

Preclinical validation of AXL receptor as a target for antibody-based pancreatic cancer immunotherapy

Running title: Anti-AXL mAb for pancreatic cancer immunotherapy

Wilhem Leconet¹, Christel Larbouret¹, Thierry Chardès¹, Gaëlle Thomas¹, Madeline Neiveyans¹, Muriel Busson¹, Marta Jarlier², Nina Radosevic-Robin³, Martine Pugnière¹, Florence Bernex¹, Frédérique Penault-Llorca³, Jean-Max Pasquet⁴, André Pèlegri¹ and Bruno Robert^{1,*}

1, INSERM-U896; IRCM; Université Montpellier1; CRLC Val d'Aurelle-Paul Lamarque. 208 rue des Apothicaires. 34298 Montpellier-Cedex5. France

2, Unité de Biostatistiques, CRLC Val d'Aurelle-Paul Lamarque. 208 rue des Apothicaires. 34298 Montpellier-Cedex 5. France

3, Département d'anatomopathologie, centre Jean-Perrin, 58 rue Montalembert, 63000 Clermont-Ferrand, France

4, INSERM-U876; Hématopoïèse Leucémique et Cible Thérapeutique; Université Victor Ségalen; Laboratoire d'hématologie CHU de Bordeaux. 33076 Bordeaux Cedex, France

***Corresponding author:** Dr Bruno ROBERT, IRCM-INSERM-U896; Campus Val d'Aurelle-Paul Lamarque, 208 rue des Apothicaires, 34298 Montpellier-Cedex 5, France. Phone: +33 467 612 418. E-mail: bruno.robert@inserm.fr

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Abstract

AXL receptor tyrosine kinases is implicated in proliferation and invasion of many cancers, particularly in pancreatic ductal adenocarcinoma (PDAC), for which new therapeutic options are urgently required. We investigated whether inhibition of AXL activity by specific monoclonal antibodies (mAbs) is efficient in limiting proliferation and migration of pancreatic cancer cells. Expression of AXL was evaluated by immunohistochemistry in 42 PDAC. The AXL role in oncogenesis was studied using the short hairpin RNA approach in a pancreatic carcinoma cell line. We further generated anti-human AXL mAbs and evaluated their inhibitory effects and the AXL downstream signaling pathways first *in vitro*, in a panel of pancreatic cancer cell lines and then *in vivo*, using subcutaneous or orthotopic pancreatic tumor xenografts. AXL receptor was found expressed in 76% (32/42) of PDAC and was predominantly present in invasive cells. The AXL-knockdown Panc-1 cells decreased *in vitro* cell migration, survival and proliferation, and reduced *in vivo* tumor growth. Two selected anti-AXL mAbs (D9 and E8), which inhibited phosphorylation of AXL and of its downstream target AKT without affecting GAS6 binding, induced down-expression of AXL by internalization, leading to an inhibition of proliferation and migration in the four pancreatic cancer cell lines studied. *In vivo*, treatment by anti-AXL mAbs significantly reduced growth of both subcutaneous and orthotopic pancreatic tumor xenografts independently of their *KRAS* mutation status. Our *in vitro* and preclinical *in vivo* data demonstrate that anti-human AXL mAbs could represent a new approach to the pancreatic cancer immunotherapy.

Key words: RTK; monoclonal antibodies; pancreatic cancer; targeted therapy

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in both men and women, with an overall 5-year survival rate of about 5% (1). At diagnosis, 80% of patients already have metastases, resulting in a median overall survival of 5-6 months. Conventional treatments, which associate surgery and radiotherapy, often in combination with chemotherapy, show a marginal clinical benefit with no real progress in patients' survival. Targeted therapies, such as antibodies or small molecule inhibitors (SMI) of receptor tyrosine kinases (RTKs) or their downstream signaling molecules have been clinically successful in various cancers (2,3), however, the results in pancreatic cancer are still limited due to the complexity of the disease. Currently, gemcitabine remains the standard pancreatic cancer treatment but with modest therapeutic gains (4).

Therefore, it is now crucial to develop new therapeutic agents based on the recent findings on molecular mechanisms involved in pancreatic cancerogenesis (5,6).

AXL belongs, with TYRO3 and MER, to the TAM family of RTKs (7). Unlike other RTKs that are stimulated by growth factors, AXL is activated by Growth Arrest-Specific factor 6 (GAS6). GAS6 binding to AXL leads to AXL dimerization, autophosphorylation and subsequent activation of signaling pathways, such as the PI3K/AKT, Mitogen-Activated Protein Kinase (MAPK), STAT and NF- κ B cascades (7). GAS6-induced AXL signaling promotes adhesion, migration, invasion, pro-inflammatory cytokine production, anti-apoptosis, proliferation and survival of cancer cells (8).

AXL was first identified as a transforming gene in chronic myeloid leukemia (9) and is expressed or upregulated in several cancers (10). AXL overexpression in breast and

pancreatic cancers is significantly associated with higher metastasis frequency and therefore with poor overall survival (11–13)

Recently, AXL has been demonstrated to be required for the epithelial-mesenchymal transition (EMT) of malignant cells, induced by various stimuli, like H-RASV12 and overexpression of SLUG (14). The cancers which have undergone EMT display increased invasiveness, metastatic capacity and multidrug resistance. Interestingly, AXL overexpression is associated with resistance either to standard chemotherapy or to tyrosine kinase inhibitors (TKI) in acute and chronic myeloid leukemia (15,16), gastrointestinal stromal tumors (17), breast (18), ovarian (19) and lung cancers (20).

In this study, we first investigated the expression of AXL in a series of PDAC and found it in the great majority of cases, similarly to the previous reports. As that indicated AXL could be a target for pancreatic cancer immunotherapy (12). We generated monoclonal antibodies (mAbs) against the extracellular domain (ECD) of AXL and tested their efficacy *in vitro* in several pancreatic cancer cell lines and *in vivo*, using xenografted nude mice. Two selected anti-AXL mAbs induced AXL down-regulation and inhibited its phosphorylation induced by GAS6, as well as the subsequent AKT activation and cell proliferation. *In vivo*, these anti-AXL mAbs reduced the growth of subcutaneous and orthotopic pancreatic tumor xenografts, independently of their *KRAS* mutation status. Finally, when compared to the standard gemcitabine treatment, the antibodies induced more complete responses and longer survival of the mice. To our best knowledge, this is the first study that evaluates anti-human AXL antibodies in both cell and animal models of pancreas cancer. The results indicate that our anti-AXL mAbs are effective candidates for the targeted therapy of this deadly disease.

RESULTS

AXL expression is predominantly observed in invasive pancreatic cancer cells

AXL was detected by IHC in 32 out of 42 PDAC (76%). In 13 out of 32 positive cases (41%) the AXL protein expression was moderate to strong and in 19 remaining cases weak. Almost all high-expressors (12 out of 13) and 9 out of 19 low-expressors, or 66% of all AXL-positive cases demonstrated presence of invasive cells (Fig. 1A) in which AXL expression was stronger than in the remaining tumor cells. These cells were localized at the periphery of the tumor and often displayed anaplastic features (Fig. 1C). AXL-positive single invasive carcinoma cells were observed in 2 cases (Fig. 1D). In another 2 cases we noted the presence of emboli containing AXL-expressing cancer cells (Fig. 1E). In contrast to cancer cells, no or weak AXL staining is observed on normal pancreatic cells (Supplementary. Fig. S1C and E).

AXL silencing reduces cell viability and migration in pancreatic cancer cell

To ascertain the role of AXL in pancreatic cancer growth, we generated two stable Panc-1 cell lines in which *AXL* was silenced by lentiviral transfection of two different anti-*AXL* shRNA (Panc-1-sh-*AXL1* and -*AXL2* cells) and a stable Panc-1 cell line transfected with empty vector (Panc-1-sh-*CONTROL*; Supplementary. Fig. S2A). Cell viability was reduced by 26 to 35% after 5 days of culture (Fig. 2A) and colony formation was decreased by 30% (Fig. 2B) in Panc-1-sh-*AXL1* and -*AXL2* cells in comparison to control cells. *AXL* silencing also inhibited cell migration, as indicated by the reduced wound healing in Panc-1-sh-*AXL1* and -*AXL2* cells in comparison to control cells after 24h (Fig. 2C). Finally, the Panc-1-sh-*AXL1* xenografted tumors grew smaller than those obtained with Panc-1 sh-*CONTROL* (Fig. 2D). These results demonstrate that AXL is involved in pancreatic cancer cell proliferation and migration.

Selected anti-AXL D9 and E8 mAbs induce AXL internalization and down-expression and inhibit MAPK and AKT phosphorylation in pancreatic cancer cells

We generated specific anti-AXL antibodies by immunizing mice with rhAXL-ECD (Supplementary. Fig. S3A-B). In order to select inhibitory antibodies, we analyzed their ability to block GAS6-mediated AXL phosphorylation in AXL-positive H1299 cells (24). D9 and E8 mAbs abolished completely AXL phosphorylation without interfering with GAS6 binding (Supplementary. Fig. S3C-D).

In order to understand the mechanism of AXL inhibition by these antibodies, we analyzed their interaction with AXL by immunofluorescence and Western blotting. We observed that D9 and E8 anti-AXL mAbs were internalized in the cytoplasm of Panc-1 cells following incubation at 37°C for 1h (Fig. 3A, lower panels). Conversely, after incubation at 4°C, they remained localized at the cell surface (Fig. 3A, upper panels). Moreover, the AXL expression was down-regulated in the four pancreatic cancer cell lines starting from 1.5 h after exposure to D9 (Fig. 3B) or E8 (Supplementary. Fig. S4A) inducing an inhibition of its phosphorylation by GAS6 (Supplementary. Fig. S4B). As AXL activation by GAS6 induces several key signaling cascades, including the AKT and MAPK pathways, we assessed the expression of phosphorylated AKT and MAPK by Western blotting in Panc-1 (mutant *KRAS*) and BxPC-3 cells (wild type *KRAS*) that were preincubated with D9 or E8 and then stimulated with rhGAS6 (Fig. 3C). GAS6-mediated phosphorylation of AXL (Y702 pAXL) and AKT (S473 pAKT) was inhibited in both cell lines by preincubation with the anti-AXL antibodies. Conversely, MAPK phosphorylation was reduced only in BxPC-3 cells, but not in Panc-1 cells, in which MAPK is constitutively activated due to the *KRAS* mutation.

Anti-AXL mAbs D9 and E8 inhibit migration and viability of pancreatic cancer cells and induce ADCC *in vitro*

As AXL plays a central role in EMT (13), we checked whether the anti-AXL mAbs D9 and E8 inhibited migration of Panc-1 cells using the wound healing assay.

In the wound-healing assay, exposure of wounded Panc-1 cultures to rhGAS6 increased cell migration in comparison to the untreated cells, resulting in the total closure of the wound gap after 24 h (Fig. 4A). Conversely, incubation with D9 or E8 after wounding inhibited cell migration, as indicated by the presence of a gap after 24 h. Addition of rhGAS6 after antibody treatment did not completely restore wound healing (Fig. 4A).

In addition, both anti-AXL mAbs significantly inhibited cell viability in a dose-dependent way in the four pancreatic cancer cell lines, independently of their *KRAS* mutation status (Figure 4B).

To evaluate the ability of anti-Axl mAbs to stimulate immune cells, we performed *in vitro* ADCC assays using hPBMC as the effector cells and MiaPaCa-2-Luc cells (Fig. 4C). Both D9 and E8 effectively induced ADCC with a potency similar to the control anti-HER2 trastuzumab.

The anti-AXL mAbs D9 and E8 reduce growth of subcutaneous pancreatic tumor xenografts

We evaluated the anti-tumor efficacy of D9 and E8 *in vivo* first using subcutaneously xenografted MiaPaCa-2 (mutant *KRAS*) or BxPC-3 (wild type *KRAS*) pancreatic cancer cells. Treatment with E8 or D9 mAbs at 15 mg/kg twice a week for 4 weeks significantly reduced growth of both MiaPaCa-2 and BxPC-3 xenografts in

comparison to the saline-treated control xenografts (MiaPaCa-2 model: $p=0.002$ for E8 and $p<0.001$ for D9; BxPC-3 model: $p=0.003$ for E8 and $p<0.001$ for D9) (Fig. 5A). At the end of the treatment with D9, tumor size was reduced by 73% in BxPC-3 xenografts and by 69% in MiaPaCa-2 xenografts; similar results were obtained with E8. The median delay to reach a tumor volume of 1000 mm^3 was much longer in mice treated with D9 (+21 days vs control) than in those treated with E8 (+7 days vs control) in MiaPaCa-2 tumors (Fig. 5B,C). BxPC-3 xenografts were more sensitive to both anti-AXL mAbs (+21 days for both D9 and E8 vs control) with a better percentage of tumor free mice (3 out of 10 for both mAbs) at the end of the experiment-(Fig. 5B,C). As previously observed (25), BxPC-3 was also sensitive to gemcitabine. We compared the efficacy of our mAbs with gemcitabine based treatment. The same effect on tumor growth was observed during treatment (Fig. 5A) but mAbs increased mice survival compared with gemcitabine and induced some complete responses (Fig. 5C).

Finally, AXL was markedly down-regulated in D9- and E8-treated tumor xenografts versus controls (Fig. 5D). Conversely, expression of cleaved caspase-3, a marker of apoptosis, was increased (Fig. 5E), whereas Ki67 index (a marker of proliferation) was unchanged (data not shown) in the antibody-treated MiaPaCa-2 tumors vs controls.

Anti-AXL mAbs treatment of orthotopic pancreatic tumor xenografts

As pancreatic cancers are characterized by poor vascularization, hypoxia and reduced penetration of antineoplastic drugs (26), we also assessed the effect of D9 mAb in an orthotopic pancreatic cancer model. SPECT-CT imaging showed that ^{125}I -labeled D9 specifically localized in orthotopic MiaPaCa-2-Luc tumor xenografts (Fig. 6A). Seven days post-graft, luminescence signals from the MiaPaCa-2-Luc cells were

detected in the pancreas region and mice were treated with 15 mg/kg D9 or saline (control) twice a week for 4 weeks. Tumor growth was quantified by measuring the bioluminescence intensity once a week (Fig. 6B). At day 35 post-graft, the bioluminescence signal was dramatically reduced in D9-treated animals in comparison to controls ($p=0.0028$) (Fig. 6B,C). Moreover, a significant reduction of the mean of tumor weight and volume were observed (72% and 84%, respectively; $p\leq 0.0001$) in D9-treated samples vs controls (Fig. 6D,E). Altogether, these *in vivo* results indicate that the anti-AXL mAbs D9 and E8 efficiently reduce growth of subcutaneous or orthotopic pancreatic tumor xenografts independently of their *KRAS* mutation status.

Discussion

A successful targeted anticancer agent has to fulfill two major criteria: to be specific for the malignant cell and to inhibit the molecular pathways driving the genesis of a given cancer. In solid tumors, the most developed category of targeted drugs are anti-EGFR antibodies or small molecule kinase inhibitors. However, in pancreas cancer, despite EGFR overexpression in up to 90% of cases, the results of the anti-EGFR therapies have been disappointing (27). In the search for a better target, the membrane RTK AXL has drawn recently much attention, by its frequent overexpression in PDAC (11,12) and a possible important role in EMT, one of the leading causes of resistance to the anti-EGFR agents (28).

AXL protein expression has been demonstrated in 55-70% of pancreatic cancers and is associated with aggressive tumor nature, higher frequency of distant metastases and poor survival (11,12). In our cohort, AXL was detected by IHC in 76% of cases (32/42), which is in concordance with previous studies. Interestingly, in 66% of the AXL-positive tumors we could observe a stronger staining in the invasive cells, either in those at the periphery of the tumor or in the emboli. This may reflect the described AXL role in cancer cell migration, invasion and dissemination from the primary site (13,14,24). Using sh-RNA tools, we confirmed that Panc-1 cell line with knocked-down expression of AXL reduced cell migration and survival *in vitro* but also impaired tumor growth as xenograft.

The frequency and pattern of AXL expression, as well as the effects of its knocking-down on cell viability and migration, make this membrane RTK a relevant target for development of the antineoplastic agents. We pursued the immunotherapy approach and generated anti-human AXL mAbs that do not cross-react with other TAM family members. In this study we show that D9 and E8, two of those anti-AXL mAbs,

induced AXL internalization and degradation. They also blocked GAS6-induced phosphorylation of AXL and of its downstream target AKT, resulting in inhibition of cell proliferation in four pancreatic carcinoma cell lines that express both AXL and its ligand GAS6.

The inhibition of GAS6-mediated AXL phosphorylation occurred through rapid internalization and dramatic down-regulation of the receptor, without competing with GAS6 for binding to AXL both in cultured pancreatic cancer cells and in xenografts.

A strong reduction in the GAS6-mediated AKT phosphorylation after exposure to D9 or E8 was observed in both wild-type and *KRAS*-mutated pancreatic cancer cell lines. Similarly, the antibodies inhibited growth of pancreatic tumor xenografts irrespective of the *KRAS* mutation status. However, MAPK phosphorylation was inhibited by anti-AXL mAbs only in *KRAS* wild type BXP-3 cells. The decreased AKT phosphorylation and the increased number of cleaved caspase-3-positive cells without changes in the Ki67 index in the antibody-treated tumor xenografts suggest that the anti-AXL mAbs D9 and E8 might reduce tumor growth mainly by apoptosis induction.

In addition, the anti-AXL mAbs D9 and E8 significantly inhibited pancreatic cancer cell migration *in vitro*, as demonstrated by the wound healing assay. Increased cell motility, migration and invasiveness are the hallmarks of EMT (29) which has been reported as a prominent feature of both *in vitro* and *in vivo* models of pancreas cancer (30). Interestingly, AXL upregulation appears to be required to maintain the expression of EMT regulators, SNAIL, SLUG and TWIST, in cancer cells (8). Furthermore, AXL expression, stimulated by vimentin, enhances breast cancer cell migration during the EMT induced by SLUG and H-RAS (14). Matrix metalloproteinase-9 (MMP-9) and MMP-2, which are enzymes involved in basement

membrane proteolysis, are up-regulated by AXL signaling in pancreatic and ovarian cancer and make their cells more invasive (31,32). These facts imply that anti-AXL antibodies, such as D9 and E8, might reduce pancreatic cancer aggressivity via interfering with EMT of its cells.

Finally, in addition to antibody binding site reactivity with the receptors, our antibodies have a Fc-dependent anti-tumor effect through activation of effector cells by ADCC. This mechanism can contribute to the therapeutic effect of mAbs observed in mice. Indeed, ADCC mechanism highly participate on antitumor effect of therapeutic mAbs (33) and recent data in patients seems to confirm this major point (34–36).

Our antibodies also inhibited tumor growth in a more relevant model, in which luciferase-positive MiaPaCa-2 cells were implanted directly in the pancreas of athymic mice. Under these conditions, tumors are less accessible, but SPECT-CT analysis clearly showed that the anti-AXL mAb D9 can reach these orthotopic xenografts and delay their growth. Recently, Mishra et al. have demonstrated that hypoxic conditions prevent GAS6-mediated AXL down-regulation in metastatic prostate cell lines, leading to increased AXL signaling (37). In the hypoxic environment of prostate cancer, AXL and GAS6 expression is increased, thus boosting the GAS6-AXL signaling and promoting tumorigenesis. As pancreatic tumors are characterized by poor vascularization and hypoxia, AXL and GAS6 could be stabilized also in such cancers and targeting AXL with specific antibodies may be a strategy to decrease oncogenic AXL signaling.

Our preclinical data clearly show that treatment with the anti-AXL mAbs D9 or E8 is sufficient to inhibit growth of pancreatic tumor xenografts and to increase survival of treated mice. Only one other group has evaluated the therapeutic impact of anti-AXL mAbs in mice, however xenografted with the A549 lung cancer or the MDA-MB-231

breast cancer cells (24,38). They reported an inhibition of tumor growth in mice treated with anti-AXL mAbs alone. This effect was further increased both in subcutaneous and metastasized xenografts when the anti-AXL mAbs were associated with chemotherapy, anti-EGFR or anti-VEGFR mAbs. Similarly, the *in vitro* efficacy of the current chemotherapy agents for lung cancer (NSCLC) is increased when AXL and/or MER are knocked down (39).

Moreover, it should be noted that our anti-AXL mAbs treatment induced some complete responses. In comparison, gemcitabine treated tumors displayed a faster re-growth when we stopped the therapy with a reduced survival time and no complete response.

This study presents two novel anti-human AXL antibodies and confirms their significant antineoplastic effect in both cellular and animal xenograft pancreas tumor models. Our results provide a rationale for clinical development of anti-AXL mAbs. As AXL activation/upregulation has been associated to cancer resistance to TKI such as erlotinib in NSCLC (40), nilotinib in chronic myelogenous leukemia (16) or lapatinib in breast cancer (18), anti-AXL antibodies could be useful in reversing the TKI-induced resistance pancreatic cancer. However, because of EMT which likely occurs very early in the development of this malignancy, anti-AXL antibodies should be considered for the first-line therapy, in combination with other cytotoxic agents.

Materials and methods

Cell lines and reagents

The BxPC-3, Capan-1 and MiaPaCa-2 cell lines were obtained from ATCC (Rockville, MD) and used within 3 months from a master cell bank. Routine authentication by typical morphology observation and mycoplasma test were conducted using MycoAlert mycoplasma detection kit (Lonza). Panc-1 cells were provided by Prof L. Buscail (INSERM U858, Toulouse, France) and were authenticated using short tandem repeat analysis. Anti-AXL shRNAs 1 and 2 (5'GGTACCGGCTGGCGTATCA3' for sh-AXL1 and 5'GAAGGAGACCCGTTATGGA3' for sh-AXL2) were provided by Dr J.M. Pasquet (INSERM U876, Bordeaux, France). All cell lines were cultured following the ATCC recommendations. Luciferase-positive MiaPaCa-2 cells (MiaPaCa-2-Luc) were generated in the laboratory. Recombinant human (rh) GAS6 and rabbit anti-human AXL antibodies were purchased from R&D Systems (Minneapolis, MN). Antibodies against phosphorylated AXL, MAPK and AKT were from Cell Signaling Technology (Beverly, MA). The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Millipore (Billerica, MA).

Immunohistochemistry (IHC)

Four-micron formalin-fixed, paraffin-embedded tissue sections were placed on poly-L-lysine coated slides, deparaffinized in xylene and rehydrated in graded alcohols. The antigen retrieval was performed by heating tissues at 95°C in 10 mM citrate buffer for 30 min. The endogenous peroxidase activity was inhibited by Dual Endogenous Enzyme-Blocking Reagent for 15 minutes (Dako, Glostrup, DK) and the nonspecific binding was reduced by the serum-free protein blocking (Dako) for 20 minutes. The sections were then incubated at room temperature with anti-Ki67 (SP6,

Thermo Fisher Scientific, Fremont, CA) diluted at 1:200, for 30 min and with anti-cleaved caspase-3 (Cell Signaling, Beverly, MA) diluted at 1:5000, for 1 h. Antibody binding was revealed using the Strepta ABComplex/HRP Duet kit (DakoCytomation, Trappes, France).

For AXL detection, the sections were incubated overnight at 4°C with rabbit polyclonal anti-AXL (ab72069, Abcam, Cambridge, UK), diluted at 1:20. Antibody binding was revealed by UltraView Universal DAB detection kit in a Benchmark XT autostainer (Ventana, Tucson, AZ). Antibody specificity to AXL receptor has been observed comparing the staining between sections of Panc-1 sh-*CONTROL* and Panc-1 sh-*AXL1* (Supplementary. Fig. S1A,B)

Antibody internalization by Immunofluorescence

100,000 cells were plated on coverslips in RPMI/10% Fetal Bovine Serum (FBS) medium at 37°C. The next day, cells were incubated or not with 20 µg/ml anti-AXL mAbs at various temperatures for 1h. Cells were then fixed in 3.7% formaldehyde in PBS, permeabilized with PBS/0.1% Triton X-100, saturated with PBS/1%BSA and finally incubated with AlexaFluor 488-conjugated goat anti-mouse IgGs (1:1000) (Life Technologies, Grand Island, NY). Coverslips were mounted with Prolong[®] Gold anti-fade and 4',6-Diamidino-2-Phenylindole (DAPI, Life Technologies) and analyzed the next day on a Zeiss Axioplan 2 Imaging microscope.

Cell viability assay

2,000 cells/well were seeded in 96-well microtiter plates. After 24 h, cells were incubated with 25 to 100 µg/ml anti-AXL mAbs for 5 days and then with a solution that contains the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the electron coupling reagent phenazine methosulfate (PMS) at 37°C for 2 h. Absorbance was

measured at 490 nm and growth inhibition was calculated based on the percentage of proliferating cells in treated samples relative to untreated cultures (0% growth inhibition) and Triton-lysed cultures (100% growth inhibition). All experiments were performed in triplicate.

Clonogenic assay

Two hundred cells were cultured in 6-well plates at 37°C in RPMI medium supplemented with 10% FBS and anti-AXL mAbs or not. At day 14, colonies were counted after fixation in a 1:3 (v/v) acetic acid:methanol solution and staining with 10% Giemsa (Sigma Chemical). All experiments were performed in triplicate.

Cell migration (wound healing) assay

Cells were seeded in 24-well plates and grown at 37°C in RPMI medium with 10% FBS. At 90% confluence, a wound was generated by scratching each monolayer with a pipette tip. Cells were then incubated 24 h in medium with 100 µg/ml anti-AXL mAbs, or not before stimulation with 200 ng/ml rhGAS6. Cell migration was verified 24 h after and then captured by a Nikon ECLIPSE TS100 microscope and an Olympus SP-510 UZ camera.

Antibody-Dependent Cellular Cytotoxicity (ADCC) assay

ADCC was evaluated with a luciferase-activity assay. In 96-well white plates, MiaPaCa-2 luc (4,000 cells/well) were preincubated with antibodies (D9, E8 or anti-HER2 trastuzumab as control) during 30 min. Ficoll-purified human peripheral blood mononuclear cells (PBMCs) from buffy coat were then added at a 10:1 effector to target cell ratio (E:T). After 24 h of incubation at 37°C, the supernatant was removed and luciferine (Promega, WI) added on the cells. Bioluminescence was determined using the Wallac Trilux 1450 Microbeta liquid scintillation and luminescence counter (Perkin-Elmer, MA). Percentage of cellular cytotoxicity was calculated using the

following formula: percentage of specific lysis = [bioluminescence in experimental point - basal bioluminescence]/[bioluminescence in total lysis - basal bioluminescence] x 100. Basal bioluminescence is obtained when MiaPaCa-2-Luc cells are incubated with hPBMC alone and bioluminescence in total lysis is obtained after a 30 min incubation of MiaPaCa-2-Luc with SDS (0.1%).

Western blotting

Western blotting was performed using protein lysates from cells or tumors as described previously (21). Blot analysis was performed using the G:BOX iChemi imaging system (Syngene).

***In vivo* studies**

All *in vivo* experiments were performed in compliance with the French regulations and ethical guidelines for experimental animal studies in an accredited establishment (Agreement No. C34-172-27). BxPC-3 (3.5×10^6) or MiaPaCa-2 (5×10^6) cancer cells were injected subcutaneously in 6 week-old female athymic mice (Harlan, Le Malourlet, France). Tumor-bearing mice were randomized in different treatment groups (n=10/group) when tumors reached a minimum volume of 100 mm^3 (day 30 post-graft for MiaPaCa-2 cells and day 14 for BxPC-3 cells) and treated with 15 mg/kg mAbs, 150 mg/kg gemcitabine or saline by intraperitoneal injection twice a week for 4 weeks. Tumors were measured using a caliper and volume was calculated using the formula $V=(\text{tumor length} \times \text{tumor width} \times \text{tumor depth})/2$, until the tumor volume reached 2000 mm^3 . For SPECT-CT studies, xenografted mice were intravenously injected with 500 μCi of ^{125}I -labeled D9 anti-AXL mAb (22). After 24 or 48 h, SPECT scans were performed at 24 projections over 360° (ROR= 45 mm, 46 s/projection) using a two-headed multiplexing multi-pinhole NanoSPECT apparatus (Bioscan Inc., Washington DC). Reconstructed data from SPECT and CT scans were

visualized and coregistered using the Invivoscope® software. For orthotopic xenografts, 1×10^6 MiaPaCa-2-Luc cells were injected in the pancreas of athymic mice and, 7 days post-graft, the mice (n=10 per group) were treated as before. Tumor growth was monitored by bioluminescence detection as described previously (23). At day 35 post-graft, mice were sacrificed and explanted pancreatic tumors weighted and measured.

Statistical analyses

A linear mixed regression model was used to determine the relationship between tumor growth and the number of days after implantation. The fixed part of the model included variables corresponding to the number of days after implantation and different groups. Interaction terms were built into the model. Random intercept and random slope were included to take into account time effect. The coefficients of the model were estimated by maximum likelihood and considered significant at the 0.05 level. Survival rates were estimated from the date of the xenograft until the date when the tumor reached a volume of 1000 mm^3 using the Kaplan–Meier method. Median survival was presented with 95%-confidence intervals. Survival curves were compared using the log-rank test. Statistical analysis was carried out using the STATA 10.0 software.

Conflict of interest

Wilhem Leconet, Christel Larbouret, Thierry Chardes, André Pèlerin and Bruno Robert are inventors on anti-AXL mAb patents related to this work. The others authors declare that they have no competing interests.

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Legends

Figure 1. AXL expression in pancreatic cancer cells. AXL protein was detected by IHC in 32 out of 42 PDAC (B – AXL-negative case; C,D,E – AXL-positive cases). Its strongest expression was observed in the invasive cells, localized at the periphery of the tumors and frequently of anaplastic morphology (C). In 2 cases AXL-expressing single invasive cells were observed (D; arrows). Two another cases had AXL-positive cells in the emboli (E; arrow).

Figure 2. AXL knockdown in the Panc-1 pancreatic cancer cell line affects cell viability, colony formation, migration and impairs tumor growth. Analysis of cell viability after five days in culture by MTS (A), colony formation after 14 days by Giemsa staining (B), cell migration after 24h of wound healing (C), and tumor growth (D) using Panc-1 sh-*AXL1*, sh-*AXL2* or sh-*CONTROL* cell lines. *** $p < 0.001$

Figure 3. The E8 and D9 anti-AXL mAbs are internalized, down-regulate AXL and inhibit the AKT signaling pathway and the MAPK pathway in a *KRAS*-wt cell line. (A) Internalization of the anti-AXL D9 and E8 mAbs in Panc-1 cells was detected by immunofluorescence. Cells were incubated or not with the anti-AXL mAbs at 4°C or 37°C for 1 hour and then with FITC-conjugated goat anti-mouse IgGs. (B) AXL expression in Capan-1, MiaPaCa-2, BxPC-3 and Panc-1 cells was analyzed by Western blotting at different time points after incubation with 100 µg/ml D9 and quantified in comparison to GAPDH level. (C) Panc-1 and BxPC-3 cells were preincubated with 100 µg/ml D9 and E8 for 1.5h and then stimulated with 200 ng/ml rhGAS6 for 30 minutes. After cell lysis, phosphorylation of AXL (Y702), AKT (S473) and MAPK (T202, Y204) was analyzed by Western blotting. GAPDH served as loading control.

Figure 4. Anti-AXL mAbs inhibited migration, viability and induced ADCC. (A) Wound healing assay using Capan-1 cells: 24h post-injury, wound healing was evaluated in untreated cells and in cells stimulated or not with 200 ng/ml GAS6, following or not preincubation with 100 µg/ml E8 or D9 anti-AXL mAbs, as indicated. (B) Cell viability was assessed by the MTS assay after incubation with 25, 50 or 100 µg/ml of E8 or D9 anti-AXL mAbs for 5 days. Results are presented as the percentage of viability inhibition relative to the untreated (0% growth inhibition) and Triton-lysed cultures (100% growth inhibition). Each value represents the mean +/- SEM (n=6). (C) Concentration-dependent evaluation of anti-AXL-dependent ADCC activity against MiaPaCa-2-Luc cells by human PBMCs. MiaPaCa-2-Luc cells were first incubated with two concentrations of anti-AXL D9 or E8 or with positive control anti-HER2 trastuzumab (10 and 100 ng/mL) during 30 min at 37°C. Then the cells were incubated with hPBMC during 24 h (37°C) at an E:T ratio of 10:1. Viability of MiaPaCa-2-Luc cells was evaluated by measuring bioluminescence in the presence of luciferin. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5. Preclinical evaluation of the E8 and D9 anti-AXL mAbs in athymic mice subcutaneously xenografted with MiaPaCa-2 or BxPC-3 cells. (A) At day 30 (MiaPaCa-2 cells) and day 14 (BxPC-3 cells), the mice were treated with 15 mg/kg of E8 or D9 mAb, gemcitabine (150 mg/kg) or saline (n=10/group), twice per week for four weeks. Results are presented as the mean tumor volume ± SEM for each group. (B) Modified Kaplan-Meier survival curves represent the percentage of mice with tumor volume < 1000 mm³ as a function of time after-graft. (C) Benefit in days and percent of tumor free mice in both MiaPaCa-2 and BxPC-3 therapies summarized. (D) E8 or D9 mAb, but not saline (CTRL), induced AXL down-regulation in both tumor xenografts 7 days after the start of treatment. GAPDH

served as loading control. (E) Immunohistochemistry analysis shows that cleaved caspase-3 expression is increased in MiaPaCa-2 tumor xenografts treated with D9 or E8, but not in saline-treated controls. **** $p < 0.01$; *** $p < 0.001$**

Figure 6: Effect of the anti-AXL mAb D9 in an orthotopic model of pancreatic cancer. MiaPaCa-2-Luc cells were injected in the pancreas of nude mice. (A) SPECT-CT imaging was carried out 24 and 48 h after injection of I^{125} -labeled D9 (day 7 post-graft). (B) Tumor growth (n=10 per group) was evaluated by measuring the emitted luminescence once a week after luciferin injection. Bioluminescence intensity (p/s) is presented as a function treatment duration (days). (C) Representative bioluminescence imaging (following intraperitoneal injection of 0.1 mg/g Luciferin) of mice at the beginning (day 7) of the treatment with 15 mg/kg D9 twice a week for 4 weeks, and at day 35 post-graft. (D,E) At day 35, all mice were sacrificed and pancreatic tumors weighted and measured. **** $p < 0,01$; *** $p < 0.001$**