1 Introduction

The biological consequences of AXL activation are complex and remain largely unknown. AXL belongs to the subfamily of receptor tyrosine kinases (RTKs) that include TYRO3 and Mer (TAM family) enzymes. Dysregulation of AXL or its sole ligand, growth arrest-specific 6 (GAS6), is implicated in the pathogenesis of a variety of human cancers including solid tumours, leukaemias and other lymphoid neoplasms. Furthermore, AXL plays an important role in tumour metastasis and invasion, as well as drug resistance.5-9

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Abstract
TAM family members (TYRO3, AXL and MERTK) play essential roles in the resolution of inflammation and in infectious diseases and cancer. AXL, a tyrosine kinase receptor, is commonly overexpressed in several solid tumours and numerous hematopoietic malignancies including acute myeloid leukaemia, acute lymphocytic leukaemia, chronic myeloid leukaemia, chronic lymphocytic leukaemia and multiple myeloma. AXL significantly promotes tumour cell migration, invasion and metastasis, as well as angiogenesis. AXL also plays an important role in inflammation and macrophage ontogeny. Recent studies have revealed that AXL contributes to leukaemic phenotypes through activation of oncogenic signalling pathways that lead to increased cell migration and proliferation. To evaluate the mechanisms underlying the role of AXL signalling in tumour metastasis, we screened a phage display library to generate a novel human monoclonal antibody, named DAXL-88, that recognizes both human and murine AXL. The concentrations of DAXL-88 required for 50% maximal binding to human and murine AXL were 0.118 and 0.164 μg/mL, respectively. Furthermore, DAXL-88 bound to human AXL with high affinity (K_D ~ 370 pM). DAXL-88 blocked the interaction between AXL and its ligand, growth arrest-specific gene 6 (GAS6), with a half maximal inhibitory concentration of 2.16 μg/mL. Moreover, DAXL-88 inhibited AXL/GAS6-dependent cell signalling, which is implicated in cell migration and invasion. In conclusion, the novel anti-AXL DAXL-88 high-affinity antibody blocks the interaction between AXL and GAS6 and inhibits tumour cell migration and invasion induced by GAS6. Thus, DAXL-88 offers promise for the development of targeted therapeutic strategies in solid tumours, leukaemias and other lymphoid neoplasms.
AXL is commonly overexpressed in several solid tumours including colon cancer, lung adenocarcinoma, pancreatic cancer, ovarian adenocarcinoma, renal cell carcinoma,\textsuperscript{10-13} and numerous hematopoietic malignancies including acute myeloid leukaemia, acute lymphocytic leukaemia, chronic myeloid leukaemia, chronic lymphocytic leukaemia and multiple myeloma.\textsuperscript{2,3,6,8} AXL also plays an important role in inflammation and macrophage ontogeny.\textsuperscript{14,15} Overexpression of AXL in breast cancer cells exhibiting drug resistance has been reported. However, silencing of AXL was combined with doxorubicin was shown to suppress the invasive and metastatic potential, and chemoresistance of breast cancer cells, and increase their elimination.\textsuperscript{16}

The AXL RTK is characterized by an extracellular domain that closely resembles the cell adhesion molecules implicated in GAS6 binding.\textsuperscript{17} The intracellular domain consists of a prototypical tyrosine kinase domain that selectively modulates AXL downstream effector signalling pathways.\textsuperscript{18} An AXL decoy protein blocks the AXL-GAS6 interaction to inhibit tumour cell migration, downregulating the phosphorylation of AXL and its antitumour function.\textsuperscript{19} In addition, AXL knockdown impairs tube formation in endothelial cells; this effect was shown to be enhanced by anti-vascular endothelial growth factor in MDA-MB-231 cells.\textsuperscript{20} Thus, targeting AXL as a research tool should enable exploration of the potential utility of AXL signalling blockade in cancer therapy.

Although AXL small-molecule tyrosine kinase inhibitors (TKIs) show therapeutic benefits in some cancers, studies of AXL TKIs have revealed they elicit increased clinical off-target toxicities and drug resistance.\textsuperscript{9,19} The development of high-affinity antibodies to block the interaction of AXL and GAS6 is therefore an important task.

Antibody engineering generally involves the display of mouse or human antibodies, or antigen-binding fragments (Fabs), on the surface of microorganisms. Numerous human antibodies currently in development have been generated by phage display technology, which enables the in vitro expression of human immunoglobulin genes. Phage display is a powerful method for isolating Fabs and whole IgG with high affinity that retain specific biological functions. With this technique, the initial antibody can be identified, avoiding the need for immunization in animals. Although knockdown of AXL by RNAi and aptamer techniques has provided insight into the role of AXL in tumour cells,\textsuperscript{20,23} research on therapeutic antibodies is lacking.

We sought to evaluate the mechanisms underlying inhibition of the interaction of AXL-GAS6, especially its roles in modulating tumour metastasis. To this end, we used phage display to obtain a phage-derived monoclonal antibody (mAb), named DAXL-88, that recognized both human and murine AXL and blocked the interaction between AXL and GAS6, thereby inhibiting tumour cell migration and invasion. Here, we describe the generation of DAXL-88 and characterize its effects on tumour cell migration and invasion, as well as its blockade in AXL signalling.

\section{MATERIAL AND METHODS}

\subsection{Reagents and antibodies}

The following reagents were used in this study: recombinant GAS6 human protein (885-GSB; R&D Systems), human AXL-ECD-His and mouse AXL-ECD-His (10279-H08H and 50126-M08H; Sino Biological), 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB; 00-4201-56; Invitrogen), 0.1% crystal violet solution (#G1064; Solarbio), Taq Blend (BTQ-201; Toyobo) and trypsin-EDTA (0.25%; 25200-072; Thermo Fisher Scientific). The anti-AXL (#4939, clone number: C2B12), anti-p-AXL (#5724, clone number: D12B2), anti-AXL antibodies (AF154; R&D Systems) and PE mouse anti-human IgG (555787; BD Biosciences) and PE mouse anti-human IgG (KLP, 01-10-06), goat anti-human IgG-Horseradish peroxidase (HRP) (#A24464; Thermo Fisher Scientific), peroxidase-conjugated streptavidin (554066; BD Biosciences) and PE mouse anti-human IgG (555787; BD Biosciences); goat anti-AXL antibodies (AF154; R&D); goat IgG (H + L) PE-conjugated antibody (F0107; R&D Systems). Dulbecco’s modified Eagle’s medium (DMEM) and McCoy’s 5A medium (DMEM) and McCoy’s 5A medium (C11995500BT and 16600-082; Gibco) were also used.

\subsection{Generation of phage-derived anti-AXL antibodies}

Human phage antibody libraries with synthetic diversity in the selected complementarity-determining regions (H1, H2 and H3), mimicking the natural diversity of the human IgG repertoire, were used for panning. Fabs were displayed bivalently on the surface of M13 bacteriophage particles.\textsuperscript{21} The phage antibody libraries were panned against human AXL-ECD-His in alternate rounds. Phage antibodies that bound to human and murine AXL-ECD-His proteins were identified by enzyme-linked immunosorbent assays (ELISAs), and antibody clone sequences were obtained to express full-length IgGs. The antibodies were purified using the ÄKTAPrime plus system (GE Healthcare).
2.3 | Cell culture

SKOV3 (AHTB-77™) and A549 (CCL-185™) cells were obtained from the American Type Culture Collection. Both cell lines were authenticated by the Beijing Zhongyuan Company. SKOV3 and A549 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and McCoy’s 5A medium (Gibco), respectively, supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and 100 U/mL penicillin-streptomyacin.

2.4 | ELISA analyses

ELISA plates were coated with 100 μL/well of 2 μg/mL AXL-ECD protein. DAXL-88 was diluted, added to the wells at 15, 5, 1.67, 0.56, 0.187, 0.062, 0.02, 0.007, 0.002 and 0.0008 μg/mL and incubated for 1 hour at 37°C. After three washes, goat anti-human IgG-HRP was added as the secondary antibody and incubated for another 30 minutes at 37°C. Binding signals were visualized using the TMB substrate, and the absorbance was measured with an ELISA reader at 450 nm. Each ELISA experiment was repeated three times.

Competitive ELISA plates were coated with 100 μL/well of 0.5 μg/mL GAS6 protein. DAXL-88, mixed with 2 μg/mL Biotin-AXL-ECD protein, was diluted, added to the wells at 150, 50, 16.7, 5.6, 1.87, 0.62, 0.2, 0.07, 0.02 and 0.008 μg/mL and incubated for 1 hour at 37°C. After three washes, peroxidase-conjugated streptavidin was added as the secondary antibody and incubated another 30 minutes at 37°C. Binding signals were visualized using the TMB substrate as before. Each competitive ELISA experiment was repeated three times.

2.5 | Binding kinetics assay

The binding kinetics and affinity of DAXL-88 to human AXL-ECD-His were measured using the Fortebio biofilm interferometry technique. DAXL-88 was captured with an Anti-Human IgG Fc Capture chip and diluted to 10 μg/mL with running buffer (phosphate-buffered saline + 0.1% Tween 20 + 0.02% bovine serum albumin) for a loading time of 90 seconds. The analyte, AXL-ECD-His, was diluted to 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0 nmol/L in running buffer to determine the binding time of DAXL-88 to the analyte (300 seconds), followed by the dissociation time (600 seconds). The chip was regenerated with 10 mmol/L glycine HCl, pH 1.7, for 5 seconds. Data were fitted to a 1:1 binding model to determine the equilibrium dissociation constant, K_D.

2.6 | Flow cytometry

Tumour cells were trypsinized and counted, and 10^6 cells per reaction were used. Cells were exposed to DAXL-88 for 30 minutes at 4°C, washed three times with buffer, then incubated with secondary antibodies (1:50, phycoerythrin mouse anti-human IgG) for 30 minutes at 4°C. At the end of the assay, cells were again washed three times and the expression of AXL analysed. To detect the cell surface binding of DAXL-88 with AXL, cell surface fluorescence intensity was analysed using a BD Biosciences flow cytometer. The flow cytometry experiments were repeated three times.

2.7 | Migration and invasion assays

After trypsin digestion, cells were counted and resuspended in serum-free DMEM. Migration assays were performed by seeding 5 × 10^4 cells into 8-μm-pore BD Falcon 24 FluoroBlok Transwell inserts. To assess GAS6-dependent migration,^16,20,23 GAS6 (200 ng/mL) was added to the lower chamber containing the migration medium (DMEM with 10% foetal bovine serum) in the presence or absence of DAXL-88 (100 μg/mL). Invasion assays were carried out using Matrigel Invasion Chambers (Corning Biocoat). After trypsin digestion, cells were counted and resuspended in serum-free DMEM. Invasion assays were performed by seeding 1 × 10^5 cells into 8-μm-pore 24-well plates. After 6 hours of migration and 24 hours of invasion, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. Both assays were repeated three times.

2.8 | Western blot

Cells were lysed with ice-cold radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitor cocktail (Roche Diagnostics) for 30 minutes. Protein concentration was quantified using a bicinchoninic acid kit (Applygen Technologies). Lysates were resolved by electrophoresis in 10% sodium dodecyl sulphate-polyacrylamide gels and analysed by Western blot. Blots were incubated with the primary antibody (1:1000 dilution) overnight at 4°C. Then, HRP-conjugated secondary antibodies were incubated with the blots for 1 hour at room temperature. Immunoreactivity was detected and visualized using an enhanced chemiluminescence detection system (SuperSignal Westpico Trial Kit; Thermo Fisher Scientific) and autoradiography. Western blot experiments were repeated three times.

2.9 | Data analyses

Statistical analyses were performed using Prism software (GraphPad). Data are presented as mean ± SE values from multiple independent experiments. In the migration assay, we performed an analysis of variance followed by Dunnett’s multiple comparisons post hoc test to compare all other groups with the GAS6 group. P values < 0.05 were considered to indicate statistically significant differences.
3 | RESULTS

3.1 | Generation of the DAXL-88 mAb

We used phage-surface display antibody libraries with synthetic diversity in the selected complementarity-determining regions to mimic the natural diversity of human IgG antibodies. After three rounds of screening, 18 antibody clones were reformatted to express full-length IgGs (Figure 1A). These IgGs were then screened for their ability to interact with human AXL-88 (Figure 1B). Based on the sequence information of the antibody clones, the expression level of the antibody and the binding ability to AXL, we selected five clones for blocking experiments to verify whether the antibody could inhibit the interaction between AXL and GAS6 (Figure S1). Competitive ELISA results showed that DAXL-88 was a candidate antibody with good blocking activity. A DAXL-88 clone was selected and purified. DAXL-88 was found to bind to human AXL with high affinity, with a $K_D$ of about $3.70 \times 10^{-10}$ M (Figure 1C). ELISA results showed that DAXL-88 bound to both human and murine AXL-ECD. The concentrations for a 50% maximal effect (EC$_{50}$) were 0.1175 and 0.1642 μg/mL for human and murine AXL-ECD, respectively (Figure 1D,E).

3.2 | Characteristics of the DAXL-88 mAb

We designed two human AXL-ECD GAS6-non-binding mutant proteins, named AXL-ECD-Fc-M3/M4 (AXL-ECD-Fc-M3 [E56R, T77R] and AXL-ECD-Fc-M4 [E59R, T77R]), to detect and model the interaction between DAXL-88 and AXL. An ELISA showed that DAXL-88 bound to both human AXL-ECD mutants, with EC$_{50}$s of 0.09448 and 0.1066 μg/mL for AXL-ECD-Fc-M3 (E56R, T77R) and AXL-ECD-Fc-M4 (E59R, T77R), respectively (Figure 2A,B). This indicated that the interaction between DAXL-88 and AXL did not involve the key AXL/GAS6 sites directly.

To determine whether DAXL-88 affected the functions of AXL, we first evaluated its effect on the binding of GAS6 to AXL. DAXL-88 blocked the interaction of GAS6 and AXL in a concentration-dependent manner, as indicated by the ELISA (Figure 2C). The half maximal inhibitory concentration (IC$_{50}$) was 2.16 μg/mL. In addition, DAXL-88 bound to AXL-positive cells SKOV3 and A549 but not AXL-negative cell 293T (Figure 2D). DAXL-88 did not...
DUAN et al. exhibit cross-reactivity with AXL-negative cells (as shown in Figure S2).

3.3 Inhibitory function of DAXL-88 in tumour cells

3.3.1 DAXL-88 blocks GAS6-mediated tumour cell migration and invasion

To investigate the potential functions of DAXL-88, we performed transwell migration and invasion assays using the AXL-positive SKOV3 (human ovarian cancer) and A549 (non-small-cell lung cancer) cell lines19,20 (Figure 2D). The addition of GAS6 to the bottom chamber significantly increased the migration and invasion of both cell lines compared with that of the control cells (Figure 3A,B). The combination of DAXL-88 and GAS6 inhibited tumour cell migration and invasion by more than 40% in both cell lines compared with that in the GAS6-alone groups (Figure 3A,B). Transwell assay results revealed that, compared with that in the GAS6 control groups, DAXL-88 effectively abolished A549 and SKOV3 cell migration induced by GAS6 (Figure 3A). A similar inhibitory function of DAXL-88 was found in MDA-MB-231 (human breast cancer) cells (Figure S3). The invasion assay yielded similar results: compared with that in the GAS6 control groups, DAXL-88 inhibited A549 and SKOV3 invasion induced by GAS6 (Figure 3B).

3.3.2 DAXL-88 inhibits downstream signalling of AXL induced by GAS6

GAS6 induces autophosphorylation of tyrosine residues in the intracellular tyrosine kinase domain of AXL by binding to the extracellular domain of AXL, subsequently activating the mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk) and phosphoinositide 3-kinase/Akt signalling pathways. This activity modulates cell survival, proliferation, migration, invasion and drug resistance.9,11,27 Accordingly, we investigated the effect of DAXL-88 on GAS6-induced AXL signalling pathways in SKOV3 cells by observing the expression of p-AXL and downstream signalling. To observe the effect of the antibody, tumour cells were pretreated with 10, 1, 0.1 and 0.01 μg/mL of DAXL-88 for 12 hours before stimulation with GAS6 30 minutes. Compared with GAS6-treated
FIGURE 3  Inhibitory functions of DAXL-88 in tumour cells. A, Cell migration was assessed in tumour cells treated with or without growth arrest-specific gene 6 (GAS6) (200 ng/mL), followed by incubation with or without DAXL-88 (100 μg/mL) for 4-6 h. B, Cell invasion was assessed in tumour cells treated with or without GAS6 (200 ng/mL), followed by incubation with or without DAXL-88 (100 μg/mL) for 24 h. DAXL-88 was added to the cell culture medium to bind with AXL on the cell surface, thus inhibiting free GAS6 binding with AXL; N = 3 and ****P < 0.0001 vs GAS6 control group. C, SKOV3 cells were pretreated with various concentrations of DAXL-88 for 12 h, followed by treatment with or without GAS6 (200 ng/mL) for 30 min. The expression of AXL, Akt and extracellular signal-regulated kinase (Erk) as well as phosphorylated AXL (p-AXL), Akt (p-Akt) and p-Erk were analysed by immunoblotting. D, SKOV3 cells were pretreated with or without DAXL-88 (1 μg/mL) for 12 h, followed by stimulation with or without GAS6 (200 ng/mL) for the indicated times. The expression of AXL, p-AXL, Axl, p-Akt, Erk and p-Erk were analysed by immunoblotting (n = 3)
Our results showed that DAXL-88 blocked AXL function by inhibiting the binding of GAS6 to its receptor on AXL (Figure 2C) as well as by downregulating the expression of AXL, leading to inactivation of AXL and its downstream signalling (Figure 3C,D). We found that DAXL-88 blocked the interaction of AXL and GAS6, but not directly through the key AXL sites of E^{56}, E^{59} and T^{77} (Figure 2A,B). The ability of DAXL-88 to decrease oncogenic downstream signalling of AXL in cancer cells represents an important mechanism underlying its inhibitory effects; accordingly, the Western blot result showed that DAXL-88 reduced the AXL expression level slightly in SKOV3 cells after 12 hours of treatment. These results clearly demonstrate that the binding of DAXL-88 with AXL on the cell surface blocked the interaction between GAS6 and AXL in cancer cells, inhibited the phosphorylation of GAS6-induced p-AXL and downregulated AXL/GAS6 downstream signalling. In future studies, we will examine this downregulating effect in other cancers, as AXL overexpression has been implicated in cancer progression and drug resistance in numerous other malignancies.7,6,29-38

The activation of AXL is involved in the progression of several tumours; in particular, the AXL/GAS6 signalling pathway plays an important role in tumour metastasis and drug resistance. Our in vitro results show that inhibition of AXL by DAXL-88 significantly suppressed SKOV3 and A549 cell migration and invasion induced by GAS6 (Figure 3A,B). These results suggest that DAXL-88 is likely to influence tumour metastasis by inhibiting tumour cell migration and invasion.

Using computer-guided homology modelling and molecular docking methods, a three-dimensional model structure of the DAXL-88 Fv fragment was constructed (Figure 4A). Furthermore, the three-dimensional structures of the DAXL-88 Fv fragment and human AXL-ECD complex were modelled (Figure 4B). Based on the complex crystal structure of AXL-GAS6, the key domains of AXL identified by GAS6 were determined to be E^{70}-W^{89} and K^{204}-R^{210} (data from the Protein Data Bank: PDB4RA0 and PDB2C5D). The E^{70}-W^{89} domain of AXL was a strong binding site, while the K^{204}-R^{210} domain was a weak binding site for GAS6. Based on the modelled three-dimensional structure of the AXL-DAXL-88 Fv fragment, the important sites of AXL identified by DAXL-88 were Q^{122}, E^{129} and H^{210} (Figure 4A). The results showed that DAXL-88 blocked the interaction of AXL and GAS6, but not directly through the key AXL sites of E^{56}, E^{59} and T^{77} (Figure 2A,B). This implied that the mechanism by which DAXL-88 blocks the binding between AXL and GAS6 may depend on steric hindrance (Figures 2A,B and 4). The theoretical and assay results showed that the binding residues of AXL identified by DAXL-88 were not the key domains (key AXL sites of E^{56}, E^{59} and T^{77}) identified by GAS6. We will further investigate the key sites of interaction between DAXL-88 and AXL to explore the mechanism by which DAXL-88 blocks this interaction.
In conclusion, we have developed a human mAb that blocks the interaction between AXL and GAS6. This anti-AXL mAb exerts its inhibitory effect through multiple mechanisms, including suppression of tumour cell migration and invasion, and downregulation of AXL downstream signalling. In further research, we will seek to examine the changes in additional molecules (including SRC, p38 and STAT1)\(^\text{11}\) in AXL downstream signalling using DAXL-88 and explore its inhibitory effect in breast, pancreatic and other cancer cell lines. We will also focus on the effect of DAXL-88 in drug-resistant cell lines and explore its ability to restore drug sensitivity in drug-resistant cells. Because of its high affinity to the human AXL protein, DAXL-88 may have considerable value in the construction of CAR-T cells. Furthermore, CAR-T cells targeted to AXL may be applied in the treatment of leukaemia and other lymphoid neoplasms.

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**CONFLICT OF INTEREST**

The authors declare that no competing interests exist.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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