 400 poses with the lowest HADDOCK scores were selected for optimization of the protein-protein interface

with simulated annealing. After further refinement of these poses in an explicit 8-Å water layer environment, clustering analysis was conducted, and the lowest score poses for each of the top 20 largest clusters were selected. One additional filter was performed by building the atomistic model of the full-length receptors interacting with SARS-CoV-2 S and excluding any conformation leading to potential steric clashes.

In the next stage, molecular dynamics simulations were carried out to assess the stability of these docking poses. Simulation systems were established using CHARMM⁴² with selected complexes solvated in a periodic 210×210×210-Å³ cubic TIP3P water box and neutralized with extra K⁺ or Cl⁻ ions. The systems were subjected to 11-ns NPT simulations with the CHARMM36 m protein force field⁴³ using OpenMM⁴⁴. Non-bonded interactions were truncated at 12 Å with smooth switching from 10 Å, and electrostatic interactions were calculated using the particle meshed Ewald (PME) method. MD trajectories evolved at 300 K with a 2-fs timestep in which bonds involving H atoms were constrained. To provide a rough estimation of the binding affinity between the candidate receptors and full-length SARS-CoV-2 S, MM/PBSA calculations were carried out using the last 1-ns MD trajectories.

$$S = \langle \Delta MM \rangle + \langle \Delta G_{PB} \rangle + \gamma \langle \Delta ASA \rangle$$

where ΔMM , ΔG_{PB} , and ΔASA are differences in non-bonded interaction energy, solvation free energy based on PB calculations, and solvent accessible surface area as the protein-protein complexes form, respectively; γ equals 0.00572 kcal/mol/Å²; and the brackets indicate the ensemble average.

Western blotting and immunoprecipitation

Cells were harvested and lysed in NETN buffer on ice for 30 min. After measuring the protein concentration using a BCA kit (Thermo Fisher Scientific, USA), 5× loading buffer (Beyotime, China) was added, and the mixture was boiled for 15 min. After 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were transferred onto PVDF membranes (Millipore, USA). The membranes were incubated with the indicated primary antibodies at 4°C overnight, washed three times with PBS-T buffer (1 × PBS, 0.05% V/V TWEEN 20) for a total of 15 min and incubated with secondary antibodies (1:2000, Cell Signaling Technology, CST, USA) for 1 h at room temperature. Signals were detected using an ECL kit (Pierce, USA).

The following primary antibodies were used: anti-MYC (1:2000, CST, #2276, USA); anti-FLAG (1:2000, CST, #14793, USA); anti-AXL (1:1000, Abcam, ab219651, USA); anti-LDLR (1:1000, Abcam, ab52818, USA); anti-ACE2 (1:1000, Abcam, ab15348, USA); anti-EGFR (1:1000, Abcam, ab52894, USA); anti-GAPDH (1:2000, Abcam, ab181602, USA); anti-EEA1 (1:2000, CST, C45B10, USA); anti-APPL1 (1:2000, CST, D83H4, USA); anti-CLTC (1:2000, CST, D3C6, USA) and anti-STX6 (1:2000, CST, C34B2, USA).

For IP and co-IP assays, 1×10^7 cells were lysed in NETN buffer on ice for 30 min. The cell lysates were precleared by incubation with 5 µl of magnetic beads (Millipore, USA) for 1 h and then with the indicated antibodies at 4°C with rotation overnight. After centrifugation, the supernatant was incubated with 10 µl

of magnetic beads at 4°C with rotation for 1 h. The beads were washed three times with cold NETN buffer using a magnetic separator (Millipore, USA), followed by elution with 40 µl of protein lysis buffer (Beyotime, China). Ten microlitres of 5 × loading buffer (Beyotime, China) was added, and the mixture was boiled for 15 min and subjected to Western blotting.

Immunofluorescence

Macrophages were collected from mice as indicated and seeded in a cell culture dish (NEST, USA). The cells were fixed and permeabilized at 4°C for 30 min. After incubation with an anti-MYC antibody (1:2000, CST, #2276, USA) and anti-FLAG antibody (1:2000, CST, #14793, USA) at 4°C overnight, the cells were washed with PBS twice and stained with goat-anti-rabbit FITC-labelled IgG or goat-anti-mouse rhodamine-labelled IgG (1:200, Proteintech, China) at 4°C for 2 h, followed by DAPI staining (Sigma-Aldrich, USA). The cells were viewed using an Olympus IX73 Microscope Imaging System (Olympus, Japan).

SARS-CoV-2 virus pseudotype production and infection

The SARS-CoV-2 virus pseudotype was packaged and used to infect cells as previously described ⁴⁵. Briefly, HEK293T cells were cultured in 10-cm plates pre-coated with poly-L-lysine and incubated with DMEM supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. The next day, the cells were co-transfected with psPAX2, pLenti-GFP, and SARS-CoV-2 S plasmids using Jetprime DNA transfection reagents (Polyplus, France) according to the manufacturer's instructions. Supernatants were collected at 48 and 72 h post-transfection, mixed with PEG overnight, passed through a 0.45-µm filter, centrifuged at 500× g for 5 min, aliquoted and stored at -80 °C.

To transduce cells with the SARS-CoV-2 virus pseudotype, HEK293T, H1299 or A549 cells were seeded into 24-well plates, transfected with the indicated plasmids or siRNAs overnight, and then infected with the SARS-CoV-2 virus pseudotype for 24 h. Cells were washed 3 times and viewed using an Olympus IX73 Microscope Imaging System (Olympus, Japan). To evaluate SARS-CoV-2 virus pseudotype infection titration, H1299 cells were cultured in 24-well plates. 25-200 µg/ml human recombinant HIS-AXL (RP-HIS-AXL) or HIS-ACE2 (RP-HIS-ACE2) were mixed with SARS-CoV-2 virus pseudotype (10⁷ PFUs, MOI 5) for 30 min and then added to the culture medium of H1299 cells. Cells were washed after 2 h post-infection and incubated with fresh medium. Cell were recovered 24 hr, and viral RNA was assayed by RT-qPCR.

Quantitative real-time PCR

Treated cells were harvested, and total RNA was extracted using TRIzol Reagent (Invitrogen, USA). RNA from each sample was reverse-transcribed to cDNA using the PrimeScriptTM RT Master Mix kit (Takara, Japan). RT-qPCR was performed using the Q5 real-time PCR system (Applied Biosystems, USA) with SYBR Green Master Mix (TOYOBO, Japan). The obtained data were normalized to GAPDH expression levels in each sample. The primers for RT-qPCR were as follows: ACE2: F: CGAAGCCGAAGACCTGTTCTA, R: GGGCAAGTGTGGACTGTTCC; AXL: F: GTGGGCAACCCAGGGAATATC, R: GTACTGTCCCGTGTGCGAAAG; LDLR: F: TCTGCAACATGGCTAGAGACT, R: TCCAAGCATTCGTTGGTCCC;

EGFR: F: AGGCACGAGTAACAAGCTCAC, R: ATGAGGACATAACCAGCCACC; GAPDH: F: GGAGCGAGATCCCTCCAAAAT, R: GGCTGTTGTCATACTTCTCATGG.

Declarations

Data analysis and ethics statement

Statistical significance between two groups was determined by unpaired two-tailed Student's t test. Differences were considered to be significant at $p < 0.05$. This study was approved by the Ethics Committee of Westlake University.

Data availability

The MS proteomics data have been deposited in ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (accession number: PXD018908). Source data for all figures and tables will be available upon publication. All other data supporting the findings of this study are available from the corresponding authors on reasonable request.

Project Name: LC-MS/MS proteomic analysis of SARS-CoV-2 Spike glycoprotein

Project accession: PXD018908

Project DOI: 10.6019/PXD018908

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Acknowledgements

We thank Drs. Hongtao Yu, Dangsheng Li and Tian Xu for their valuable advice and critical reading. We thank Drs. Qiyun Zhu, Peihui Wang and Qi Xie for kindly sharing valuable reagents. This work was supported by the Natural Science Foundation of China (91954103, 21803057 and 31930059) and the Natural Science Foundation of Zhejiang Province (LEZ20C010001, LR19B030001).

Author contributions

X.L., J.H. and Q.Z. conceived of the project. S.W., X.D., T.Z., R.Y. and P.W. performed all the experiments. Z.Q., Y.H., S.X. and J.H. analysed the data. S.W., J.H. and X.L. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

ACE2 expression is low in the human lung and trachea. (a) Schematic of the SARS-CoV-2 virion surface S mediated receptor recognition and membrane fusion. During viral infection, the trimeric S protein is cleaved into S1 and S2 subunits and S1 subunits are released in the transition to the post-fusion conformation. RBD domain on S1 directly binds to ACE2, while S2 is responsible for membrane fusion. (b, e) An overview of the human cell landscape at the single-cell level in (b) lung and (e) trachea cells. (c, d, f,

g) ACE2 expression levels in (c, d) pulmonary and (f, g) bronchial cells were evaluated using the human cell landscape at the single-cell level. Gene expression for each cell type was visualized using (c, f) tSNE and (d, g) violin plots. (h) Twelve lung, liver, kidney, stomach, and neuro cell lines were infected with the GFP-labelled SARS-CoV-2 virus pseudotype. The infection efficiency was evaluated using microscopy images taken at 24 h post-infection.

Figure 2

Schematic of the integrated proteomics-computational workflow for identifying potential SARS-CoV-2 receptors in human pulmonary and bronchial epithelial cells. (i) The SARS-CoV-2 spike glycoprotein was constructed into an SFB tag-fused expression vector. (ii) A549, H1299 and BEAS-2B cells stably expressing the SARS-CoV-2 spike glycoprotein or control H5N6 haemagglutinin glycoprotein were generated via transient transfection and puromycin selection. (iii) Interacting protein complexes were tandemly affinity purified and identified by LC-MS profiling. (iv) High-confidence interacting proteins were generated with the MUSE statistical model. (v) The results were further enriched using a hierarchical computational protocol combining protein-protein docking, molecular modelling, and MM/PBSA binding

affinity calculations from MD simulations of protein complexes. (vi) Three human receptor proteins (red), AXL, LDLR and EGFR, were bound the SARS-CoV-2 S N-terminal domain (green) or RBD (blue) with reasonable affinity.

Figure 3

The SARS-CoV-2 spike glycoprotein interacts with host AXL. (a) Validation of the interaction between SARS-CoV-2 S and ACE2, AXL, LDLR or EGFR. HEK293T cells were transfected with FLAG-tagged SARS-CoV-2 S and MYC-tagged ACE2, AXL, LDLR or EGFR for 24 h. The cells were lysed, and the lysates were incubated with FLAG-M2 resin; 5% lysate was used as the input control. Blots with antibodies recognizing the FLAG- or MYC-epitope tags are shown. (b) Co-localization assay of SARS-CoV-2 S and ACE2, AXL, LDLR or EGFR. HEK293T cells were transfected with the indicated constructs and subjected to immunofluorescence with an anti-FLAG antibody against SARS-CoV-2 S (red), anti-MYC antibody against candidate receptors (green) and DAPI (blue) and visualized by microscopy. (c) In vitro pull-down assay of SARS-CoV-2 S and AXL. FLAG-tagged SARS-CoV-2 S and His-tagged AXL were purified and co-incubated for 1 h. Blots with antibodies recognizing the FLAG- or His-epitope tags are shown. (d, e) AXL is highly expressed in A549, H1299 and BEAS-2B cell lines. Expression of ACE2, AXL, LDLR and EGFR was examined in HEK293T, A549, H1299 and BEAS-2B cell lines by (d) Western blotting and (e) RT-qPCR. (f-i)

AXL expression levels in (f, g) pulmonary and (h, i) bronchial cells were evaluated using the human cell landscape at the single-cell level. Gene expression for each cell type was visualized using (f, h) tSNE and (g, i) violin plots.

Figure 4

AXL facilitates SARS-CoV-2 entry into host cells. (a) AXL facilitates SARS-CoV-2 virus pseudotype infection as potently as ACE2. HEK293T cells were transfected with ACE2, AXL, LDLR or EGFR, infected with the GFP-labelled SARS-CoV-2 virus pseudotype, and visualized by microscopy at 24 h post-infection. (b) The fluorescence intensities in (a) were quantitated as indicated. (c) AXL facilitates viral particle entry into cells. 293T cells were transfected with MYC-tagged AXL plasmids and infected with the GFP-labelled SARS-CoV-2 virus pseudotype. The cells were fixed, incubated with anti-MYC (red) and DAPI (blue) and visualized by confocal microscopy at 24 h post-infection. (d-f) AXL specifically facilitates SARS-CoV-2

virus pseudotype infection. (d) HEK293T cells were transfected with AXL, MER or FGFR, and expression was evaluated by Western blotting with antibodies recognizing the MYC epitope tag. (e) Cells were infected with the GFP-labelled SARS-CoV-2 virus pseudotype and visualized by microscopy at 24 h post-infection. (f) The fluorescence intensities in (e) were quantitated as indicated. (g) The AXL N-terminal domain is required for binding with SARS-CoV-2 S. MYC-tagged wild-type or truncated AXL plasmids were transfected with FLAG-tagged SARS-CoV-2 S; co-IP assays were performed using an anti-MYC antibody, and epitope-tagged proteins were detected using Western blotting. (h) The AXL N-terminal domain is required for SARS-CoV-2 virus pseudotype infection. HEK293T cells were transfected with full-length AXL or its N-terminal deletion mutant, infected with the GFP-labelled SARS-CoV-2 virus pseudotype and visualized by microscopy. (i) The fluorescence intensities in (h) were quantitated as indicated. (j) AXL-facilitated SARS-CoV-2 virus pseudotype particles utilize the host endocytosis system to enter host cells. HEK293T cells were transfected with MYC-tagged AXL and infected with the GFP-labelled SARS-CoV-2 virus pseudotype. The cells were fixed, incubated with the indicated protein antibodies (red) and DAPI (blue) and visualized by confocal microscopy.

a

c

b

d

e

f

g

Figure 5

AXL is required for SARS-CoV-2 viral infection of human pulmonary epithelial cells. (a-d) Knocking down AXL using siRNA impaired SARS-CoV-2 viral infection. (a, b) H1299 and (c, d) A549 cells were transfected with siRNAs against ACE2, AXL, LDLR and EGFR, with NC as a negative control, and infected with the SARS-CoV-2 pseudotype virus for 24 h. (a, c) Microscopy images were taken at 24 h post-infection. (b, d) Knockdown efficiencies were evaluated using RT-qPCR. (e-g) Knocking out AXL completely inhibited

SARS-CoV-2 viral infection. (e) ACE2- or AXL-knockout A549 cells were established using the CRISPR-Cas9 system, and knockdown efficiencies were evaluated using Western blotting with an antibody against ACE2 or AXL, respectively. (f) Wild-type A549 and ACE2-, AXL-KO cells were infected with the GFP-labelled SARS-CoV-2 virus pseudotype and visualized by microscopy at 24 h post-infection. (g) The fluorescence intensities in (f) were quantitated as indicated.

Figure 6

Soluble human ACE2 and AXL inhibit SARS-CoV-2 infections independent to each other. (a) Soluble human ACE2 blocks SARS-CoV-2 virus pseudotype infection in cells overexpressing ACE2. HEK293T cells were transfected with ACE2 for 24 h. Human recombinant His-ACE2 were mixed with the GFP-labelled SARS-CoV-2 virus pseudotype for 30 min and then added to the culture medium. Cells were washed after 2 h post-infection and incubated with fresh medium. Cell were recovered 24 h and visualized by microscopy. (b) The fluorescence intensities in (a) were quantitated as indicated. (c-f) Soluble human AXL blocks SARS-CoV-2 virus pseudotype infection in cells overexpressing AXL. (c) HEK293T cells were transfected with AXL for 24 h. Human recombinant His-AXL were mixed with the GFP-labelled SARS-CoV-2 virus pseudotype for 30 min and then added to the culture medium. Cells were washed after 2 h post-infection and incubated with fresh medium. Cell were recovered 24 h and visualized by microscopy. (d) The fluorescence intensities in (c) were quantitated as indicated. (e-g) Soluble human recombinant AXL, but not ACE2, blocks SARS-CoV-2 virus pseudotype infection in H1299 cells. (e) Human recombinant His-AXL were mixed with the GFP-labelled SARS-CoV-2 virus pseudotype for 30 min and then added to the culture medium of H1299 cells. Cells were washed after 2 h post-infection and incubated with fresh medium. Cell were recovered 24 h and visualized by microscopy. (f) The fluorescence intensities in (e) were quantitated as indicated. (g) 25-200 µg/ml Human recombinant HIS-AXL (RP-His-AXL) or HIS-ACE2 (RP-HIS-ACE2) were mixed with SARS-CoV-2 virus pseudotype for 30 min and then added to the culture medium of H1299 cells. Cells were washed after 2 h post-infection and incubated with fresh medium. Cell were recovered 24 h, and viral RNA was examined by RT-qPCR. (h) Schematic of the AXL-mediated SARS-CoV-2 receptor recognition and/or membrane fusion.

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