Supplementary Data

Methods

Construction of fully human scFv-Fc genes. The gene encoding scFv C21 was amplified by PCR from the original phagemid vector pHEN1 (from the phage scFv display library) as an EcoRI/BglII fragment using primers with the following sequences: EcoR1 Forward (5'-AAAAGAATTCGATGCAGGTGCAGCTGGTG-3') and BglII Reverse (5'-TTTTAGATCTACCTAGGACGGTCAGCTT-3'). The PCR products were purified utilizing QIAquick PCR Purification Kit (QIAGEN) and digested with the restriction enzymes EcoRI and BglII (New England BioLabs). The resulting fragments were cloned into the pFUSE-IgG2-hFC2 vector (InvivoGen) to generate the construct pFUSE-scFv C21 utilizing the DNA Ligation Kit, Mighty Mix (TAKARA Bio USA) according to the manufacturer's instructions. In order to obtain the gene encoding the human IgG1 Hinge-CH2-CH3 (Fc), the latter DNA fragment was amplified by PCR from a human IgG1 expression vector (kindly provided by Dr. J. Schlom, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD) as a BglII/ NheI fragment using the primers with the following sequences: BglII Forward (5'-

TTTTAGATCTGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGC

ACCTGAACTCCTGGGGGGACCGTCAGTCTTCC-3') and NheI Reverse (5'-TTTTGCTAGCACTCATTTGCCCGGAGACAG-3'). The PCR products were purified as described above, digested with the restriction enzymes BglII and NheI (New England BioLabs) and cloned into the pFUSE-scFv C21 vector to generate the final construct pFUSE-scFvC21-IgG1Hinge-CH2-CH3 (pFUSE-scFv-FcC21), which encodes scFv-FcC21. Using a similar approach, the control anti-anti-id scFv119 was cloned into pFUSE-hIgG1-FC2 vector

(InvivoGen) to generate the construct pFUSE-scFv119-IgG1Hinge-CH2-CH3 (pFUSE-scFv-Fc119), which encodes scFv-Fc119.

Fully human scFv-Fc antibody expression and purification. Expression plasmids pFUSE-scFv-Fc were transfected into the mouse myeloma cell line P3X63Ag8.653 using electroporation performed with the Gene Pulser II Electroporation System (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. The transfected cells were selected in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (PAA) and with Zeocin (Invitrogen) (50µg/mL). Zeocin – resistant cells were then single-cell-subcloned by limiting dilution. The spent supernatants of subcloned cells were screened by ELISA for expression of human Fc and for reactivity of scFv-Fc antibodies with the corresponding antigens. The stable transfectant, which secretes active scFv-Fc antibody into the culture medium, was gradually adapted to hybridoma serum free medium (Invitrogen) supplemented with FCS at a 5% concentration to facilitate antibody purification. The fully human scFv-Fc antibodies were purified from either spent culture supernatant or mouse ascites, using HiTrap protein A HP column (GE healthcare) according to the manufacturer's instructions. The purity and activity of scFv-Fc preparations were determined by SDS-PAGE and antigen binding assays, respectively.

Binding assays. The ELISA to test the reactivity of soluble scFv antibodies with cells was performed as described (1). Results are expressed as absorbance of optical density (O.D.) at 450nm (OD_{450}).

Flow cytometry analysis of cells stained with scFv C21/scFv-FcC21 antibodies was performed as described (1). Briefly, cells ($5x10^5$) were incubated for 1 hr at 4^oC with 12.5µL of scFv PP and with 0.5µg of mAb 9E10 (both diluted in a total volume of 100µL of 2% BSA-PBS) or 1µg of scFv-FcC21 (diluted in a total volume of 100µL of 2% BSA-PBS). Cells were then washed twice

with 0.5% BSA-PBS and incubated for 30 mins at 4^{o}C with an optimal amount of RPE-labeled F(ab')$_2$ fragments of goat anti-mouse IgG antibodies or RPE-labeled F(ab')$_2$ fragments of goat anti-human IgG Fcγ antibody. Following two washes, cells were fixed in 2% formaldehyde and analyzed with a CyAn™ ADP LX 9 Color flow cytometer (Dako). A total of 10,000 cells were counted using a forward and side scatter gate to eliminate aggregates and debris for each sample. Results are expressed as relative fluorescence intensity.

The competition assay to map the epitope recognized by scFv C21 was performed by mixing biotinylated scFv C21 (0.25μg/well) with two fold dilutions of mouse mAb or scFv PP or by mixing two fold dilutions of scFv PP with an amount of biotinylated mAb which gave an absorbance of 1.0 at 450nm. The mixture (100μL/well) was incubated for 1hr at 4^{o}C with CSPG4^{+} cells (2 x 10^5/50μL of RPMI 1640 medium) in a 96-well tissue culture plate (Falcon 3072, Becton Dickinson). Binding of biotinylated scFv antibodies and biotinylated mAb to target cells was measured by sequential incubation with SA-HRP and substrate as described (2). The results are expressed as percent inhibition by mAb or scFv antibodies of scFv antibody or mAb binding to CSPG4^{+} cells. The percent inhibition was calculated using the formula: % inhibition = [(OD$_{450}$ in the absence of inhibitor – OD$_{450}$ in the presence of inhibitor)/OD$_{450}$ in the absence of inhibitor] x 100.

shRNA knockdown. CSPG4^{+}MV3 cells were seeded at the density of 6x10^4 per well in a 6-well plate and incubated in RPMI1640 medium plus 10% FBS for 24 hours at 37ºC in a 5% CO_2 atmosphere prior to viral infection. Cells were transduced with CSPG4-specific shRNA (Oligo sequence:

CCGGCAATGCATCAGCCGTAGTGAACTCGAGTTCACTACGGCTGATGCATTGTTTTT TG) or the control shRNA, i.e., ABCB5-specific shRNA (Oligo sequence:

CCGGGCGGCATTATCGAGACCATATCTCGAGATATGGTCTCGATAATGCCGCTTTTT G) lentiviral particles ($1x10^6$ per well) in presence of polybrene (2μg/ml) as described (3). Following a 18 hr incubation at 37ºC, culture medium was removed and replaced with fresh culture medium. Following an additional incubation for up to 72 hr at 37ºC, cells were collected for further analysis. The knockdown of cell surface CSPG4 expression was monitored by flow cytometric analysis of cells stained with CSPG4-specific mAb 225.28 (4).

Binding kinetics. scFv-FcC21 at different concentrations (7.6, 22.3, and 30.4 nM) were pre-incubated with RPE-labeled $F(ab)_2$ fragments of goat anti-human IgG, Fcγ fragment specific antibody (RPE-secondary antibody) at 1 to 4 molar ratio on ice for 30 min to form complex. To each of the complex containing tube, 3ml of a $CSPG4^+$ MV3 cell suspension ($1x10^6$ cells/ml) were then added. The binding (MFI: Mean fluorescence intensities) of scFv-FcC21/RPE-secondary antibody complex to $CSPG4^+$ cells was immediately measured by flow cytometry constantly for 10 minutes. scFv-Fc119 was measured in the same manner as a specificity control. Following Trautmann et al's method (5), the rate constants k_{on} and k_{off} were derived from the plot of $1/\tau on$ as a function of [A] (**Fig1D**). Here we used the MATLAB software (MathWorks, Inc.) to calculate the rate constants k_{on} and k_{off}. The dissociation constant of scFv-FcC21 was calculated based on the formula:$k_d = k_{off} / k_{on}$.

Internalization assay. $CSPG4^+$MV3 cells were seeded at the density of 5.0×10^4 cells per well in a 8-well Millicell EZ slide (Millipore) overnight at 37ºC. Then cells were incubated with 100μl/well of either Cy3 labeled scFv-FcC21 (10μg/ml) or Cy3 labeled scFv-Fc119 (10μg/ml) for 5, 20, 45 or 60 minutes. Following two washes with PBS, cells were fixed with 2% PFA/PBS for 20 min and stained with DAPI for 30 seconds. Then cells were washed again twice with PBS, mounted and analyzed with a confocal fluorescence microscope.

Treatment with Glycosidase. CSPG4^{+} MV3 cells (5×10^{5}/ 50µl RPMI 1640 medium) were incubated with or without 2µl of each PNGase F, O-Glycosidase and α-2(3,6,8.9)-Neuraminidase (Enzymatic Protein Deglycosylation Kit, Sigma) for 24 hr at 37˚C in a 5% CO_2 atmosphere. The treated cells were then stained with scFv-FcC21 and analyzed by flow cytometry (Cyan, Beckman Coulter).

IHC staining of normal TMA. Frozen human normal tissue microarray (TMA) slides were fixed by 4% formaldehyde /PBS for 20 minutes at room temperature. IHC staining of TMA slides with scFv-FcC21 was performed as described (6). Pictures of stained tissue microarrayslides were taken using OLYMPUS BX51 microscope (OLYMPUS UK Ltd) at a magnification ×200 for review (6).

ADCC and CDC assays. ADCC assay was performed as previously described (7) using PBMC purified from human blood as effector cells. Briefly, CSPG4^{+} MV3 cells, which are positive for EGFR as well, were labeled with 50 µCi of ^{51}Cr (Perkin Elmer) and resuspended at a density of $0.4x10^{6}$ cells/ml. ^{51}Cr labeled cells were mixed with 10-fold diluted scFv-FcC21 (100-0.1 µg/ml 50µl/well) in a 96-well tissue culture- U-bottom assay plate (BD Falcon). EGFR-specific mAb Cetuximab which is known to induce ADCC (8) and scFv-Fc119 were used as positive and negative controls, respectively. Following a 30 min incubation at 4°C, PBMC (40:1 E:T) were added and incubation was continued for an additional 4 hrs at 37 °C in a CO_2 incubator. ^{51}Cr release was determined by counting the cell free supernatant using a Packard TopCount™Microplate Scintillation Counter (Conroe). The experiment was performed twice in triplicates.

CDC assay was performed as previously described (7). Briefly, ^{51}Cr labeled MV3 and Raji cells were resuspended at a density of 1x10^{6}cells/ml. MV3 cells were incubated with 10-fold diluted scFv-FcC21 (100-0.1 µg/ml, 50µl/well) in the presence of human serum complement (Quidel) diluted 4 folds in RPMI 1640 medium, 10mM HEPES, 0.1% BSA. scFv-Fc119 was used as a negative control. CD20$^+$ Raji cells and CD20-specific mAb Rituximab which is known to mediate CDC (9) were used as positive controls for the assay. Following a 2 hr incubation at 37 °C in a CO_2 incubator, ^{51}Cr release was determined by counting the cell free supernatant using a Packard TopCount™Microplate Scintillation Counter. The experiment was performed twice in triplicates.

Antitumor activity of scFv-FcC21 in mice bearing established human melanoma cell-derived lung metastases. Eight wk old, female SCID mice were injected intravenously (i.v.) with the human melanoma MV3 cells (1.4x10^6 cells/mouse-MV3 model) or M21 cells (2.0x10^6 cells/mouse-M21 model). Fifteen days following the i.v. injection of cells, 2 randomly chosen mice from each model were sacrificed; lungs were harvested, formalin fixed and paraffin embedded (FFPE). FFPE tissue sections were stained with hematoxylin and eosin (H &E) and examined microscopically to confirm the establishment of lung metastases. The remaining 26 mice in the MV3 model were randomly divided into 2 groups of 13 mice each. The remaining 12 mice in the M21 model were randomly divided into 2 groups of 6 mice each. One group of mice from each model was injected i.v. with scFv-FcC21 antibody (100µg/ mouse) every 48 hrs for a total of 3 injections. The other group of mice from each model was injected i.v. with the isotype control scFv-Fc119, utilizing the same schedule. Twenty-four hrs following the last injection, mice (7/each group in the MV3 model and 6/each group in the M21 model) were sacrificed. Lungs were collected and subjected to process of FFPE. H&E stained lung tissue sections were

examined microscopically for metastasis. The remaining 6 mice /each group (MV3 model) without further treatment were monitored for survival.

Figure legends

sFig.1. Selective reactivity in ELISA of soluble scFv C21 with CSPG4$^+$ human cell lines.

Cultured human melanoma cells Colo38, FO-1, SK-MEL-28 and Melur, all of which express CSPG4, and human prostate carcinoma cells PC3, human bladder carcinoma cells T24, human breast carcinoma cells T47D and human B lymphoid cells JY, LG-2 and LKT13, all of which do not express CSPG4 and rat neuroblastoma cells B49, which express a CSPG4 homologne (10). were incubated at 4^0C for 2 hr with 50 μl of SNT scFv C21 (▨) and with biotinylated mAb 9E10 (2.5 μg/ml 1% BSA-PBS). Binding of scFv fragments was detected using SA–HRP. Results are expressed as absorbance at 490 nm. Human anti-anti-id scFv 119 (□) was used as a specificity control.

sFig.2. No detectable ADCC and CDC mediated by scFv-FcC21. A, ^{51}Cr labeled MV3 cells (target cells), human PBMC (effector cells) (40:1 E:T) in the presence of the indicated concentrations of each mAb were used in the ADCC assay. **B,** ^{51}Cr labeled MV3 cells (target cells) or **C,** ^{51}Cr labeled Raji cells (target cells) in the presence of human serum complement were used in the CDC assay.

sFig.3. Reduction by scFv-FcC21 of established M21 cell-derived experimental lung metastases *in vivo*. Lungs of mice bearing human melanoma M21 cells derived metastases were collected and fixed in 10% formalin and paraffin-embedded. The sections of FFPE tissue (5 sections/slide/ mouse) were subjected to the following analysis: the sizes/areas of metastatic nodules (total nodules examined: 32 in scFv-FcC21treated group and 48 in scFv-Fc119 treated

group using 5 randomly selected high power fields (×200)/each section) (OLYMPUS BX51 microscope) were measured and calculated by the SPOT IMAGING SOFTWARE Advanced (Diagnostic Instruments, Inc.) The values shown are the mean tumor area of each group (**A);** the number of proliferating tumor cells was detected by staining p-Histone H3 protein in lung tissue FFPE sections and determined by counting 5 fields/slide/mouse (×200). The values shown are the mean number of mitotic tumor cells in each group **(B)**.

sTable.1. Lack of cross-reactivity of scFv-FcC21 with normal human organ tissues. Normal human multiple organ frozen tissue microarray (TMA), constructed from 20 normal human tissues, was stained with scFv-FcC21(2μg/ml). Frozen sections of M14/CSPG4 ($CSPG4^+$) and M14 ($CSPG4^-$) cell pellets were stained simultaneously as positive and negative controls, respectively. Immunohistochemistry results on TMA (2 cores, per tissue from the same donor) and cell pellet-derived sections are shown.

*: uninterpretable due to high background staining.

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