Abstract

**Purpose:** Because of its high expression on various types of tumors and its restricted distribution in normal tissues, chondroitin sulfate proteoglycan-4 (CSPG4) represents an attractive target for the antibody-based therapy of several solid tumors. We tested whether T cells transduced with a CSPG4-specific chimeric antigen receptor (CAR) inhibited the growth of CSPG4-expressing tumor cells both *in vitro* and *in vivo*.

**Experimental Design:** We first independently validated by immunohistochemistry (IHC) the expression of CSPG4 in an extensive panel of tumor arrays and normal tissues as well as queried public gene expression profiling datasets of human tumors. We constructed a second-generation CSPG4-specific CAR also encoding the CD28 costimulatory endodomain (CAR.CSPG4). We then evaluated human T lymphocytes expressing this CAR for their *ex vivo* and *in vivo* antitumor activity against a broad panel of solid tumors.

**Results:** IHC showed that CSPG4 is highly expressed in melanoma, breast cancer, head and neck squamous cell carcinoma (HNSCC), and mesothelioma. In addition, in silico analysis of microarray expression data identified other important potential tumors expressing this target, including glioblastoma, clear cell renal carcinoma, and sarcomas. T lymphocytes genetically modified with a CSPG4-CAR controlled tumor growth *in vitro* and *in vivo* in NSG mice engrafted with human melanoma, HNSCC, and breast carcinoma cell lines.

**Conclusions:** CAR.CSPG4-redirected T cells should provide an effective treatment modality for a variety of solid tumors. *Clin Cancer Res;* 1–10. ©2013 AACR.

Introduction

Chondroitin sulfate proteoglycan-4 (CSPG4), also known as high-molecular-weight (HMW) melanoma-associated antigen and melanoma-associated chondroitin sulfate proteoglycan (MCSF), is a well-characterized cell surface proteoglycan first identified on human melanoma cells (1). Subsequent studies showed it to be highly expressed on other solid tumors such as mesothelioma (2) and triple negative breast carcinoma (3), all of which often show an aggressive clinical course. In contrast, CSPG4 has a restricted distribution in normal tissues (4).

CSPG4 participates in tumor migration, invasion, angiogenesis, and metastasis (5). It interacts with α4β1 integrins to directly modulate cell adhesion, motility, and metastasis as demonstrated by its ectopic expression in tumor cells (6). Given its restricted expression in normal tissues, high expression on various types of solid tumors and its role in the biology of tumor cells, CSPG4 is an attractive target for immunotherapy.

CSPG4 has been targeted with monoclonal antibodies (mAb) in models of melanoma, mesothelioma, and breast carcinoma, resulting in the inhibition of tumor growth and survival in addition to thwarting the metastatic capability of tumor cells (7). Recent advances in potentiating the anti-tumor effects of a specific mAb rely on coupling its antigen-binding specificity with the effector function and long-term persistence of T lymphocytes to generate specific chimeric antigen receptors (CAR) (8–10). These molecules are obtained by fusing the extracellular antigen-binding domain of the mAb with the intracellular signaling domains derived from the CD3-ζ chain of the T-cell receptor, in tandem to costimulatory endodomains to support survival and proliferative signals (11–13). Because CAR-modified T cells function independently of a patient’s MHC and can...
Translational Relevance
Adoptive transfer of CAR-redirected T lymphocytes represents a promising therapy for patients with lymphoid malignancies. Here we extend the applicability of this strategy to a broad array of solid tumors by targeting the CSPG4 antigen; this antigen is overexpressed by numerous tumor types while having negligible expression in normal tissues. Our study provides preclinical evidence that CSPG4-directed T cells can control the growth of human melanoma, head and neck squamous cell carcinoma, and breast cancer both ex vivo and in vivo in xenograft models.

Materials and Methods
Cell lines
The previously described SENMA, CLB, and P1143 tumor cell lines were generated in our laboratory from melanoma biopsies (18). MDA-MB-231 was originally obtained from American Type Culture Collection (ATCC) and authenticated by the analysis of short tandem repeat sequences performed at MD Anderson Cancer Center (Houston, TX). UACC-812, PCI-30, and PHI cell lines were provided by Dr. Ferrone and these cells, when maintained in culture for several passages, retained the same phenotypic expression of CSPG4 as the early cell passages. Previously described melanoma cell lines PLAODE, NE-18732, NE-18588, NE-8959, NE-4405, and NE-371952 were only used to confirm the expression of CSPG4 in a broad array of melanoma cell lines (18). All these cells, including SENMA, CLB, and P1143, when maintained in culture for several passages, retained the same phenotypic expression of CSPG4 as the early cell passages. SENMA, CLB, UACC-812, MDA-MB-231, and PCI-30 cell lines were cultured in Dulbecco’s Modified Eagle Medium (Invitrogen) or RPMI 1640 (P1143, UACC-812, and PHI; Cambrex) medium supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Thermo Fisher Scientific Inc.), 200 IU/mL penicillin, 200 mg/mL streptomycin (Invitrogen), and 2 mmol/L GlutaMAX (Invitrogen) at 37°C in a 5% CO₂ atmosphere. Tumor cell lines were transduced with a γ retroviral vector-encoding eGFP to obtain GFP⁺ tumor cells (>98% GFP⁺). Primary epithelial cells from normal small airway, kidney, and prostate were purchased from ATCC and kept in culture according to ATCC recommendation.

Tissue microarrays and immunohistochemistry
Antigen retrieval was performed by placing the samples in 1× Dako Citrate Buffer followed by incubation at 90°C in a pressure cooker for 45 minutes. After blocking with normal goat serum diluted in Tris-buffered saline, samples were incubated with the CSPG4 mAb [Abcam; anti-NG2 antibody (LHM 2), ref. no. ab104535: 1:300 dilution] either overnight at 4°C or at room temperature for 1 hour. Detection of CSPG4 was then assessed using the VECTASTAIN ABC Kit (Vector Laboratories, Inc., ref. no. PK-4001) following the manufacturer’s protocol. Tissue arrays were obtained from Cybrdi Inc. for breast cancer (CC08-10-001) and HNSCC (CC34-01-001), whereas melanoma (ME2082b), neuroblastoma (MC602), mesothelioma (T392), and normal tissue (FDA 808b) arrays were obtained from US Biomax, Inc. Each array contained a range of 4 to 192 cores of tumor or 5 to 18 cores of normal tissue samples in duplicate, triplicate, or quadruplicate in the case of mesothelioma. Expression of CSPG4 in tumor cells was scored in blind fashion by the pathologist Dr. M. Ittmann based on both intensity (0–3+) and extent of staining (1–3+). A multiplicative staining score was calculated by multiplying the intensity and extent scores to yield scores on a 10-point scale from 0 to 9 (19). In microarrays with multiple cores per patient, the individual scores were averaged to obtain a final score. In some cores, tumor was not identified because of artifacts. In the vast majority of cases, immunohistochemistry (IHC) showed uniform staining (3+) within a given core and in most cases cores from different patients were highly concordant. Areas of necrosis or acellular keratin were not included in the scoring. Cases were divided based on staining scores into 3 groups: negative/weak (0–3), moderate (4–6), or strong (7–9).

Generation of the CSPG4-specific CAR and transduction of T lymphocytes
The hybridoma 763.74 was generated from a BALB/c mouse immunized with cultured human melanoma cells (20). The scFv 763.74 was isolated from the hybridoma (21) and then cloned in frame with the human IgG1-CH₂CH₃ domains, the CD28 costimulatory endodomain, and the CD3ζ chain into the SFG retroviral backbone (CAR. CSPG4), as previously described (22, 23). The control CAR specific for the CD19 antigen (CAR.CD19) has been previously described (13). Transient retroviral supernatant was generated by cotransfection of 293T cells with the RD114 envelope (RDF plasmid), the MoMLV gag-pol (PegPam3-e plasmid), and the retroviral vector, as previously described (23). For the generation of CAR-T cells, peripheral blood mononuclear cells (PBMC) were isolated from buffy coat preparations (Gulf Coast Regional Blood Center, Houston, TX) using Ficoll-Paque (Amersham Biosciences). PBMCs were activated with OKT3 and CD28 (BD Biosciences).
PharMingen) mAbs, transduced with the retroviral supernatant by day 3 of culture and then expanded in complete medium containing 45% RPMI 1640 and 45% Click’s medium (Irvine Scientific) supplemented with 10% FCS, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L GlutaMAX. Cells were fed with interleukin (IL)-2 (50 U/mL; PeproTech) twice a week for 2 weeks (23).

**Flow cytometry**

Conjugated CD3, CD4, CD8, CD45RO, CD62L, and CCR7 mAbs (BD Biosciences) were used to identify T lymphocytes, whereas the CSPG4 mAb (Miltenyi-Biotech Inc.) was used to label tumor cells. CAR expression in T lymphocytes was assessed using an antibody recognizing the human IgG1-CH2CH3 fragment (Jackson ImmunoResearch) (23). Analyses were performed on a FACsCaliber flow cytometer using the BDFACs CellQuestPro software (BD Biosciences).

**Cytotoxicity and coculture assays**

The cytotoxic activity of control and CAR.CSPG4+ T lymphocytes was determined using a standard 51Cr release assay at different effector-to-target (E:T; 40:1, 20:1, 10:1, and 5:1) ratios using a γ counter (Perkin-Elmer) (23). For the coculture experiments, control and CAR.CSPG4+ T lymphocytes were plated at 1 × 10⁶ cells/well in 24-well plates at different E:T ratios according to the kinetic growth of each tumor cell line. Tumor cell lines with a slow kinetic growth were plated at higher tumor ratio (T cells:tumor cells 3:1) compared with tumor cell lines with a fast kinetic growth (T cells:tumor cells 5:1). Supernatant was collected at 24 hours of culture to measure IFN-γ and IL-2 release using specific ELISAs (R&D Systems). Following 72 hours of culture at 37°C, adherent tumor cells and T cells were collected and residual tumor cells and T cells assessed by fluorescence-activated cell-sorting (FACS) analysis based on GFP and CD3 expression, respectively.

**Carboxyfluorescein diacetate succinimidyl ester assay**

One week posttransduction, control and CAR.CSPG4+ T lymphocytes were labeled with 1.5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and plated with irradiated tumor target (SENMA) at an E:T ratio of 5:1. CFSE dilution was measured on CD4+ and CD8+ cells by flow cytometry by day 4 of coculture.

**Xenogenic mouse models**

*In vivo* experiments were performed in accordance with Baylor College of Medicine’s Animal Husbandry guidelines. Antitumor activity of control and CAR.CSPG4+ T lymphocytes was evaluated using NOG/SCIDγc−/− (NSG) mice (Jackson Lab, Bar Harbor, ME) engrafted with tumor cells. Eight- to nine-week-old mice were subcutaneously injected with 0.5 × 10⁶ SENMA, 3 × 10⁶ UACC-812, or 3 × 10⁶ PCI-30 cells resuspended in Matrigel (BD Biosciences). On days 4, 6, and 8 following tumor cell injection, 1 × 10⁷ control or CAR.CSPG4+ T lymphocytes were injected intravenously by tail vein. In summary, for the melanoma xenograft model 3 different preparations of CAR.CSPG4 T cells were generated from 3 different donors. Three doses, given 2 days apart, of 1 × 10⁷ were infused intravenously into 5 mice per group. In total, 15 animals were treated for each group. The endpoint of the experiment was to examine differences in tumor volume up to day 30 after tumor injection. For the xenograft models of breast cancer and HNSCC, 2 different preparations of T cells generated from 2 different donors were used. Two doses, given 2 days apart, of 1 × 10⁷ were infused intravenously into 5 mice per group. In total, 10 animals were treated per group. In all tumor models, mice were sacrificed at 30 days or in accordance with our institution’s guidelines for the handling of sick animals. Weekly manual caliper measurements were performed posttreatment to evaluate tumor growth. Tumor volume was calculated using the modified ellipsoidal formula: tumor volume (mm³) = (width)² × length/2.

**Statistical analysis**

*In vitro* data are presented as mean ± SD and a paired Student t test was used to determine statistical significance. The *in vivo* data are presented as mean ± SEM and a paired Student t test was used to identify significant differences between CAR- and control-treated groups. Public gene expression profiling datasets of human tumors were queried for CSPG4, including data from The Cancer Genome Atlas (TCGA Data Portal; http://tcga-data.nci.nih.gov/tcga), Bittner multicancer dataset (unpublished, from http://www.oncomine.org/www.oncomine.org; refs. 26, 27) and Gene-Atlas U133A dataset (http://niogps.org).

**Results**

**CSPG4 is expressed on a variety of solid tumors**

As CSPG4 was originally identified as a melanoma-associated antigen, we first independently validated its expression using IHC in a melanoma tissue array containing multiple primary cutaneous and visceral melanomas and metastatic lesions. Examples of either strong or negative/low staining are shown in Fig. 1A. Consistent expression of the antigen was documented in all types of lesions, regardless of their primary or metastatic origin, or their cutaneous and visceral source. We therefore analyzed melanomas as a whole group. Overall, 59% of melanomas showed strong staining and 25% displayed moderate staining (Fig. 1B). We then extended the analysis to include multiple samples of additional solid tumors including breast cancer, HNSCC, neuroblastoma, and mesothelioma. For the breast cancer array, staining was seen in invasive ductal and lobular carcinomas as well as in the small number of Paget disease and ductal *in situ* carcinoma cases present on the array. Staining for representative invasive ductal carcinomas (either strong or negative/low) are shown in Fig. 1A and summarized in Fig. 1B, whereas other lesions were not sufficient in number for a comparative analysis. Staining for CSPG4 in invasive ductal carcinoma was remarkable with 77% of cases showing moderate or strong staining. HNSCC most predominantly (50%) expressed moderate staining for CSPG4, with only 20% showing strong staining. Although neuroblastoma exhibited the weakest overall...
Figure 1. CSPG4 expression in primary solid tumors and tumor-derived cell lines. A, representative IHC and scoring of analyzed solid tumor tissue arrays. Representative melanoma and breast carcinoma samples are shown at ×200 magnification. B, scoring summary of a panel of solid tumors that includes melanoma, breast carcinoma (Breast CA), HNSCC, neuroblastoma (NeuroB), and mesothelioma (MesoT). C, CSPG4 mRNA expression by TCGA in a variety of solid tumors. Box plots show median, 25%–75% range, 5%–95% range, and minimum/maximum. D, CSPG4 mRNA expression analysis, comparing tumor versus corresponding normal tissues, for astrocytoma/glioblastoma (GBM), HNSCC, clear cell renal carcinoma, and melanoma. Indicated P values were calculated by t test. E, CSPG4 expression in the indicated array of melanoma cell lines as assessed by flow cytometry (FACS). F, FACS analysis of CSPG4 expression in the selected mesothelioma (MILL and PHI), HNSCC (PCI-30), and breast cancer–derived (MDA-MB-231 and UACC-812) cells lines. Dotted and bold lines indicate isotype and CSPG4 mAbs, respectively.
staining, there was still a fraction of cases with moderate to strong expression. Finally, despite the limited number of mesotheliomas, these lesions all consistently expressed CSPG4. We concluded that, at the protein level, all these malignancies exhibited variable but in most cases significant expression of CSPG4. To examine the expression of CSPG4 in a broader array of tumors, we examined publicly available databases for mRNA expression data. As shown in Fig. 1C, examination of TCGA datasets showed overexpression of CSPG4 transcripts in melanoma and in glioma as anticipated based on the previously reported expression of the protein (28). Concordant with our protein expression data, CSPG4 mRNA expression was increased in HNSCC. We also found increased mRNA expression in clear cell renal carcinomas by in silico analysis, and overall, despite some intratumor variability, significant increased mRNA levels in all these tumor types relative to the corresponding normal tissues (Fig. 1D). Examination of the large Bittner multicancer dataset (www.oncomine.org) confirmed high CSPG4 mRNA expression in melanoma, clear cell renal carcinoma, HNSCC, multiple sarcoma types (chondrosarcoma, leiomyosarcoma, liposarcoma), gastrointestinal stromal tumors, skin, and vulvar squamous cell carcinomas (Supplementary Fig. S1). Of note, several sarcoma cell lines have been previously reported to express CSPG4 protein (3). A number of other common malignancies such as colorectal, ovarian, and endometrial carcinoma did not show increased CSPG4 transcripts, consistent with the mRNA expression from the TCGA datasets. CSPG4 protein expression in an array of normal tissues was negative (Supplementary Fig. S2). In addition to the normal tissues represented in Supplementary Fig. S2, we evaluated CSPG4 expression on a total of 33 different types of tissues, all of which were negative. Using the public Novartis GeneAtlas (http://biogps.org) and TCGA databases, CSPG4 mRNA expression was observed in a number of normal tissues. However, the levels of expression are remarkably lower than those of cancer tissues (Supplementary Fig. S3).

We next examined CSPG4 expression in a series of cell lines from a variety of tumor types analyzed above. Expression of CSPG4 was detected in 8 of the 9 melanoma cell lines screened (Fig. 1E). Importantly, CSPG4 was detected on tumor cell lines representative of the above-identified solid tumors such as mesothelioma (MILL and PHI), HNSCC (PCI-30), and breast cancer [MDA-MB-231 (adenocarcinoma) and UACC-812 (ductal carcinoma); Fig. 1F], all consistent with our analysis of human tumor samples.

**T lymphocytes expressing the CSPG4-specific CAR are cytotoxic against CSPG4**-tumor cell lines but not against primary normal tissues

To target CSPG4 tumors we generated a CSPG4-specific CAR containing the CD28 costimulatory endodomain (CAR.CSPG4; Fig. 2A). T lymphocytes from 4 healthy donors were engineered to express the CAR.CSPG4 using a γ retroviral vector. Transduction efficiency was 80% ± 3%, and both CD4 and CD8 T cells stably expressed the CAR (26% ± 9% and 51% ± 16%, respectively), as assessed by phenotypic analysis by day 7 of culture (Fig. 2B). The majority of CAR.CSPG4 T cells were CD45RO (76% ± 7%) and a fraction retained CD62L expression (51% ± 7%) and CCR7 (13% ± 2%), indicating that they were mainly composed of effector-memory T cells (Fig. 2C). The expression of CAR.CSPG4 by T cells was comparable to that obtained with a previously described CD19-specific CAR (CAR.CD19) (Supplementary Fig. S4; ref. 29), which was used as an irrelevant-CAR control population.

Cytotoxic activity of control and CAR.CSPG4 T cells, after 1 to 2 weeks of culture, was assessed against K562, to measure natural killer cell-mediated activity, and against the melanoma-derived cells lines P1143 (as CSPG4 target) and SENMA (as CSPG4 target; Fig. 1E) at various E:T ratios (Fig. 3A). CAR.CSPG4 but not control T lymphocytes significantly lysed the CSPG4 target (59% ± 5% vs. 11% ± 8% at 20:1 ratio; P < 0.01), whereas both CAR.CSPG4 and control T cells showed minimal activity against K562 (12% ± 9% vs. 13% ± 11%) and the CSPG4 target (<10% in both cases). The antitumor activity of CAR.CSPG4 T lymphocytes was also evaluated in a 72-hour coculture assay (Fig. 3B and C). CAR.CSPG4 and control T lymphocytes were cocultured with GFP-expressing tumor cell lines at an E:T ratio ranging from 5:1 to 3:1 according to the kinetic growth of each cell line. CAR.CSPG4 T cells significantly controlled the growth of all CSPG4 cell lines tested: SENMA (residual tumor cells = 0.1% ± 0.06%), CLB (0.1% ± 0.1%), UACC-812 (6% ± 6%), MILL (3% ± 5%), MDA-MB-231 (3% ± 3%), PH (4% ± 3%), and PCI-30 (0.5% ± 0.5%), but not of the CSPG4 target P1143 (residual tumor cells 38% ± 10%). As expected, all tumor cell lines tested rapidly grow in the presence of control T lymphocytes (residual tumor cells for: SENMA = 62% ± 3%, CLB = 70% ± 6%, UACC-812 = 47% ± 15%, MILL = 50% ± 8%, MDA-MB-231 = 42% ± 11%, PH = 29% ± 6%, PCI-30 = 17% ± 3%, and P1143 = 45% ± 10%). In all cases, the effects of CAR.CSPG4 T cells were significantly greater than those of control T cells (from P < 0.05 to P < 0.001). T cells expressing the control CAR.CD19 showed cytotoxic activity neither against CSPG4 nor CSPG4 targets (Supplementary Fig. S4). As illustrated in Fig. 3, commercially available primary normal epithelial cell lines (small airway, kidney, and prostate) derived from tissues found to express low levels of CSPG4 mRNA (Supplementary Fig. S3) did not express detectable levels of the protein by flow cytometry (Fig. 3D), and were not lysed by CAR.CSPG4 T cells when tested in 51Cr release assays (Fig. 3E).

**CAR.CSPG4**-T lymphocytes secrete Th1 cytokines and proliferate in response to CSPG4 tumors

Because CAR.CSPG4 contains the CD28 costimulatory endodomain, we studied CAR.CSPG4 T lymphocyte proliferation in response to CSPG4 tumor cells using a CFSE dilution assay. When CFSE-labeled control and CAR.CSPG4 T cells were cultured with irradiated SENMA tumor cells for 96 hours, a significant CFSE dilution occurred for CAR.CSPG4 T cells, with both CD4 and CD8 T cells proliferating at a higher percentage (66% ± 12% and
68% ± 8%, respectively) compared with control CD4 and CD8 T cells (8% ± 7% and 14% ± 10%, respectively; \( P < 0.05 \) and \( P < 0.01 \), respectively; Fig. 4A and B). T cells transduced with the control CAR.CD19 also containing the CD28 endodomain did not show significant proliferation in response to CSPG4 targets (Supplementary Fig. S4). We also evaluated whether the inclusion of a “late” costimulatory endodomain, such as 4-1BB, in addition to CD28 provided these T cells with additional proliferative and cytotoxic activity, but found no further benefits (Supplementary Fig. S5).

We finally quantified the IL-2 and IFN-\( \gamma \) cytokines released in response to the antigen, by coculturing control and CAR.CSPG4 or CAR.CSPG4+ tumor cells. As expected, CAR.CSPG4+ T lymphocytes secreted significantly more IL-2 than control T cells only in the presence of CSPG4+ tumor cells (Fig. 4C). A positive trend, although not statistically significant, was observed when CAR.CSPG4+ T lymphocytes were cultured with the CSPG4+ tumor cell lines PHI, MILL, UACC-812, and MDA-MB-231 (data not shown). Similarly IFN-\( \gamma \) production was significantly higher than control T cells when CAR.CSPG4+ T lymphocytes were cultured with CSPG4+ tumor cells (Fig. 4D).

**CAR.CSPG4+ T lymphocytes control the growth of human melanoma, HNSCC, and breast carcinoma cells engrafted in immunodeficient mice**

To assess the *in vivo* relevance of our *in vitro* results, we engrafted NSG mice subcutaneously with cell lines derived from representative melanoma tumor (SENMA), HNSCC (PCI-30), or breast carcinoma (UACC-812). Four to seven days later (depending on the kinetics of the tumor growth), mice were infused via tail vein injection with either control or CAR.CSPG4+ T lymphocytes, and tumor growth quantified by sequential tumor volume measurements. In all 3 models, CAR.CSPG4+ T lymphocytes inhibited tumor growth significantly better than control T lymphocytes (Fig. 5). By day 30, melanoma tumors reached a volume of 879 ± 124 mm\(^3\) in mice receiving CAR.CSPG4+ T lymphocytes versus 8,359 ± 958 mm\(^3\) in mice receiving...
control T cells ($P < 0.01$; Fig. 5A), and this corresponded to improved overall survival (Supplementary Fig. S6). Although HNSCC and breast carcinoma tumors were not as aggressive as melanoma in vivo, we observed that in both models CAR.CSPG4$^+$ T lymphocytes controlled tumor growth. By day 30, the size of HNSCC tumors was $19 \pm 10$ mm$^3$ in treated mice versus $190 \pm 75$ mm$^3$ in control mice ($P < 0.001$; Fig. 5B) and the size of breast carcinoma tumors was $28 \pm 13$ mm$^3$ in treated mice versus $166 \pm 64$ mm$^3$ in control mice ($P < 0.001$; Fig. 5C).

**Discussion**

The involvement in several signaling pathways associated with cell proliferation, survival, migration, and suggested high expression in various types of cancers highlight the critical role that CSPG4 has in promoting tumor growth and simultaneously make it an attractive target for immuno-therapy. By IHC, we independently validated CSPG4 protein expression in several solid tumors with poor prognosis, such as melanoma, breast cancer, mesothelioma, and HNSCC. In *silico* analysis of microarray expression data confirmed overexpression of CSPG4 in tumors that we validated by IHC as compared with normal tissues, and also disclosed CSPG4 overexpression in other important malignancies including glioblastoma, clear cell renal carcinoma, and sarcomas suggesting that targeting this antigen may have a major impact on a broad array of solid tumors.

Because CSPG4-specific mAbs can control tumor growth of CSPG4$^+$ tumor cells, in both melanoma and breast cancer tumor models (7), we proposed to improve the therapeutic benefits of this antibody-based approach by generating a CAR that targets the CSPG4 molecule. In contrast to mAb-based therapy, CAR-T cells should produce...
long-lasting effects, as engineered T cells can expand at the tumor site upon antigen stimulation if an appropriate costimulatory endodomain, derived from CD28, CD137, or CD134, is incorporated within the CAR (13, 30). In contrast to a previous report (21), we found that the CSPG4-specific CAR obtained from the same 763.74 single chain has potent antitumor activity. We traced these striking differences to 2 critical components we have introduced in our construct. First, the scFv in our CAR is coupled with the CD3-ζ endodomain of the TCR rather than the FcεRI-gamma chain, which is known to promote a much weaker and less durable signaling (31, 32). Second, we incorporated the CD28 costimulatory endodomain within the CAR, to accomplish sustained IL-2 production and proliferation in response to CSPG4+ tumor cells, thus recapitulating previous observations for other CAR molecules (13). Of note, the inclusion of a second costimulatory endodomain derived from CD137 did not further improve the function of our CAR in vitro, supporting the concept that there is no single optimal configuration that is applicable to all CAR molecules, but that CAR receptor optimization remains largely empirical and required for each molecule.

The most critical improvement in the field by our work is the applicability of CAR.CSPG4+ T cells not only to target melanoma (17), but more broadly to other solid tumors generally characterized by poor prognosis with conventional treatments such as breast carcinoma, HNSCC, and mesothelioma. We demonstrated that CAR.CSPG4+ T cells produce IFN-γ and promote tumor elimination not only when challenged with tumor cells with high CSPG4 expression but also with tumor cell lines characterized by moderate/low CSPG4 expression, such as the breast carcinoma tumor cell lines UACC-812 and MBA-MB-231. This further supports the advantages of antibody specificity coupled with the T-cell effector function, as mastered by CAR-modified T cells, which can target tumors neglected by naked corresponding antibodies because of the suboptimal expression of the targeted antigen (33, 34).

Antitumor effects mediated by CAR.CSPG4+ T cells significantly limit tumor growth in xenograft mouse models of melanoma, HNSCC, and breast carcinoma, strongly validating our in vitro findings. The lack of sustained and complete tumor eradication in these models was not caused by selection of CSPG4-negative tumor cells, as harvested...
tumors retained the expression of the antigen (Supplementary Fig S6), but conversely are likely to be attributed to an intrinsic limitation of the models, as T cells do not persist long term in these immunodeficient mice (Supplementary Fig S6).

To fully translate this approach, the differential expression of CSPG4 in tumor cells versus normal tissues needs to be ensured to limit potential toxicities (35, 36). We found the expression of CSPG4 absent or negligible in normal tissue arrays as assessed by IHC. The analysis of publically available datasets indicates that there is some level of CSPG4 mRNA expression in several normal tissues. However, when we compared normal tissues with cancer tissues, the cancers show consistent and dramatically higher expression of CSPG4 at mRNA levels. Our in vitro analyses illustrate that primary epithelial cells derived from some of these tissues do not express significant amount of the protein and are not targeted by CAR.CSPG4⁺ T cells. Even though the tissue screening, bioinformatics analysis, and lack of toxicity by in vitro experiments support the relevance of CSPG4 as a targetable antigen in cancer patients, we cannot fully exclude that the low levels of mRNA in normal tissues, as reported in public datasets, may promote sufficient protein expression in specific physiologic conditions to become a target for CSPG4-specific CAR-T cells. The clinical translation of this approach may thus benefit from the inclusion of a suicide gene within the vector cassette, to allow the rapid elimination of CAR-modified T cells in case of undesired toxicity (29, 37).

In summary, we provide ample data to support the use of CAR.CSPG4⁺ T cells to treat a broad range of solid CSPG4⁺ tumors for which the prognosis remains poor with conventional treatments. The combination of this approach with other biologic agents may further increase their activity and thus clinical benefits.

Disclosure of Potential Conflicts of Interest
Center for Cell and Gene Therapy has a collaborative research agreement with Celgene and bluebird bio.

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T Lymphocytes Redirected against the Chondroitin Sulfate Proteoglycan-4 Control the Growth of Multiple Solid Tumors both In Vitro and In Vivo

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