

## Cancer Therapy: Preclinical

## Antibodies to TWEAK Receptor Inhibit Human Tumor Growth through Dual Mechanisms

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## Abstract

**Purpose:** Targeted therapeutics have significantly changed the outcome for patients diagnosed with cancer. Still, effective therapeutic intervention does not exist for many cancers and much remains to be done. The objective of this study was to identify novel genes that potentially regulate tumor growth, to target these gene products with monoclonal antibodies, and to examine the therapeutic potential of these antibodies.

**Experimental Design:** Using cDNA microarray analysis, we identified genes overexpressed in several solid malignancies. We generated a mouse monoclonal antibody, 19.2.1, and its humanized counterpart, PDL192, to one such target, TweakR (TWEAK receptor, Fn14, TNFRSF12A, CD266), and characterized the antitumor activities *in vitro* and in mouse xenograft models.

**Results:** Both 19.2.1 (mouse IgG2a) and PDL192 (human IgG1), like TWEAK, the natural ligand of TweakR, inhibited the growth of several TweakR-expressing cancer cell lines in anchorage-dependent and anchorage-independent assays *in vitro*. Both antibodies showed significant antitumor activity in multiple mouse xenograft models. PDL192 and 19.2.1 also induced antibody-dependent cellular cytotoxicity (ADCC) of cancer cell lines *in vitro*. A chimeric version of 19.2.1 containing the mouse IgG1 Fc region (19.2.1×G1) exhibited significantly less ADCC than 19.2.1. However, 19.2.1×G1 showed differential activity *in vivo*, with activity equivalent to 19.2.1 in one model, but significantly less efficacy than 19.2.1 in a second model. These results indicate that PDL192 and 19.2.1 mediate their antitumor effects by signaling through TweakR, resulting in reduced tumor cell proliferation, and by ADCC. *Clin Cancer Res*; 16(2); 497–508. ©2010 AACR.

Members of the tumor necrosis factor (TNF) receptor superfamily are type I transmembrane proteins that are categorized by intracellular domains that define their downstream functions. One receptor subfamily, including TNFR1, Fas, TRAIL-R1 (DR4), and TRAIL-R2 (DR5), contains a death domain motif that interacts with cellular adaptor proteins to activate the extrinsic apoptotic pathway (1). A second subfamily, including CD30, CD40, LTβR, 4-1-BB, and TweakR (TWEAK receptor, Fn14, TNFRSF12A, CD266), lacks the death domain, but contains a motif that binds and activates TNF receptor (TNFR)-associated factors and cytoplasmic proteins that regulate pleiotropic responses, including proliferation, differentiation, and immunoregulatory functions (2). Given the significance of the TNFR superfamily members in these various biological functions, both the receptors and their

ligands are attractive targets for the development of targeted therapeutics for the treatment of oncologic and immunologic diseases. Indeed, therapeutics against several ligand and receptor superfamily members are either approved or in development for the treatment of oncology and autoimmune diseases (3–5).

The pleiotropic nature of TweakR activation has been characterized primarily through investigating the functions of its only known ligand, TWEAK (6). TWEAK has been shown to play various roles in angiogenesis, including the ability to stimulate the growth of vascular cells *in vitro* and *in vivo* (7). In addition, TWEAK is involved in inflammation, largely through its ability to stimulate the secretion of cytokines and chemokines from a variety of cell types (8–10). In different contexts, TWEAK has also been shown to promote or to inhibit cellular differentiation (11–13) and has growth-inhibitory effects on some tumor cells, including the ability to induce apoptosis in some cell lines (14–16).

Antitumor activity of TWEAK *in vitro* is suggestive of therapeutic potential, given that TweakR is overexpressed in several human cancers (17–19). However, TWEAK also functions as a survival factor in glioblastoma cell lines and promotes their invasion and migration (20). TweakR is also expressed in breast cancers and appears to play a role in the invasive potential of this disease (12, 21). Thus,

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### Translational Relevance

Targeted therapeutics have significantly changed the prognosis for many cancer patients. However, for many tumor types, the current lack of effective treatment requires the continued development of novel therapeutics. PDL192 is a novel, humanized antibody to TWEAK receptor (TweakR, Fn14, TNFRSF12A, CD266) and is currently in a phase 1 safety study in patients with solid tumors. PDL192 and the mouse parental antibody 19.2.1 exhibit potent antitumor activity in multiple mouse xenograft models. TweakR is expressed in a range of solid tumor types, suggesting that PDL192 has the potential to benefit patients across multiple indications. PDL192 and 19.2.1 exert their antitumor activity through two distinct mechanisms: direct inhibition of tumor cell growth and antibody-dependent cellular cytotoxicity. The ability of PDL192 to inhibit tumor growth through multiple mechanisms enhances the potential efficacy of the antibody and thus increases the likelihood of benefit to patients with solid tumors.

TWEAK binding to TweakR seems to exert multiple functions on tumor cells. The functional relevance of this pathway in oncology suggests that an agent targeted to this pathway might be a potent cancer therapeutic.

In this report, we show that TweakR mRNA and protein are overexpressed in several solid tumor types, compared with their cognate normal tissues. We describe the development of the anti-TweakR monoclonal antibody, 19.2.1, and its humanized counterpart, PDL192. Both have antitumor effects, including the ability to inhibit the growth of several TweakR-expressing cancer cell lines *in vitro* and the ability to induce antibody-dependent cellular cytotoxicity (ADCC). We also show that 19.2.1 and PDL192 inhibit the growth of multiple TweakR-expressing human xenograft tumors in mouse models and that the *in vivo* antitumor activity of the antibodies is driven by dual mechanisms.

### Materials and Methods

**Cell lines and reagents.** All tumor cell lines were obtained from American Type Culture Collection, except the SN12C cell line, which was obtained from Developmental Therapeutics Program/Division of Cancer Treatment and Diagnosis Tumor Repository at the National Cancer Institute and the HSC3 cell line, which was purchased from the Health Sciences Research Resources Bank at the Japan Health Sciences Foundation. A253 salivary gland cancer cells were maintained in McCoy's media; H520 lung cancer cells and SN12C renal cancer cells were maintained in RPMI; A375 melanoma cells and MiaPaCa2 cells were maintained in DMEM; and HSC3 and Calu6 cells were maintained in EMEM. H520 cells were transfected with a TweakR expression construct to generate

H520-TweakR. All cells were maintained and growth assays were done in the appropriate growth media containing fetal bovine serum (10%), unless otherwise indicated. All cell culture media and serum were purchased from Hyclone (Thermo Fisher Scientific).

The anti-TweakR monoclonal antibody 374.2 was generated by immunizing BALB/c mice with a synthetic peptide comprising amino acids 37 to 49 (SRGSSWSADLDKC) of the human TweakR coding region. Hybridomas were screened for TweakR specificity by Western blot and immunohistochemistry (IHC) on samples with known levels of TweakR expression.

The hybridoma expressing the anti-TweakR monoclonal antibody 19.2.1 was derived from spleens of BALB/c mice immunized *i.p.* with NIH-3T12 cells overexpressing TweakR. Hybridomas were identified as specific for TweakR by ELISA and flow cytometry using samples with known levels of TweakR expression.

19.2.1 was humanized to PDL192 using previously described methods (22). The human V region frameworks used as acceptors for 19.2.1 complementarity-determining regions were chosen based on sequence homology, and the computer programs ABMOD and ENCAD (23) were used to construct a molecular model of the variable regions. Amino acids in the humanized V regions predicted to have contact with complementarity-determining regions were substituted with the corresponding residues of 19.2.1.

The mouse IgG2a Fc region of 19.2.1 was replaced with mouse IgG1 Fc sequences to create 19.2.1×G1.

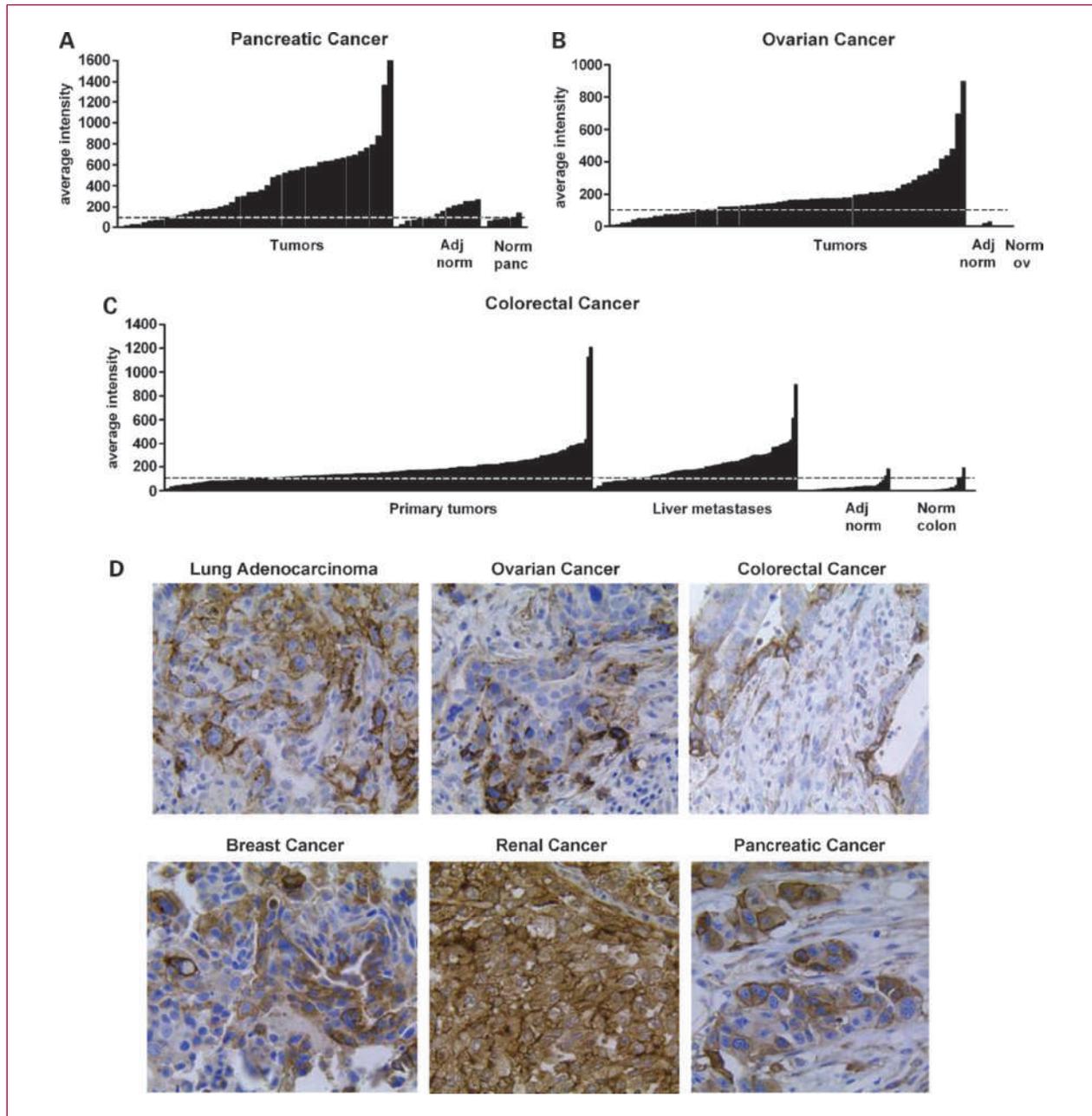
The hybridoma expressing the murine IgG1 control antibody (TIB191) was obtained from American Type Culture Collection. The human IgG1 control antibody, MSL109, is the fully human antibody to cytomegalovirus (24). Antibodies were purified using standard Protein G or Protein A affinity purification methods.

TWEAK was purchased from R&D Systems.

**Gene expression profiling.** Messenger RNA isolated from primary and metastatic cancers and from normal human adult tissues was hybridized to Eos Hu03, a customized Affymetrix gene chip containing ~59,000 probe sets representing 46,000 genes, EST clusters, and predicted exons as described previously (25).

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue samples or microarrays were either purchased from Asterand, Zoion Diagnostic, NELSG, or US Biomax, or obtained from the Cleveland Clinic. Five-micrometer sections were deparaffinized, and BORG antigen retrieval (Biocare Medical) was performed, followed by blocking with Background Sniper (Biocare Medical). Slides were incubated with the anti-TweakR antibody, 374.2 (10 µg/mL), or a control mouse IgG1 antibody, and antibody binding was detected using the MACH4 detection system (Biocare Medical). Slides were then incubated with diaminobenzidine (DAKO) for 5 min and were counterstained with hematoxylin. The slides were scored by a consulting board-certified pathologist.

**Cell growth assays.** Anchorage-dependent proliferation assays were done by seeding 100 to 500 cells in 96-well



**Fig. 1.** TweakR is overexpressed in human tumor samples. *A* to *C*, gene expression profiling. Messenger RNA isolated from primary and metastatic tumors, matched adjacent normal tissue, and cognate tissues from normal donors was converted to cRNA and hybridized to the Eos Hu03 gene chip. The Y axis on each graph represents the intensity of RNA hybridization to the TweakR probe set; the X axis indicates the sample identity. Dashed line, the estimated level of background (100 intensity units). TweakR expression above background was observed in 79% (37 of 47) of pancreatic tumors, 57% (8 of 14) of adjacent normal pancreatic tissue, and 17% (1 of 6) of normal pancreatic tissues (*A*); 73% (48 of 66) of ovarian cancers, 0% (0 of 4) of adjacent normal ovarian tissue, and 0% (0 of 2) of normal ovarian tissue (*B*); 75% (128 of 171) of primary colorectal tumors, 76% (62 of 82) of colorectal tumors that had metastasized to the liver, 5% (2 of 36) of adjacent normal colorectal tissues, and 7% (2 of 29) of normal colorectal tissues (*C*). *D*, TweakR protein was detected by IHC in formalin-fixed, paraffin-embedded tumor samples using a mouse anti-TweakR antibody, 374.2. Examples of TweakR staining in pancreatic, ovarian, colorectal, breast, lung, and renal cancers are shown.

plates in the presence of TWEAK or antibodies. Five to 13 d later, cell viability was measured by exposing cells to Alamar Blue for 2 h at 37°C. Fluorescence was emitted for excitation at 544 nm and emission was measured at

590 nm. Relative viability was calculated by dividing the fluorescence of TWEAK-treated or anti-TweakR antibody-treated samples with that of control antibody-treated cells. In most cases, assay results represent the readings

from six replicate wells. Each assay was done at least twice, with one representative assay shown.

Tumor cell lines were grown in soft agar by adding a suspension of 200 to 1,000 cells in 0.32% SeaPlaque agarose (Cambrex) in cell growth media to 96-well plates containing a solid base of 0.53% SeaPlaque agarose in cell growth media. The wells were then overlaid with cell growth media containing TWEAK or antibodies. Six to 14 d later, the colonies were stained with 0.001% crystal violet at 4°C for 24 h, after which they were imaged and counted using the Discovery-1 software (Molecular Devices). The total colony area was quantified for each well, and relative growth was calculated by dividing the mean total area per well of treated samples by that of the control samples. Each assay was done at least twice, with six replicate wells per assay; one representative assay is shown. Statistical differences between treatments were measured using a two-tailed unpaired *t* test. Differences were considered significant if  $P \leq 0.05$ .

**Caspase assay.** HSC3 cells were seeded into 96-well plates, after which TWEAK or antibodies were added and incubated for 5 d. Caspase-3/7 activation was assessed using the Caspase-Glo 3/7 Assay System (Promega Corp.) according to the manufacturer's instructions.

**Human tumor xenograft models.** Tumor cells, suspended in RPMI, were inoculated s.c. into the right flank of 6-wk-old severe combined immunodeficient mice (IcrTac:ICR-Prkdc<sup>scid</sup>, Taconic) at  $1 \times 10^7$  cells per mouse. Animals were randomized into groups when the mean tumor volume reached 80 to 130 mm<sup>3</sup>. Antibodies were administered i.p. Tumor volumes ( $L \times W \times H/2$ ) were measured on dosing days and twice per week thereafter. Groups were removed from the study when at least one tumor in the group reached the allowable limit (1,500 mm<sup>3</sup>). For all xenograft studies, tumor volumes were measured on each dosing day; the group means + SEM are displayed. The statistical significance of the differences between groups was determined by *t* test using the SAS statistical software. Mean tumor volumes between groups were considered significantly different if  $P \leq 0.05$ . All animal work was carried out under the NIH guidelines "Guide for the Care and Use of Laboratory Animals" using the Institutional Animal Care and Use Committee-approved protocols.

**ADCC assay.** <sup>51</sup>Cr-labeled H520 cells overexpressing TweakR were preincubated with antibodies at 4°C for 30 min, after which human peripheral blood mononuclear effector cells (PBMC) from healthy human donors or

**Table 1.** Summary of the immunostaining of TweakR across 1,655 samples from 22 solid tumor subtypes

Cancer		IHC score					≥2+	% pos.
		0	1+	2+	3+	4+		
NSCLC-adenocarcinoma	176	47	26	25	39	39	103/176	59
NSCLC-SCC	113	50	16	19	15	13	47/113	42
NSCLC-large cell	53	12	6	6	14	15	35/53	66
NSCLC-BAC	20	3	2	5	4	6	15/20	75
Pancreatic	93	19	18	30	18	8	56/93	60
Breast-ductal-primary	327	145	76	32	43	31	106/327	32
Breast-LN metastases	59	40	9	6	3	1	10/59	17
Breast-lobular	29	19	9	0	1	0	1/29	3
Renal	67	26	21	11	2	7	20/67	30
Head and neck	30	10	11	6	2	1	9/30	30
CRC-primary	150	71	47	24	3	5	32/150	21
CRC - liver metastases	16	2	6	7	1	0	8/16	50
Esophageal	49	22	14	5	3	5	13/49	27
Bladder	31	19	5	5	2	0	7/31	23
HCC	20	12	4	1	2	1	4/20	20
Ovarian	148	102	17	17	7	5	29/148	20
Melanoma	40	21	11	3	3	2	8/40	20
Gastric-adenocarcinoma	68	38	17	9	4	0	13/68	19
Sarcoma	36	24	5	2	1	4	7/36	19
Prostate	61	58	3	0	0	0	0/61	0
Small cell lung	32	32	0	0	0	0	0/32	0
Bone metastases	37	9	8	5	12	3	20/37	54
Total	1,655	781	331	218	179	146	543/1,655	33

NOTE: IHC score is based on the percentage of epithelial cells stained in the tumor portion of the sample: 0, <10% positive; 1+, 11% to 25% positive; 2+, 26% to 50% positive; 3+, 51% to 75% positive; 4+, >75% positive.

Abbreviations: SCC, squamous cell carcinoma; BAC, bronchioloalveolar carcinoma; LN, lymph node; CRC, colorectal; HCC, hepatocellular carcinoma.

isolated mouse splenocytes were added, for a final effector/target cell ratio of 50:1. After a 4-h incubation at 37°C, supernatant was collected and counted in a Gamma counter. Experiments with human effectors were done using PBMC from at least two different donors.

$$\text{Percent specific lysis} = \frac{(\text{cpm sample} - \text{cpm spontaneous release})}{(\text{cpm maximum release} - \text{cpm spontaneous release})}$$

cpm spontaneous release = cpm from cells incubated in the absence of antibody or effector cells.

cpm maximum release was obtained from cells solubilized in 1% Triton X-100.

## Results

**TweakR is overexpressed in multiple solid tumor types.** TweakR was identified in a microarray screen as overexpressed in multiple solid tumor types, compared with matched adjacent normal tissue or normal tissues from nondiseased donors. We observed high levels of TweakR mRNA levels in pancreatic (Fig. 1A) and ovarian cancers (Fig. 1B), as well as in both primary and metastatic colorectal cancers (Fig. 1C). TweakR mRNA overexpression was also observed in breast cancer, non-small cell lung cancer (NSCLC; both adenocarcinoma and squamous cell carcinoma), renal cancer, esophageal cancer, head and neck cancer, bladder cancer, uterine cancer, stomach cancer, cervical cancer, and melanoma, but not in hematologic malignancies (data not shown).

Human tumor samples were also screened for TweakR protein expression by IHC (Fig. 1D), and Table 1 summarizes the immunostaining of TweakR across 1,655 samples from 22 solid tumor subtypes. The IHC data corroborated the microarray analysis with TweakR protein being detected on a range of solid tumor types and overexpressed compared with the cognate normal tissue (data not shown). TweakR was detected in most tumor types screened, including pancreatic cancer (60%) and multiple subtypes of NSCLC (average of 55%). Additionally, as shown by microarray, TweakR protein was expressed in both primary colorectal cancer tumors (21%) as well as in liver metastases of colorectal cancer (50%). Moreover, TweakR was detected in 54% of bone metastases, derived from a variety of primary tumor tissues. Thus, TweakR mRNA and protein are overexpressed in a wide variety of solid tumors, including primary tumors and tumors that have metastasized to other sites.

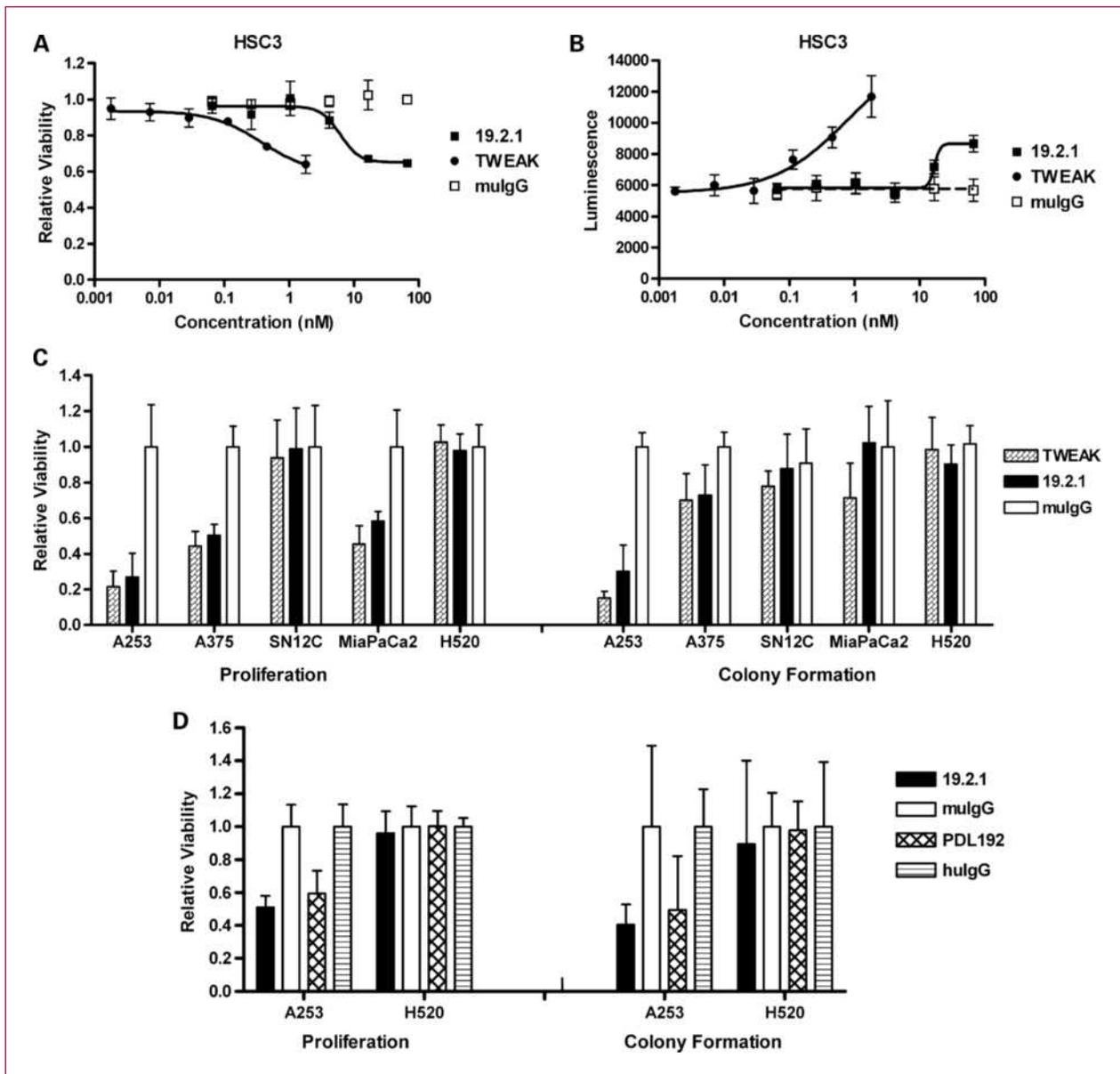
**Anti-TweakR antibodies inhibit the growth of multiple tumor cell lines in vitro.** Our findings show that TweakR is overexpressed in a variety of cancers, and data from other laboratories have shown that TWEAK treatment results in apoptosis of some cancer cell types, including HSC3 oral squamous carcinoma cells (16). These results, taken together, suggest that TweakR is a compelling target for antibody therapy in oncology. We generated a mouse monoclonal antibody, 19.2.1, which binds specifically to

TweakR, and tested 19.2.1 for its ability to kill HSC3 cells in a manner similar to TWEAK. When HSC3 cells were incubated with 19.2.1 or TWEAK, a dose-dependent decrease in cell viability was observed (Fig. 2A). However, on a molar basis, 19.2.1 was less potent than TWEAK ( $EC_{50} = 6.15 \text{ nmol/L}$  versus  $0.38 \text{ nmol/L}$ ). A control antibody had no effect on cell viability.

The mechanism of apoptosis in HSC3 cells by TWEAK occurs through the activation of caspase-3 and caspase-8 (16). We found that 19.2.1 treatment, similar to TWEAK, resulted in a dose-dependent increase in activated caspase-3/7 in HSC3 cells (Fig. 2B). However, as observed in the proliferation assay, 19.2.1 was found to be less potent than TWEAK, with both a reduced  $EC_{50}$  ( $16.83 \text{ nmol/L}$  versus  $0.78 \text{ nmol/L}$ ) and a reduced  $V_{\max}$  of caspase-3/7 activation. These findings indicate that although 19.2.1 stimulates signaling through TweakR, the level is reduced compared with that induced by the natural agonist ligand, TWEAK. 19.2.1 was also found not to interfere with TWEAK signaling, as treatment of cells with the combination of 19.2.1 and TWEAK exhibited similar levels of caspase-3/7 activation as cells treated with TWEAK alone (Supplementary Fig. S1).

We next determined if other tumor cell lines that express TweakR might be sensitive to TWEAK and/or 19.2.1 treatment. TWEAK and 19.2.1 were tested for their effect on cell viability in two different *in vitro* assays: an anchorage-dependent proliferation assay and an anchorage-independent colony formation assay. In the proliferation assay, both TWEAK and 19.2.1 inhibited the growth of several TweakR-expressing cell lines, including the A253 salivary gland cell line, the A375 melanoma line, and the MiaPaCa2 pancreatic cancer line (Fig. 2C). Neither 19.2.1 nor TWEAK inhibited the growth of either SN12C renal cancer cells, which express TweakR, or H520 lung cancer cells, which do not express TweakR. In the colony formation assay, treatment with TWEAK or 19.2.1 significantly inhibited the growth of the A253 and A375 cell lines (Fig. 2C). As expected, neither TWEAK nor 19.2.1 had an effect on colony growth of the nonexpressing cell line, H520.

**19.2.1 and its humanized counterpart, PDL192, have comparable in vitro characteristics.** The overexpression of TweakR in multiple tumor types and the ability of 19.2.1 to inhibit the growth of tumor cells suggested that this antibody might have potential as a cancer therapeutic. To create a suitable agent for therapeutic investigation, 19.2.1 was humanized to generate PDL192. The affinities of 19.2.1 and PDL192 for TweakR, as measured by surface plasmon resonance, were found to be similar (see Supplementary Fig. S2). The 19.2.1 antibody was found to have an association rate ( $k_a$ ) of  $2.5e^6 \pm 3.4e^5$  (1/Ms), a dissociation rate ( $k_d$ ) of  $1.7e^{-2} \pm 1.3e^5$  (1/s), and a resultant affinity ( $K_D$ ) of  $7.1 \pm 1.4 \text{ nmol/L}$ . Similar kinetics [ $k_a = 2.6e^6 \pm 7.5e^5$  (1/Ms),  $k_d = 1.5e^{-2} \pm 4.7e^{-3}$  (1/s)] and affinity ( $K_D = 5.5 \pm 0.3 \text{ nmol/L}$ ) were found for the PDL192 antibody. PDL192 was assessed for its ability to inhibit the growth of tumor cells in the proliferation and colony formation assays and was found to be comparable with 19.2.1 (Fig. 2D).



**Fig. 2.** 19.2.1 and PDL192, anti-TweakR antibodies, kill HSC3 cells by inducing caspase-3/7 activation, and inhibit the growth of multiple tumor cell lines. **A**, HSC3 oral squamous carcinoma cells were treated with TWEAK, 19.2.1, or a mouse IgG2a control antibody (*mulgG*) at various concentrations. Five days later, cell viability was assessed using Alamar blue. Points, mean; bars, SD. **B**, HSC3 cells were treated with TWEAK, 19.2.1, or a mouse IgG2a control antibody. Caspase-3/7 activity was measured 5 d later. **C**, TweakR-expressing A253, A375, SN12C, and MiaPaCa2 cell lines, and the TweakR-nonexpressing H520 cell line were incubated with TWEAK (100 ng/mL), 19.2.1 (10  $\mu$ g/mL), or a mouse IgG2a control antibody in proliferation and colony formation assays. In the proliferation assay, treatment of A253, A375, and MiaPaCa2 cells with TWEAK or 19.2.1 resulted in a statistically significant decrease in viability ( $P \leq 0.05$ ) compared with control-treated cells. In the colony formation assay, A253 and A375 growth was significantly inhibited by TWEAK or 19.2.1 ( $P \leq 0.05$ ) compared with control-treated cells. Columns, mean for each treatment group; bars, SD. **D**, A253 and H520 cells incubated with 19.2.1, PDL192, a mouse IgG2a control antibody, or a human IgG1 control antibody (*hulgG*; 10  $\mu$ g/mL) in proliferation and colony formation assays. Columns, mean for each treatment group; bars, SD.

Therefore, the humanization process retained both the binding and the signaling functions of 19.2.1.

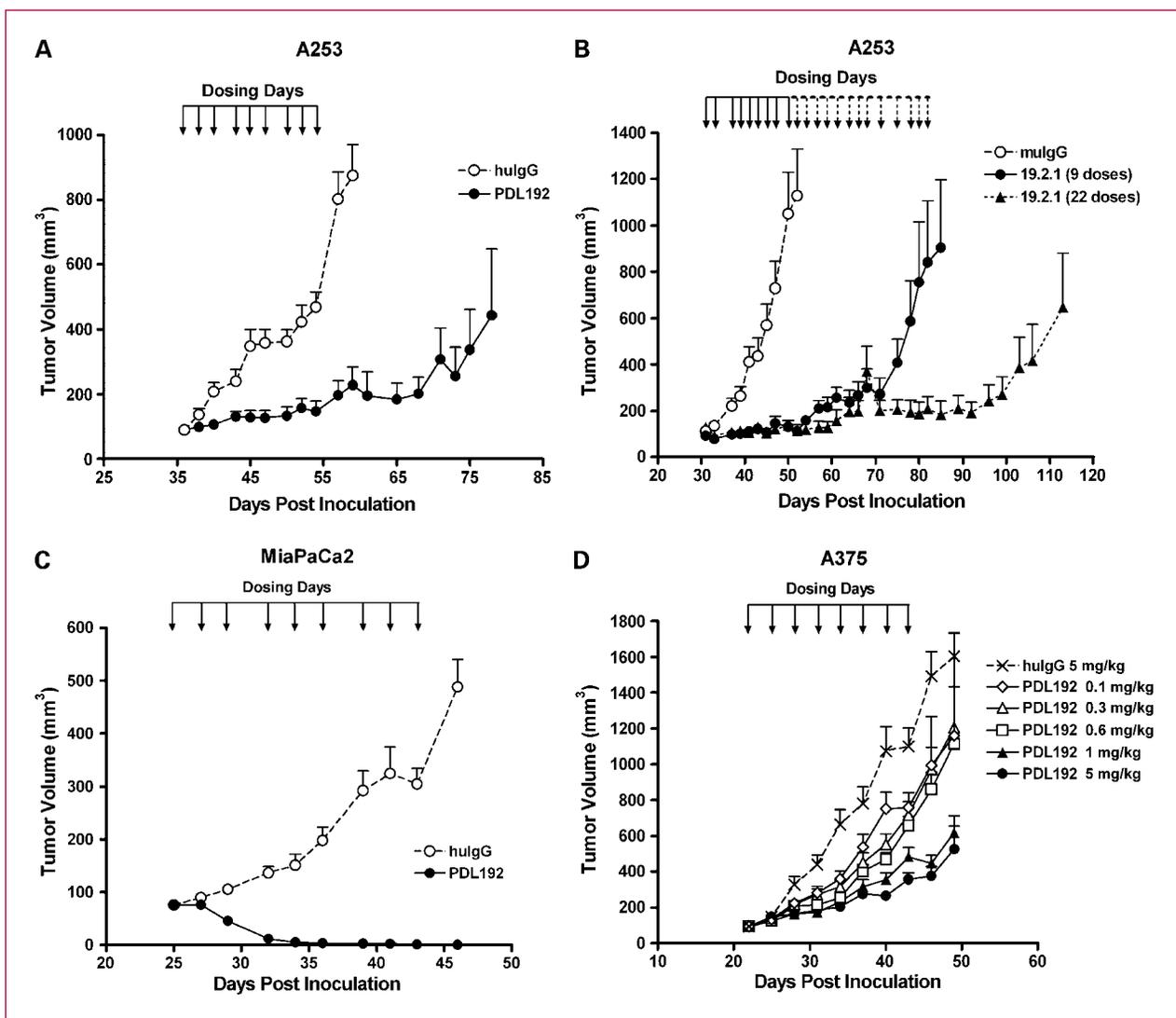
**PDL192 and 19.2.1 inhibit tumor growth in multiple xenograft models.** Given the ability of PDL192 and 19.2.1 to inhibit the growth of several TweakR-expressing cell lines *in vitro*, we next assessed their antitumor

activities in mouse xenograft models. In the A253 model, compared with control-treated tumors, PDL192 treatment significantly inhibited tumor growth during the dosing period and for several weeks thereafter (Fig. 3A). Similar activity was observed with 19.2.1 in the A253 model using two different dosing schedules

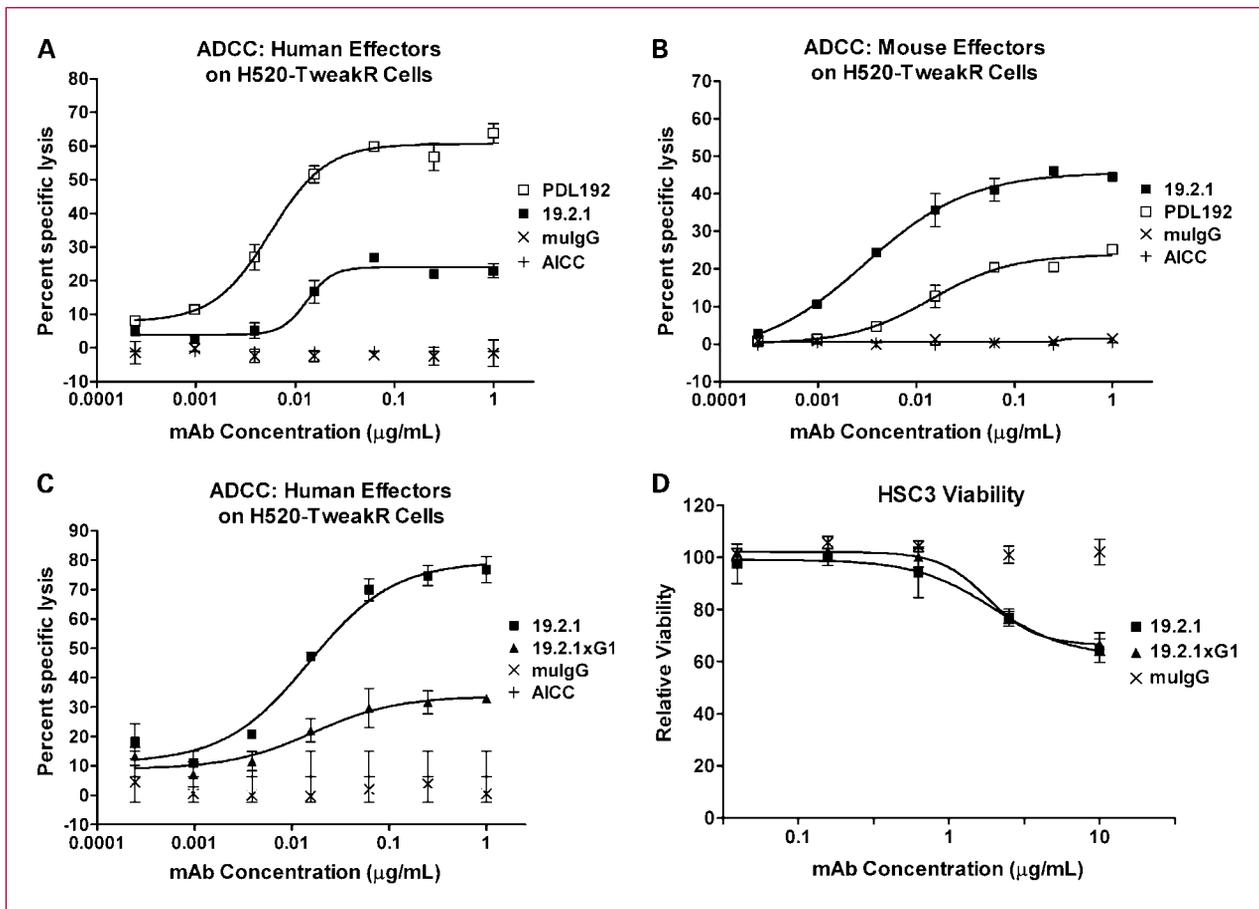
of 3-week (9 doses) or 7-week (22 doses) duration (Fig. 3B). In several xenograft models, including the MiaPaCa2 model, PDL192 treatment resulted in tumor regression (Fig. 3C). In this model, complete tumor eradication was observed in seven of eight animals treated with PDL192 (data not shown). Although PDL192 and 19.2.1 exhibited potent antitumor activity in the majority of xenograft models, in some models, such as the Calu6 model of lung cancer or the HCT116 model of colorectal cancer, the antibodies exhibited moderate or no growth-inhibitory activity, respectively (see Supplementary

Fig. S3). TweakR protein was expressed in all of these xenograft models, as determined by IHC (Supplementary Fig. S4).

We also performed a dose-ranging study using the A375 xenograft model to determine the range of PDL192-mediated antitumor activity and to correlate activity with the levels of PDL192 in the circulation (Fig. 3D). Compared with treatment with the control antibody, PDL192 treatment resulted in statistically significant inhibition of growth at every dose level tested. However, the strongest antitumor activity was observed



**Fig. 3.** PDL192 and 19.2.1 inhibit the growth of xenograft tumors. A and C, mice bearing established A253 (A) or MiaPaCa2 (C) tumors were treated with PDL192 or a human IgG1 control antibody (*hulgG*) thrice per week at 10 mg/kg. Dosing groups contained eight animals each. B, mice bearing established A253 tumors were treated with 19.2.1 at 5 mg/kg or a mouse IgG2a control antibody (*mulgG*) at 10 mg/kg thrice per week. Dosing groups contained 5 animals for each of the two 19.2.1 treatment groups and 10 animals for the mouse IgG2a control antibody control group. Tumor volumes were measured on each dosing day; points, mean; bars, SEM. D, mice bearing established A375 tumors were treated every 3 d with PDL192 at various dose levels or a human IgG1 control antibody at 5 mg/kg. Dosing groups contained 20 animals each. Differences in tumor volumes were significant ( $P \leq 0.05$ ) between the control antibody and all dose levels of PDL192 on days 28 to 46. Differences in tumor volumes between the 1 and 5 mg/kg dose levels were not statistically significant, nor were the tumors in the 0.1, 0.3, and 0.6 mg/kg dose groups significantly different from each other ( $P > 0.05$ ).



**Fig. 4.** PDL192 and 19.2.1 promote ADCC. A to C,  $^{51}\text{Cr}$  labeled H520 cells overexpressing TweakR were incubated with PDL192, 19.2.1, 19.2.1xG1, or a mouse IgG2a control antibody (*mulgG*) in the presence of freshly isolated human PBMC (A and C) or mouse splenocytes (B). Percent specific lysis was calculated as described in Materials and Methods. Points, mean; bars, SD. D, HSC3 cells were incubated with 19.2.1, 19.2.1xG1, or a mouse IgG2a control antibody for 5 d, after which cell viability was assessed using Alamar blue. Relative viability was calculated by dividing the fluorescence of treated cells with that of untreated cells. Points, mean; bars, SD.

at the two highest dose levels tested, 1 and 5 mg/kg every three days. In contrast, minimal-to-moderate activity was observed at the three lowest doses, 0.1, 0.3, and 0.6 mg/kg every 3 days. The serum concentration of PDL192 was also measured at various time points during the study to determine the concentration of antibody required for biological activity. The median pooled trough/peak concentrations were 0.001/0.087, 0.004/1.4, and 0.027/3.0 μg/mL at the 0.1, 0.3, and 0.6 mg/kg dose levels, respectively. At these lower dose levels, the area under the curve at the eighth dose were 4.7, 35.7, and 98.2 μg×h/mL. At higher dose levels, where maximal biological activity was observed, the median pooled trough/peak concentrations were 2.4/5.9 and 62/86 μg/mL, whereas the area under the curve at the eighth dose were 297 μg×h/mL and 5,530 μg×h/mL at the 1 and 5 mg/kg dose levels, respectively. Optimal antitumor activity was observed at similar dose levels in other xenograft models tested (see Supplementary Fig. S5). Thus, PDL192 exhibits potent antitumor activity at clinically achievable drug concentrations.

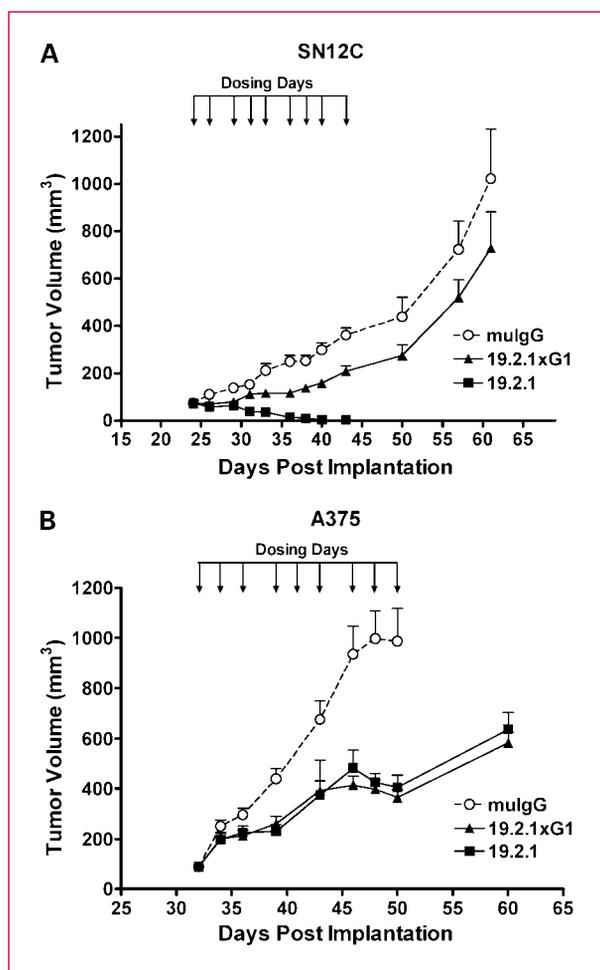
**PDL192 and 19.2.1 exhibit ADCC.** The ability of 19.2.1 and PDL192 to inhibit tumor cell growth directly is one desirable characteristic of an anticancer therapeutic. In addition, some therapeutic monoclonal antibodies in oncology may exert their effect through ADCC, which is mediated in part through the interaction of the Fc portion of the antibody and activating Fc receptors expressed on the surface of immune effector cells (26). PDL192 (human IgG1) and 19.2.1 (IgG2a) are both predicted to confer ADCC due to their isotypes (27, 28). We thus tested the ability of the antibodies to induce ADCC *in vitro* using both human and mouse effector cells. For this assay, we used a cell line overexpressing TweakR (H520-TweakR) as target cells to maximize the ability to detect ADCC when the antibodies and effectors are derived from different species. PDL192 and 19.2.1 killed the TweakR-expressing cells in a dose-dependent manner (Fig. 4A). As expected, PDL192 exhibited higher ADCC than 19.2.1 when human effectors were used; however, when mouse effectors were used, 19.2.1 had

higher ADCC (Fig. 4B). Neither PDL192 nor 19.2.1 exhibited complement-mediated cytotoxicity (data not shown).

**Both ADCC and direct tumor growth inhibition contribute to the antitumor activity of PDL192 and 19.2.1.** Given our findings that PDL192 and 19.2.1 exhibited both the ability to inhibit tumor cell growth directly and to induce ADCC *in vitro*, we next determined whether one or both of these functions was responsible for their antitumor activity *in vivo*. A mouse IgG1 version of 19.2.1 (19.2.1×G1) was generated, which was predicted to have

reduced ADCC based on the reduced affinity of the mouse IgG1 Fc to activating Fc receptors (27, 29). As expected, 19.2.1×G1 displayed reduced ADCC *in vitro*, compared with 19.2.1 (Fig. 4C). However, the two antibodies had similar abilities to bind TweakR-expressing cells (data not shown). In addition, 19.2.1×G1 killed HSC3 cells in a dose-dependent manner similar to 19.2.1 (Fig. 4D). These results suggest that only ADCC was affected in the 19.2.1×G1 chimera and that the direct binding and functional component of the antibody was left intact.

We compared the antitumor activities of 19.2.1 and 19.2.1×G1 in two different xenograft models. In the SN12C model of renal cancer, 19.2.1 treatment resulted in complete tumor eradication in all animals (Fig. 5A). In contrast, although 19.2.1×G1 treatment resulted in significant antitumor activity ( $P < 0.05$ ), its effect was significantly reduced compared with the IgG2a isoform ( $P < 0.001$ ; Fig. 5A). The modest tumor growth inhibition observed by 19.2.1×G1 in the SN12C model is consistent with the lower ADCC activity of this antibody (Fig. 4C). Thus, in the SN12C xenograft model, ADCC seems to be a major component of the antitumor activity of 19.2.1. Similar results were observed with an FcγR-binding mutant form of PDL192 (data not shown). In the A375 model of melanoma, compared with the isotype control antibody, 19.2.1 and 19.2.1×G1 exhibited equally potent antitumor activity ( $P < 0.001$ ; Fig. 5B). This suggests that ADCC does not contribute significantly to the antitumor activity of 19.2.1 in this model. These results indicate that the anti-TweakR antibodies mediate their antitumor effects both by signaling through TweakR, resulting in tumor cell growth inhibition, and by ADCC.



**Fig. 5.** Both ADCC and direct tumor growth inhibition contribute to antitumor activity *in vivo*. Mice bearing established SN12C (A) or A375 (B) tumors were dosed with 19.2.1, 19.2.1×G1, or a mouse IgG1 control antibody (*mulgG*) at 10 mg/kg thrice per week. Tumor volumes were measured on each dosing day; points, mean; bars, SEM. In the SN12C model, each group contained nine animals; differences in tumor volumes were significant ( $P \leq 0.05$ ) between the following: 19.2.1 versus mouse IgG1 control antibody on days 26 to 61, 19.2.1×G1 versus mouse IgG1 control antibody on days 33 to 50, and 19.2.1 versus 19.2.1×G1 on days 31 to 61. In the A375 model, each group contained eight animals; differences in tumor volumes were significant ( $P \leq 0.05$ ) only between the 19.2.1 and mouse IgG1 control antibody groups (days 43-50) and between the 19.2.1×G1 and *mulgG* groups (days 36-50).

## Discussion

TweakR is a member of the TNFR superfamily. Several TNFR members have been found to be attractive targets for the development of therapeutics in oncology due to their expression in cancers and functional activity in tumor cells. Based on previous work showing the expression of TweakR in cancers and the ability of the natural ligand, TWEAK, to induce apoptosis in some human tumor cell lines, we hypothesized that antibodies targeting TweakR might have therapeutic potential. Using gene expression profiling and IHC, we show that TweakR is overexpressed in a wide range of solid tumor types. Our studies extend previous findings (12, 17, 18, 20) and show that compared with the cognate normal tissues, both TweakR mRNA and protein are overexpressed in several primary and metastatic solid tumors.

We isolated and characterized two anti-TweakR antibodies, 19.2.1 and its humanized version PDL192, which mimic some of the biological activities of TWEAK. The antibodies do not interfere with normal TWEAK signaling, and the lack of antagonism of TWEAK may be due in part to the relative affinities of the different molecules to

TweakR. TWEAK has been reported to have an affinity of 0.8 to 2.3 nmol/L (30, 31), which is somewhat higher than the affinities of 19.2.1 and PDL192, which are 7.1 and 5.5 nmol/L, respectively. In contrast, anti-TweakR antibodies with affinities equal to or higher than TWEAK antagonize TWEAK signaling effectively (Supplementary Fig. S1). Differences in affinities, however, do not seem to contribute to the differences in signaling potencies between TWEAK and 19.2.1 or PDL192, as antibodies with higher affinities do not necessarily exhibit more potent signaling activity (Supplementary Fig. S1). The higher potency of TWEAK relative to 19.2.1 and PDL192 may be due in part to the conformations of the different molecules. Activation of signaling through TNFRs is thought to occur by receptor trimerization, induced by the binding of its trimer ligand (4). In contrast, antibodies are dimers and thus would likely be less efficient at forming trimers of their receptors. Further characterization of PDL192 and TWEAK binding and signaling through TweakR is currently under investigation.

PDL192 and 19.2.1 inhibited the growth of a wide range of human tumor cell lines, both *in vitro* as well as in multiple xenograft models. We have shown that 19.2.1 inhibited tumor growth *in vivo* through two distinct mechanisms: direct growth inhibition and ADCC. We also show that preclinical activity of PDL192 in xenograft models is observed at clinically achievable drug concentrations. These findings suggest that PDL192 has potential as a therapeutic agent in a range of solid tumor types.

TweakR expression is necessary for the sensitivity of cancer cells *in vitro* to 19.2.1, PDL192, or TWEAK, as cell lines that do not express TweakR were resistant to treatment with these agents. However, the sensitivity of cell lines to TWEAK, 19.2.1, and PDL192 did not correlate with the level of TweakR expressed in the different cell lines ( $R^2 = 0.09$  and  $0.03$  for PDL192 inhibition in the anchorage-dependent and anchorage-independent assays, respectively; data not shown). A lack of correlation between expression and sensitivity to functional biological therapeutics has been observed for several other receptor targets, including the TRAIL receptors. Various explanations have been proposed for the differential sensitivity of cell lines to TRAIL, including mutations in receptor cytoplasmic death domains (32, 33), or to the differential expression of decoy receptors, O-glycosylation enzymes, or the caspase inhibitor FLIP (32, 34, 35). A similar diversity of mechanisms may also be involved in the resistance of different cell lines to the anti-TweakR antibodies.

The ability of 19.2.1 and/or PDL192 to inhibit the growth of tumor cells *in vitro* did not always correlate with the extent of antitumor activity *in vivo*. This may be due to the differential contribution of ADCC in various xenograft models. SN12C cells *in vitro* are not sensitive to 19.2.1 treatment and thus would not be predicted to be sensitive to direct antitumor effects *in vivo*. Indeed, in this model, the antitumor activity of 19.2.1 is

largely dependent on ADCC. In contrast, A375 cell growth is inhibited by 19.2.1 *in vitro*, and the antitumor activity of 19.2.1 is largely ADCC-independent *in vivo*. Thus, PDL192 and 19.2.1 seem to be able to mediate their antitumor effects by signaling through TweakR, resulting in reduced tumor cell proliferation, as well as by ADCC.

ADCC seems to be a major mechanism for several monoclonal antibodies used to treat hematopoietic malignancies, including rituximab for the treatment of non-Hodgkin lymphoma (36, 37). For solid tumors, however, the contribution of ADCC to the therapeutic potential for a monoclonal antibody is less clear, given the potential challenge of antibody and immune effector cell penetration into solid tissues. In preclinical studies, both ADCC and direct antitumor activities have been attributed to antibodies directed against HER2, epidermal growth factor receptor (EGFR), and insulin-like growth factor IR (38–40); however, the relative contribution of ADCC to the clinical benefit of these antibodies is still under investigation.

Cetuximab and panitumumab are two anti-EGFR antibodies that are effective in the treatment of colorectal cancer. In preclinical studies, both antibodies block epidermal growth factor binding and EGFR phosphorylation (39, 41), but they differ in ADCC activity. Cetuximab exerts potent ADCC, and panitumumab exerts low levels of ADCC (42, 43). In patients, the anti-EGFR antibodies have been shown not to be effective in patients with activating *Kras* and *Braf* mutations, suggesting that blocking EGFR signaling is critical for their activity (44, 45). However, polymorphisms in FcγRIIIa and FcγRIIIb have recently been found to correlate with clinical response to cetuximab and irinotecan combination treatment (46). This result suggests that the clinical activity of cetuximab and panitumumab may be achieved both through the blocking EGFR signaling and through ADCC.

The potential contribution of ADCC to the activity of trastuzumab has also been explored both preclinically and in patients. Trastuzumab downregulates HER2 protein, inhibits downstream signaling pathways, and inhibits the growth of HER2 overexpressing breast cancer cell lines *in vitro* (47). In addition, trastuzumab induces potent ADCC *in vitro*, and its antitumor activity in some xenograft models depends largely on ADCC (48). Although HER2 overexpression seems to be required for clinical response to trastuzumab treatment in breast cancer patients, response to trastuzumab is correlated with several other parameters, including baseline numbers of circulating CD16<sup>+</sup> lymphocytes, the ADCC capacity of PBMCs isolated from patients before and after trastuzumab treatment, and the presence of tumor-associated lymphoid cells after trastuzumab treatment (49, 50). Thus, ADCC may contribute to the clinical efficacy of trastuzumab in breast cancer patients.

The ability of PDL192 to inhibit tumor growth through both direct cell growth inhibition and ADCC enhances the potential efficacy of this antibody and the likelihood of

benefit to patients. PDL192 is currently in a phase I clinical study in solid tumors.

### Disclosure of Potential Conflicts of Interest

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## Antibodies to TWEAK Receptor Inhibit Human Tumor Growth through Dual Mechanisms

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