Characterization of zolbetuximab in pancreatic cancer models

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ABSTRACT
In healthy tissue, the tight junction protein Claudin 18.2 (CLDN18.2) is present only in the gastric mucosa. Upon malignant transformation of gastric epithelial tissue, perturbations in cell polarity lead to cell surface exposure of CLDN18.2 epitopes. Moreover, CLDN18.2 is aberrantly expressed in malignancies of several other organs, such as pancreatic cancer (PC). A monoclonal antibody, zolbetuximab (formerly known as IMAB362), has been generated against CLDN18.2. In a phase 2 clinical trial (FAST: NCT01630083), zolbetuximab in conjunction with chemotherapy prolonged overall and progression-free survival over chemotherapy alone and improved quality of life. In this study, the mechanism of action and antitumor activity of zolbetuximab were investigated using nonclinical PC models. Zolbetuximab bound specifically and with strong affinity to human PC cells that expressed CLDN18.2 on the cell surface. In ex vivo systems using immune effector cells and serum from healthy donors, zolbetuximab induced antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), resulting in the lysis of cultured human PC cells. The amplitude of ADCC and CDC directly correlated with cell surface CLDN18.2 levels. The chemotherapeutic agent gemcitabine upregulated CLDN18.2 expression in cultured human PC cells and enhanced zolbetuximab-induced ADCC. In mouse xenograft tumors derived from human PC cell lines, including gemcitabine-refractory ones, zolbetuximab slowed tumor growth, benefited survival, and attenuated metastases development. The results presented here validate CLDN18.2 as a targetable biomarker in PC and support extension of the clinical development of zolbetuximab to patients with CLDN18.2-expressing PC.

Introduction
Targeted therapy with monoclonal antibodies (mAbs) has become a key component in the treatment of many cancers. Monoclonal antibodies can exert their effects via various mechanisms, including blocking of growth factor signaling pathways in cancer cells and cytolsis by activation of immune effectors. Activation of immune effectors is mediated by the Fc-domain of the mAb and includes the processes of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). These cytotoxic processes are initiated when specific mAbs bind to cellular targets, identifying the cell for immune-mediated destruction. For hematologic malignancies, highly effective mAbs (eg, rituximab, daratumumab), which act predominantly via ADCC and/or CDC, have been developed. Although this mechanism of action has scarcely been implemented for solid tumors because of a lack of suitable cell surface targets, it may offer a promising approach for the treatment of a variety of cancers with high unmet medical needs.

An ideal therapeutic mAb would be expected to have tumor-specific action, high and sustained potency, applicability to a large population of patients, and low toxicity. For these desirable characteristics to be met, finding a suitable specific target is critical. Previous work has identified the tight junction protein Claudin-18 (CLDN18) as an attractive target candidate in several solid tumors (eg, gastric, pancreatic, lung, and bile duct cancers). This transmembrane protein is normally expressed as two tissue-specific splice variants: CLDN18.1, predominantly expressed in the lung, and CLDN18.2, expressed exclusively in the stomach and maintained in a substantial proportion of gastric cancers (GCs). We have previously reported that a chimeric monoclonal IgG1 antibody, zolbetuximab, was developed as a specifically CLDN18.2-targeting therapeutic mAb. In a phase 2 trial (FAST; NCT01630083), zolbetuximab in combination with chemotherapy significantly prolonged survival with acceptable safety and tolerability in patients with CLDN18.2-positive (CLDN18.2+) advanced/recurrent GC and gastroesophageal junction (GEJ) cancers. Furthermore, health-related quality of life was maintained for a longer duration in patients who received zolbetuximab plus chemotherapy compared with chemotherapy alone.

In pancreatic cancer (PC), CLDN18.2 is aberrantly expressed with a prevalence of 60–90% reported in pancreatic...
ductal adenocarcinoma, the most common form of PC.\textsuperscript{10–12} Aberrantly expressed CLDN18.2 appeared in precancerous lesions, indicating it may be valuable as an early marker of malignant transformation.\textsuperscript{12,13} Depending on disease stage and patient health, systemic treatment of PC is currently based primarily on gemcitabine monotherapy, Abraxane or combinations of highly toxic fluoropyrimidine-, paclitaxel-, or oxaliplatin-based chemotherapy regimens (eg, FOLFIRINOX).\textsuperscript{14–17} There are few approved targeted therapy options for PC: the kinase inhibitor erlotinib has marginal benefit\textsuperscript{15} and the checkpoint inhibitor pembrolizumab can be used for the small fraction of patients with mismatch repair-deficient PC per a biomarker-based label approval in the United States.\textsuperscript{16} Our earlier studies showed that aberrant transcriptional induction of CLDN18.2 in pancreatic neoplasms may render a fraction of PCs targetable by zolbetuximab.\textsuperscript{12} Thus, the tumor-specific expression of CLDN18.2 in PC, as well as evidence demonstrating the beneficial effects of zolbetuximab in GC, prompted the exploration of zolbetuximab as a potential therapeutic agent for PC.

Using in vitro and in vivo nonclinical studies, we investigated the binding characteristics of zolbetuximab to CLDN18.2 in human PC cell lines, the mechanism of action of zolbetuximab, and the potential antitumor activity of zolbetuximab in mouse xenograft models of human PC-derived tumors. In addition, we examined the combined effect of zolbetuximab and the chemotherapeutic agent gemcitabine in these nonclinical tumor models.

**Results**

**Zolbetuximab binds with high specificity and affinity to cldn18.2-expressing cells**

Findings from our group and others have demonstrated that patients with PC frequently (60–90%) express high levels of CLDN18.2 in their tumor tissue (Figure 1a). To identify PC cell lines that endogenously express CLDN18.2, we screened 26 human PC cell lines by RT-PCR; of these screened cell lines, three were considered CLDN18.2\textsuperscript{*} (Patu 8988S, Dan-G, YAPC; Figure 1b). Furthermore, protein expression was also observed in these cell lines as well as in BxPC-3 and Panc 05.04 cells (Figure 1c; Suppl Table 1). This difference of CLDN18.2 expression prevalence between tumor tissue resectates and cell lines has also been observed for gastric cancer (data not shown). An interesting observation in this context is that expression of CLDN18.2 mRNA in cell lines declines over sequential passages in cell culture (Suppl Figure 1 A and B). This implies that culturing is associated with downregulation of CLDN18.2 and may explain why cell lines with pronounced levels of endogenous CLDN18.2 are rare in the first place.

To determine if chemotherapeutic agents commonly used in the treatment of PC may affect the expression of CLDN18.2, endogenously CLDN18.2-expressing PC cell lines (Dan-G and Patu 8988S) were treated with gemcitabine and gemcitabine plus oxaliplatin (GemOx). Gemcitabine increased both CLDN18.2 mRNA and protein expression in Dan-G and Patu 8988S cells. GemOx increased protein expression in these cells, though only a slight increase of mRNA was observed in Patu 8988S cells. (Figure 1, d and e). Furthermore, flow cytometry analysis of Patu 8988S cells revealed that single-agent gemcitabine and 5-fluourouracil (5-FU) increased CLDN18.2 on the cell surface (Figure 1f); with paclitaxel and oxaliplatin treatment, protein expression was maintained on baseline level (data not shown).

**Zolbetuximab induces ADCC and CDC in human cldn18.2\textsuperscript{*} PC cells**

Having found that endogenously CLDN18.2 expressing cell lines are scarce, and for the purposes of these studies, PC cell lines were transduced with CLDN18.2 (designated as ~ CLDN18.2) to generate models for functional studies. The transfected cell lines were found to express higher CLDN18.2 than endogenously CLDN18.2 expressing cell lines (Figure 1b). Evaluation of the binding of zolbetuximab to PC cell lines expressing endogenous or transduced CLDN18.2 showed that zolbetuximab binds to PC cell lines that express robust levels of CLDN18.2 (Figure 2a and b). Zolbetuximab bound poorly to those that express low surface levels of CLDN18.2 (eg, Panc 05.04) and did not bind to cell lines that lacked CLDN18.2 (data not shown).

To determine if the specific binding of zolbetuximab to CLDN18.2\textsuperscript{*} PC cells results in immune effector mediated cell killing, cells were co-incubated with human peripheral blood mononuclear cells (PBMCs) as effectors for ADCC in zolbetuximab-supplemented culture. Zolbetuximab-mediated ADCC was strictly target-dependent and highly efficient against all tested CLDN18.2-transduced PC cells (SUIT-2; CLDN18.2, MIA PaCa-2; CLDN18.2, and BxPC-3; CLDN18.2) with specific lysis reaching > 78% (Figure 2c); the corresponding CLDN18.2-negative (CLDN18.2*) parental cell lines (SUIT-2, MIA PaCa-2, and BxPC-3) and other CLDN18.2* cells were not lysed (data not shown). All three cell lines (Patu 8988S, Panc 05.04, and Dan-G) that expressed endogenous CLDN18.2 were also efficiently killed, including those with poor binding of zolbetuximab (Panc 05.04 and Dan-G; Figure 2c).

Although CLDN18.2 was limited on the cell surface of Pan 05.04 and Dan-G cell lines, zolbetuximab was still able to induce ADCC in these cells, indicating there was sufficient epitope to activate ADCC. However, due to the sparse presence of cell-surface CLDN18.2 in these cells, the effective concentrations for half-maximal response (EC\textsubscript{50}) values were up to > 100-fold higher than some lentivirally transduced cell lines that expressed high cell-surface CLDN18.2. The EC\textsubscript{50} values ranged from ~ 20 ng/mL for strong CLDN18.2-expressing cell lines to > 10,000 ng/mL for cell lines with poor zolbetuximab binding (Suppl Figure 2a, right panel). Maximum lysis of target cells with endogenous CLDN18.2 was only slightly lower than that of target cells with transduced CLDN18.2 expression (range 48%–68% vs 78%–93%, respectively; Suppl Figure 2a, left panel).

To examine if gemcitabine, through upregulating CLDN18.2 expression, may have an augmenting effect on zolbetuximab-induced ADCC and EC\textsubscript{50} values, Dan-G cells were pretreated with gemcitabine before ADCC assay was performed. Zolbetuximab-induced ADCC was more efficient in the gemcitabine-pretreated cells, as reflected in significantly lower EC\textsubscript{50} (Figure 2d and Suppl Figure 3).
Figure 1. Expression of CLDN18.2 in human PC cell lines.

(a) Typical image of CLDN18.2+ pancreatic ductal adenocarcinoma. Staining was performed with 43-14A antibody. Magnification: 200x.

(b) Transcript (qRT-PCR) and (b) protein (western blot) levels of CLDN18.2 in PC cell lines with endogenous (light red bars) and transduced (dark red bars) CLDN18.2 expression. qRT-PCR data are mean ± SD of 1–9 independent measurements. Cell lines with a relative expression level above $1 \times 10^5$ were considered CLDN18.2+ (dotted line in a). Gray bars represent non-PC cell lines and controls.

(c) Detection of CLDN18 protein in pancreatic cancer cell lysates. Western blot analysis was performed using a CLDN18 antibody detecting the C-terminal of CLDN18.1 and CLDN18.2 (C-term, Zymed) and a loading control antibody detecting β-actin. Lysates of SKBR-3 cells were used as negative control, whereas lysates of HEK293 cells stably transfected with CLDN18.2 (HEK293-p740) were used as positive control. BxPC-3 (e) and BxPC-3 (a) represent BxPC-3 cell lines from ECACC and ATCC, respectively.

(d) Effect of treatment with Gem or GemOx on CLDN18.2 mRNA expression. mRNA was isolated from DAN-G cells (untreated, treated with Gem [1 ng/mL] or GemOx [Gem 1 ng/mL + Ox 10 ng/mL] for 2 days) or Patu 8988S cells (untreated or treated with Gem [10 ng/mL] or GemOx [Gem 10 ng/mL + Ox 100 ng/mL] for 3 days). RNA was reverse transcribed to cDNA and CLDN18.2 transcript levels analyzed by qRT-PCR. Expression levels are depicted relative to the housekeeping gene HPRT.

(e) Effect of treatment with Gem or GemOx on CLDN18.2 protein expression. CLDN18 protein expression was analyzed in total cell lysates of untreated, Gem- (1 ng/mL) or GemOx- (Gem 10 ng/mL + Ox 100 ng/mL) treated DAN-G, or Patu 8988S cells by Western blot and detected with the Zymed C-term polyclonal antibody. Actin served as loading control.

(f) Influence of Gem, and 5-FU on surface expression of CLDN18.2 in Patu 8988S pancreatic cancer cells. CLDN18.2 expression was detected using flow cytometry with zolbetuximab as primary antibody and anti-hu-IgG-APC as secondary antibody. Histogram shows CLDN18.2 expression on Patu 8988S cells treated for 3 days with DMSO (gray line), 10 ng/mL Gem, 500 ng/mL 5-FU, 100 ng/mL PTX, or 500 ng/mL Ox (red line).

Abbreviations: 5-FU, 5-fluorouracil; APC, allophycocyanin; CLDN, claudin; FITC, fluorescein isothiocyanate; Gem, gemcitabine; GemOx, gemcitabine in combination with oxaliplatin; HPRT, hypoxanthin-guanine-phosphoribosyltransferase; MFI, mean fluorescence intensity; n.d., not detectable; NTC, non-template control; PC, pancreatic cancer; PTX, paclitaxel; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation.
Figure 2. Zolbetuximab induces cytotoxicity against human PC cells.
(a) Binding dynamics of zolbetuximab (FITC-conjugated) to cell-surface CLDN18.2 on human PC cells by flow cytometry.
(b) Density of the zolbetuximab epitope (zolbetuximab molecules bound per cell) on human PC cells. Data are depicted as zolbetuximab molecules bound per cell.
(c) Specific lysis of CLDN18.2-expressing cell lines (left panel, endogenous; right panel, transduced) with negative control (MIA PaCa-2) by zolbetuximab-induced ADCC. Data are (a) mean of two or (b) mean ± SD of 3–5 independent donors per cell line.
(d) Zolbetuximab-induced ADCC EC_{50} in DAN-G cells with or without 1 ng/mL Gem pretreatment. Data are mean ± SD of 10 independent donors. P value was calculated with an unpaired t-test. (e) Specific lysis of CLDN18.2-expressing cells (left panel, endogenous; right panel, transduced) by zolbetuximab-induced CDC. Healthy human serum pool served as a complement source. Data are mean ± SD of triplicate. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; EC_{50}, half-maximal effective concentration; SD, standard deviation.
Next, to investigate the sensitivity of the PC cell lines to zolbetuximab-mediated CDC, human serum as a source of complement was added to the culture in addition to zolbetuximab. Zolbetuximab induced CDC against transduced CLDN18.2-expressing cell lines only; endogenously expressing cell lines were not killed by CDC (Figure 2e), indicating that CDC activity required higher expression levels of CLDN18.2 compared with ADCC. The EC\textsubscript{50} values for CDC ranged between 3000–7000 ng/mL (Suppl Figure 2B). The in vitro antitumor activity of zolbetuximab was strictly target-specific for both ADCC and CDC, and while it was donor dependent, it was robust across different donors (data not shown).

**Zolbetuximab exhibits in vivo antitumor activity in mouse xenograft models**

To study the in vivo antitumor activity of zolbetuximab, PC cell lines, including cell lines refractory to 50 mg/kg gemcitabine, were used for subcutaneous (SC) xenografts. Mice were treated with weekly repeat doses of systemic zolbetuximab alone and in combination with gemcitabine (Figure 3 and Suppl Figure 4). Treatment of two different xenograft tumor models with zolbetuximab as a single agent resulted in significant tumor growth retardation (Figure 3, a–c).

In the MIA PaCa-2~CLDN18.2 model, inhibition of tumor growth translated into a significant survival benefit for mice treated with zolbetuximab and gemcitabine over gemcitabine alone (Figure 3b, right panel). Further, MIA PaCa2~CLDN18.2 tumor lesions remaining in mice that survived longer with zolbetuximab treatment had significantly lower fractions of CLDN18.2\textsuperscript{+} cells compared with tumors of control or gemcitabine-treated mice (Figure 3c), indicating eradication of target-positive cells. Visual evidence could be seen in FFPE tissue sections of isotype-treated control and zolbetuximab-treated mice bearing MIA PaCa-2~CLDN18.2 xenografts. (Figure 3c, a-f).

A modified form of paclitaxel, nanoparticle albumin-bound paclitaxel (nab-paclitaxel), has been approved as first-line chemotherapy for several cancer types, including breast cancer. In the current study, nab-paclitaxel was used as a control arm in combination with gemcitabine and zolbetuximab. The results demonstrated that combining nab-paclitaxel with gemcitabine and zolbetuximab led to a significant increase in tumor growth inhibition compared to gemcitabine alone (Figure 3d).

**Figure 3.** Zolbetuximab alone and in combination with gemcitabine exhibits in vivo antitumor activity in mouse xenograft models.

(a) Tumor growth kinetics of BxPC-3~CLDN18.2 xenografts. The size of SC tumors were measured twice weekly. BxPC-3~CLDN18.2 xenograft tumors were inoculated by SC injection of \(8.5 \times 10^6\) BxPC-3~CLDN18.2 cells into the flanks of 10 female Hsd:Athymic Nude-Foxn1\textsuperscript{nu} mice per treatment group. On Day 3 after tumor cell injection, treatments were initiated with Gem (100 mg/kg IP) and were continued weekly for 6 weeks. 24 h after every injection of Gem, 200 μg zolbetuximab or saline control treatments were applied IV into the tail vein. Zolbetuximab treatment was continued semi-weekly with alternating IP and IV injections until mice were euthanized. Data are mean ± SEM. *\(P < .05\); **\(P < .01\) based on Tukey’s multiple comparisons test.

(b) Tumor growth kinetics of MIA PaCa-2~CLDN18.2 xenografts (left). The size of tumors was measured twice weekly. Data are mean ± SEM. Kaplan–Meier survival estimates (right). MIA PaCa-2~CLDN18.2 xenograft tumors were inoculated by SC injection of \(5 \times 10^6\) MIA PaCa-2~CLDN18.2 cells into the flank of 10 female Hsd: Athymic Nude-Foxn1\textsuperscript{nu} mice per treatment group. On Day 4 after tumor cell injection, treatment was initiated with Gem (50 mg/kg IP) and was continued weekly for 6 weeks. 24 h after injection of Gem, 200 μg zolbetuximab or control treatments were applied IV into the tail vein. Zolbetuximab treatment was continued semi-weekly with alternating IP and IV injections until mice were euthanized (left). \(P < .05\) (zolbetuximab vs Gem, zolbetuximab + Gem vs saline); **\(P < .01\) based on Dunn’s multiple comparisons test (right).

(c) IHC analysis of CLDN18.2\textsuperscript{+} tumor cells in MIA PaCa-2~CLDN18.2 xenografts after treatment with zolbetuximab, Gem, or both. Percentage of CLDN18.2\textsuperscript{+} tumor cells in MIA PaCa-2~CLDN18.2 xenografts (upper panel). Data points represent individual measurements with the horizontal line representing mean ± SD. ***\(P < .001\) based on one-way ANOVA followed by a Tukey’s multiple comparisons test. CLDN18.2 protein expression in zolbetuximab treated and untreated MIA PaCa-2~CLDN18.2 xenografts (lower panel). FFPE tissue sections of (a–c) isotype-treated control and (d–f) zolbetuximab-treated mice bearing MIA PaCa-2~CLDN18.2 xenografts (a, d). 1× overview, magnification 100× (b, e) and 200× (c, f). Staining was performed with Zymed anti-CLDN18 antibody. Abbreviations: ANOVA, analysis of variance; FFPE, formalin-fixed paraffin-embedded; Gem, gemcitabine; IHC, immunohistochemistry; IP, intraperitoneal; IV, intravenous; SC, subcutaneous; SD, standard deviation; SEM, standard error of the mean.
treatment for PC in combination with gemcitabine. We tested the antitumor activity of zolbetuximab and nab-paclitaxel in xenograft tumors, but found no additional benefit either for tumor volume reduction or for survival (Suppl Figure 5).

**Zolbetuximab alone and in combination with gemcitabine prevents lung metastasis formation in intravenous mouse xenograft models**

Next, the potential of zolbetuximab to prevent hematogenous dissemination of PC cells was investigated. To this aim, CLDN18.2-expressing cell lines were administered intravenously (IV) into the tail vein of mice, resulting in manifestation of lung metastases. Tumor load after treatment was assessed by two independent methods: the ratio of human to murine genomic DNA by PCR and the ratio of human MHC-I to lung cell surface by IHC. In the SUIT-2~ CLDN18.2 model, mice were treated with zolbetuximab monotherapy and control isotype antibody; in the Patu 8988S model, both zolbetuximab and the isotype were combined with gemcitabine. In both models, significantly reduced lung metastatic load was observed with zolbetuximab (Figure 4a and b).

**Discussion**

Zolbetuximab is a first-in-class mAb that specifically binds to the tight junction protein CLDN18.2 and has shown promising antitumor activity in patients with gastric and GEJ cancers. In these nonclinical studies, zolbetuximab effected potent PC cell lysis in vitro by inducing ADCC and CDC. ADCC was more pronounced and affected more cell lines than CDC. Consistent with this cytolytic ability, zolbetuximab demonstrated antitumor activity in human PC cell line-derived mouse xenograft tumors. In this regard, zolbetuximab is similar to mAbs such as rituximab and daratumumab, which mainly act by stimulating ADCC and/or CDC. In our experiments, the amplitude of zolbetuximab-induced cytotoxic effects correlated with the expression level of CLDN18.2 on the cell surface. An intriguing finding in our studies is that the chemotherapeutic agent gemcitabine upregulates CLDN18.2 expression in PC cell lines. This raises the possibility that the combination of zolbetuximab with certain chemotherapy may provide enhanced cytotoxic activity against CLDN18.2* expressing cells.

Therapeutic antibodies initially developed for solid tumors acted by inhibiting tumor growth-promoting molecules (eg, growth factor receptors, angiogenic agents). A second class of mAbs for solid tumors, the immune checkpoint inhibitors that enhance T-cell-mediated cytolysis, were developed more recently and include drugs such as pembrolizumab and nivolumab. Some mAbs, such as the anti-HER2 trastuzumab, also stimulate immune effector pathways such as ADCC, although not as their primary cytolytic mechanism. A targeted, high efficacy treatment for PC remains an unmet medical need; drugs that have failed include growth factor-inhibiting mAbs and small-molecule tyrosine kinase inhibitors. In these nonclinical studies, we demonstrate that zolbetuximab, a mAb that acts through a novel target and mechanism, has antitumor activity in animal PC models utilizing human PC cell lines.

There are a few limitations to this study. Many of the cell lines used did not endogenously express appreciable levels of CLDN18.2, as CLDN18.2 seems to be downregulated and lost under cell culture conditions. Therefore, we lentivirally transduced cell lines, the expression levels of which may not fully reflect those naturally occurring in the pancreatic cancers of human patients. Also, while we have established ADCC and CDC as the dominant cytolytic mechanisms in ex vivo assays, we cannot discount that additional mechanisms are making a contribution, especially in in vivo environments. Another
issue is that our ADCC and CDC assays were conducted with PBMC or serum pool from healthy subjects. However, in a first-in-human study, PBMC and serum from patients with GC were as capable as those from healthy donors in mediating zolbetuximab-induced cell lysis.

Our results therefore support further development of zolbetuximab as a targeted therapy for PC. In addition, these results, in conjunction with earlier findings, suggest zolbetuximab may possess a number of desirable characteristics for a therapeutic mAb: the expression of CLDN18 in a large fraction of PC (60–90% in pancreatic ductal adenocarcinoma, the most common form of pancreatic cancer) indicates that a large population of patients may be treated; the very limited expression of CLDN18.2 in normal tissue may translate into a low occurrence of adverse events; and the efficient lysis suggests it can have high potency.

Clinical data of the antitumor activity of zolbetuximab in CLDN18.2-expressing GC and GEJ cancer have been promising, which prompted investigations into the potential of zolbetuximab as a therapeutic agent for CLDN18.2-expressing PC as well. Our findings from this nonclinical study support the notion that zolbetuximab can be a therapeutic antibody not only for GC, but also for several other CLDN18.2-expressing solid tumors, such as PC.

Methods

Quantitative real-time PCR (qRT-PCR)

RNA was isolated with an RNeasy Mini Kit (Qiagen) and cDNA was synthesized with the Super Script III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. The CLDN18.2 DNA sequence was amplified from cDNA with primers #5054 as (5'-AGAGAGCTCTGCTTCAAGCGTGG-3') and #5060 as (5'-CCAGAATGTAGTCACCAGCATGTTG-3') which are selective for CLDN18.2 and do not cross-amplify DNA sequences for its closely relate d isoenzyme, CLDN18.1. The qRT-PCR reactions were prepared with SYBR Green (Qiagen) and were analyzed using the ABI-PRISM7900 Sequence Detection System (Applied Biosystems). The relative CLDN18.2 expression was calculated using constitutive HPRT as reference gene with the ΔΔCT method. Samples with a relative mRNA expression of > 1 × 10² were considered CLDN18.2⁺.

For detection of human DNA present in murine lung genomic DNA samples, the qPCR reaction was performed with primer pair #5861 5'-CGGATAATTCTTAGCTAAGCG-3' and #5862 5'-TTCCGTTTAGGTGTGCTAGTTATC-3'* specifically amplifying the alpha-satellite DNA present in human chromosome 17, but not in mouse DNA. To generate a standard curve and as positive control, Patu 8988S DNA was mixed with mouse DNA and 5-fold dilutions were prepared, resulting in 100%, 20%, 4%, 0.8%, 0.16%, 0.032%, and 0.0064% human DNA in mouse DNA, respectively. The curve was used to calculate (linear regression) the amount of human metastasis-DNA present in mouse lung tissue.

Western blot

PC cells were lysed in sodium dodecyl sulfate (SDS)-buffer (34% glycine, 250 mM Tris pH 6.8, 5% β-mercaptoethanol, 8.2% SDS) and sonicated to remove genomic DNA. 75 µg total protein were electrophoresed on a 12.5% polyacrylamide gel, then blotted on a nitrocellulose membrane (90 min, 160 mA). Blots were incubated overnight at 4°C with primary antibodies (0.25 µg/mL rabbit anti-Claudin 18 [C-term, Zymed] or 0.1 µg/mL mouse anti-β-actin [Sigma] as loading control). Binding of the primary antibody was detected with labeled secondary antibodies (goat anti-rabbit IgG Fc or rabbit anti-mouse; diluted 1:1000). Protein expression was detected by the addition of 1–3 mL detection solution (Pico and Dura Detection System [Pierce]) and scanning the blots in a LAS-3000 detection box (Fujifilm).

Flow cytometry

Cells were harvested with Trypsin/EDTA (Sigma-Aldrich Co.), counted, centrifuged for 5 min (300 × g), and the pellet was resuspended in 200 µL of FACS buffer (antiMACS Running Buffer, Miltenyi Biotec). Then 100 µL of cell suspensions were plated in 96-well plates. Zolbetuximab was diluted 0.05 mg/mL in 50 µL FACS buffer and added to the cells for 30 min at 4°C as a primary antibody for detection of CLDN18.2. The plate was centrifuged (2 min, 670 × g) and then washed twice with 200 µL FACS buffer. After centrifugation, the pellet was resuspended in 100 µL of FACS buffer. Secondary goat anti-human antibody (Fc-specific, F(ab')2 conjugated with APC, Jackson ImmunoResearch Labs, Inc.) was diluted in FACS buffer and 20 µL was added to each well. The final dilution of secondary antibody in each well was 1:100. The plate was incubated for 30 min at 4°C in the dark. After incubation, the plate was washed twice with 200 µL FACS buffer and the pellet was resuspended in 200 µL FACS buffer and filtrated through a 70 µm nylon mesh filter. Then, 100 µL FACS buffer containing 5 µL 7-AAD (BD Biosciences) was added to the cell suspension for measurement using FACSVerse (BD Biosciences). The obtained data were analyzed using FlowJo software version 10.2 (FlowJo LLC, Ashland, OR, USA). Because the experiment was conducted once, the results are therefore from single experiments (no replicates).

Epitope density

The Quantum™ Simply Cellular™ anti-human IgG kit (Bangs Laboratories, Inc.) was used according to the manufacturer’s recommendation. Cells were stained with a dilution series of FITC-conjugated zolbetuximab or FITC-conjugated isotype control antibody (rituximab). For calibration, the different beads were incubated with an excess of the respective FITC-conjugated antibodies. Mean fluorescent intensity (MFI) was measured by flow cytometry using a FACS Array Bioanalyzer (BD).

Antibody-dependent cellular cytotoxicity (ADCC) analysis

Peripheral blood mononuclear cells as ADCC effector cells (E) were isolated from human peripheral blood samples or Buffy coats from healthy donors (Transfusionszentrale, Mainz) by standard Ficoll density gradient centrifugation (Ficoll-Paque
Whitney U-test (two-tailed) for comparison of two treatment groups. All differences of not-normally distributed data were calculated with a Mann–Whitney U-test (two-tailed) for comparison of two treatment groups or by a one-way ANOVA followed by Tukey’s multiple comparisons test for multiple comparison. Differences in survival between groups were calculated with a log-rank (Mantel–Cox) test. All statistical tests were two-sided and differences were considered significant if \( P < .05 \).
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Disclosure of Potential Conflicts of Interest

Özlem Türeci was a co-founder, shareholder, and former CEO of the privately owned company Ganymed Pharmaceuticals GmbH. Dr. Türeci has received consultancy fees from Astellas Pharma, Inc and has licensed patents relevant to the work, which have been acquired by Astellas Pharma, Inc. Rita Mitnacht-Kraus and Stefan Wöll are former employees of Ganymed Pharmaceuticals GmbH and have patents relevant to the work, which have been acquired by Astellas Pharma, Inc. Ugur Sahin was a co-founder of Ganymed Pharmaceuticals GmbH and is the founder, shareholder, and CEO of the privately owned company BioNTech. Tomohiro Yamada is an employee of Astellas Pharma, Inc. Prof. Sahin has received consultancy fees from Ganymed Pharmaceuticals GmbH and has licensed patents relevant to the work, which have been acquired by Astellas Pharma, Inc

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