Therapeutic efficacy of antibody-drug conjugates targeting GD2-positive tumors

Supplementary Methods

Production of chimeric monoclonal antibody 14.18

The sequences coding for the heavy chain (HC) and light chain (LC) variable domains of the model chimeric antibody were obtained from data reported by Bolesta et al. (2005) and codon-optimized for efficient expression in mammalian cells. The coding sequences of the HC and LC variable domains and secretion signal sequences were then fused to the cDNA of human gamma 1 and kappa constant regions, and cloned into pcDNA3.3-TOPO TA and pOptiVEC-TOPO TA vectors (Invitrogen) using the XhoI/NheI and XbaI/NotI restriction sites, respectively. The chimeric HC and LC expression units were coupled with selection markers.

Parental CHO DG44 (dfhr-/-) cells were cultured in a protein-free medium CD DG44 supplemented with 8 mM GlutaMAX-I and 0.05% Pluronic F-68 (all from Gibco). The plasmid DNA was purified using Qiagen MaxiPrep Kit (Qiagen) and linearized using the unique MluI site. For transfection, $0.5x10^6$ cells, 0.6 µg of plasmid DNA, and 0.5 µg of FreeStyle MAX transfection reagent (Thermo Fisher Scientific) were used per 1 ml of transfection mixture. For batch selection of the stable clones, transfectants were selected in a shaker culture in the medium containing 200 µg/ml of G418 (Sigma-Aldrich) and lacking hypoxanthine/thymidine. Cell pools resistant to the first selection step were subjected to four steps of methotrexate amplification (25-500 nM). Single-cell clones were isolated from the heterogeneously amplified pools by limiting dilution cloning. Selected clones were scaled to shake flasks and cultured in CD OptiCHO medium (Thermo Fisher Scientific) supplemented with 8 mM GlutaMAX-I.

Purification of recombinant proteins was performed on Protein G sepharose (Thermo Fisher Scientific). The prepared culture medium was applied to the column at a rate of 1 ml per minute. Elution was carried out sequentially with three buffer solutions: 0.1 M sodium citrate, pH 3.75; 0.1 M sodium citrate, pH 3.0; and 0.1 M glycine, pH 2.0. The eluates were collected separately, and pH was immediately adjusted to neutral values. Ch14.18 antibodies were then transferred to phosphate buffered saline (PBS) in Amicon Ultra-4 10 kDa centrifugal filters (Merck) and sterilized through a 0.22 µm membrane filter. Protein concentration was calculated at a wavelength of 280 nm by BioDrop µLITE spectrophotometer (BioDrop, UK).

Direct ELISA

Nunc MaxiSorp high protein-binding capacity 96 well ELISA plates (Thermo Fisher Scientific) were coated with gangliosides GD2, GM2, GD1b, and GD3 at concentration of 0.25 µg in 100 µl of 96% ethanol per well. Following air drying, plate wells were blocked with 100 µl 2% BSA in PBS supplemented with 0.1% Tween-20 (PBS-T) per well for 2 h at RT. Chimeric antibody ch14.18 or control mouse antibody 14G2a (Santa Cruz Biotechnology) (100 µl per well in PBS-T) were added in triplicates at different concentrations. Following incubation for 1.5 h and washing with PBS-T, and anti-human or anti-mouse Fc-specific HRP-labeled antibodies (both 1:6000; Santa Cruz Biotechnology) were added to the wells. After 40 min incubation and further washing, 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was added, and the color reaction optical density (OD) was measured at 450 nm by Multiscan FC microplate reader (Thermo Fisher Scientific). Percent of cross-reactivity was calculated as the ratio of TMB color reaction OD_{450} in GM2-, GD1b-, or GD3-coated wells to OD_{450} in GD2-coated wells.

MTT assay

ADC-induced decrease in cell viability was analyzed by colorimetric MTT (Sigma-Aldrich) assay. Tumor cells were cultured in 96 well flat-bottom tissue culture plates (10^4 cells/well, Greiner) with serial dilutions of anti-GD2 ADCs, antibodies (ch14.18 or 14G2a), MMAE, or MMAF for 72 h under standard conditions. Following incubation, the MTT solution (in 250 µg/ml final concentration) was added to each sample for 4 h, and the formazan precipitate was dissolved in DMSO. OD was assessed by Multiscan FC microplate reader at a wavelength of 540 nm. Cell viability was calculated as $(OD_{\text{treated cells}} - OD_{\text{blank}})/(OD_{\text{control cells}} - OD_{\text{blank}}) \times 100\%$, where OD_{blank} represents OD in control wells containing no cells. Dose–response curves were generated using SigmaPlot software. All MTT experiments were reproduced at least three times.

Tissue sample processing for *ex vivo* biodistribution studies.

Blood was collected into heparin treated tubes by retro-orbital bleeds of anaesthetized mice, followed by plasma analysis. Euthanasia was performed with isoflurane. In order to measure biodistribution in tissues with minimal blood contamination, residual blood was removed by transcardial perfusion with 10 ml of heparinized PBS. Tissues were dissected and homogenized, followed by incubation in a hydrochloric acid-ethanol solution overnight at 4 °C. Samples were cleared by centrifugation, and fluorescence intensity was analyzed by GloMax-Multi Detection System fluorometer (Promega) with excitation at 625 nm and absorbance at 660–720 nm. Organs and blood plasma from intact mice were used for subtraction of autofluorescence.

Supplementary Data

Table S1.

Extinction coefficients employed for the calculation of drug-antibody ratio (DAR) by UV-VIS spectroscopy.

Molecule	Extinction coefficients (cm^{-1} M^{-1}) calculated at given wavelength			
	253 nm	**256 nm**	**280 nm**	**494 nm**
ch14.18	84960	93810	205310	26550
MC-VC-PABC-MMAE	21920	-	3220	-
MC-VC-PABC-MMAF	21900	-	2740	-
DOX	-	25180	8620	-
FAM-maleimide	-	-	14770	74380

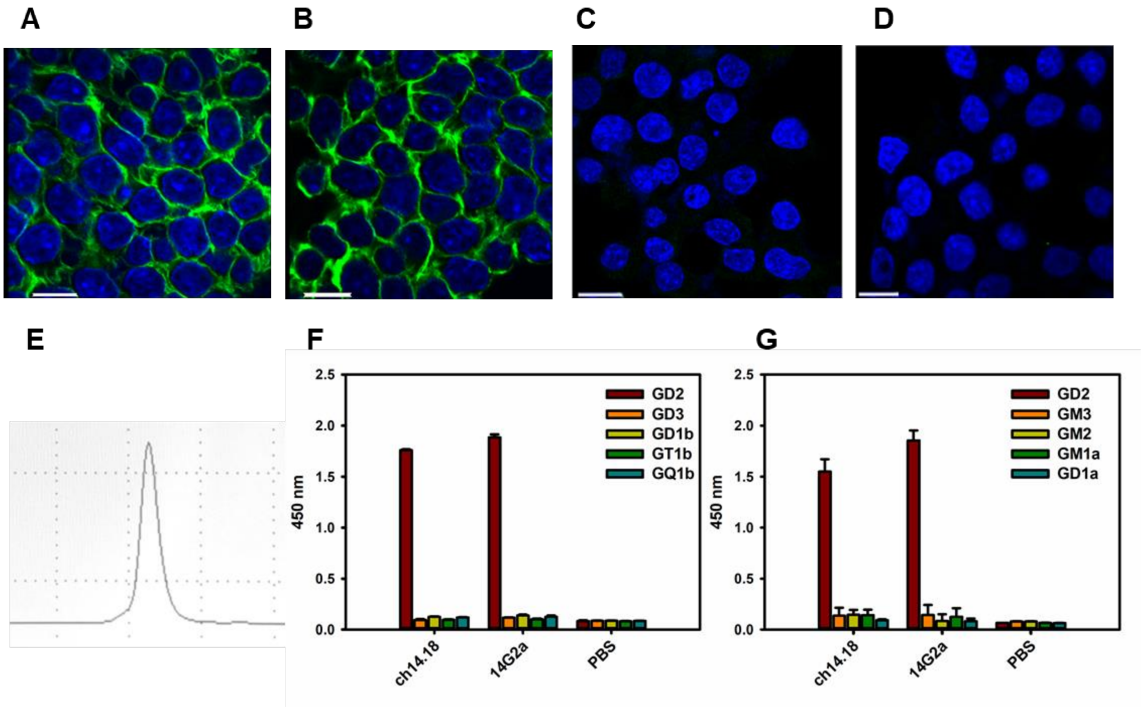

Figure S1.

A-D, Confocal images of GD2-positive mouse lymphoma EL-4 (A, B) and GD2-negative mouse melanoma M3 (C, D) cells stained with antibody conjugates 14G2a-AF488 (A, C) and ch14.18-FAM (B, D). Following staining with fluorescently labeled GD2-specific antibodies and DAPI

(4',6'-diamidine-2-phenylindole, Molecular Probes), cells were fixed and analyzed on the confocal microscope C1 (Nikon, Japan). Staining with anti-GD2 conjugates is shown in green color; the nuclei are counterstained with DAPI (shown in blue). Bar scale: 10 μm.

E, Size-exclusion chromatographic analysis of ch14.18 antibody performed in PBS eluent at 0.75 ml/min flow rate with detection at 280 nm (Superdex 200 10/300 GL column, Cytiva; Beckman System Gold high-performance liquid chromatography system).

F, Evaluation of cross-reactivity of ch14.18 and 14G2a antibodies to b-series gangliosides in direct ELISA. GD2, GD3, GD1b, GT1b, or GQ1b gangliosides were adsorbed on the plate. Ch14.18 or 14G2a antibodies were added in 0.1 μg/ml concentration.

G, Evaluation of cross-reactivity of ch14.18 and 14G2a antibodies to a-series gangliosides in direct ELISA. GD2, GM3, GM2, GM1a, or GD1a gangliosides were adsorbed on the plate. Ch14.18 or 14G2a antibodies were added in 10 μg/ml concentration.

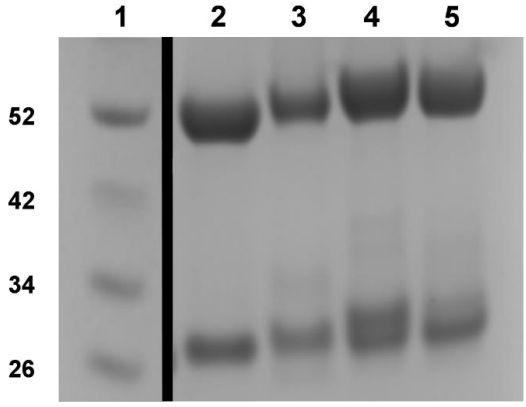

Figure S2.
12% reducing polyacrylamide gel electrophoresis; increased molecular weight of LC and HC observed for the resulting ADCs relative to the initial antibody molecule. 1, molecular weight protein markers; 2, ch14.18 antibody; 3, ch14.18-DOX DAR 4.6; 4, ch14.18-MMAE DAR 4.4; 5, ch14.18-MMAF DAR 4.1.

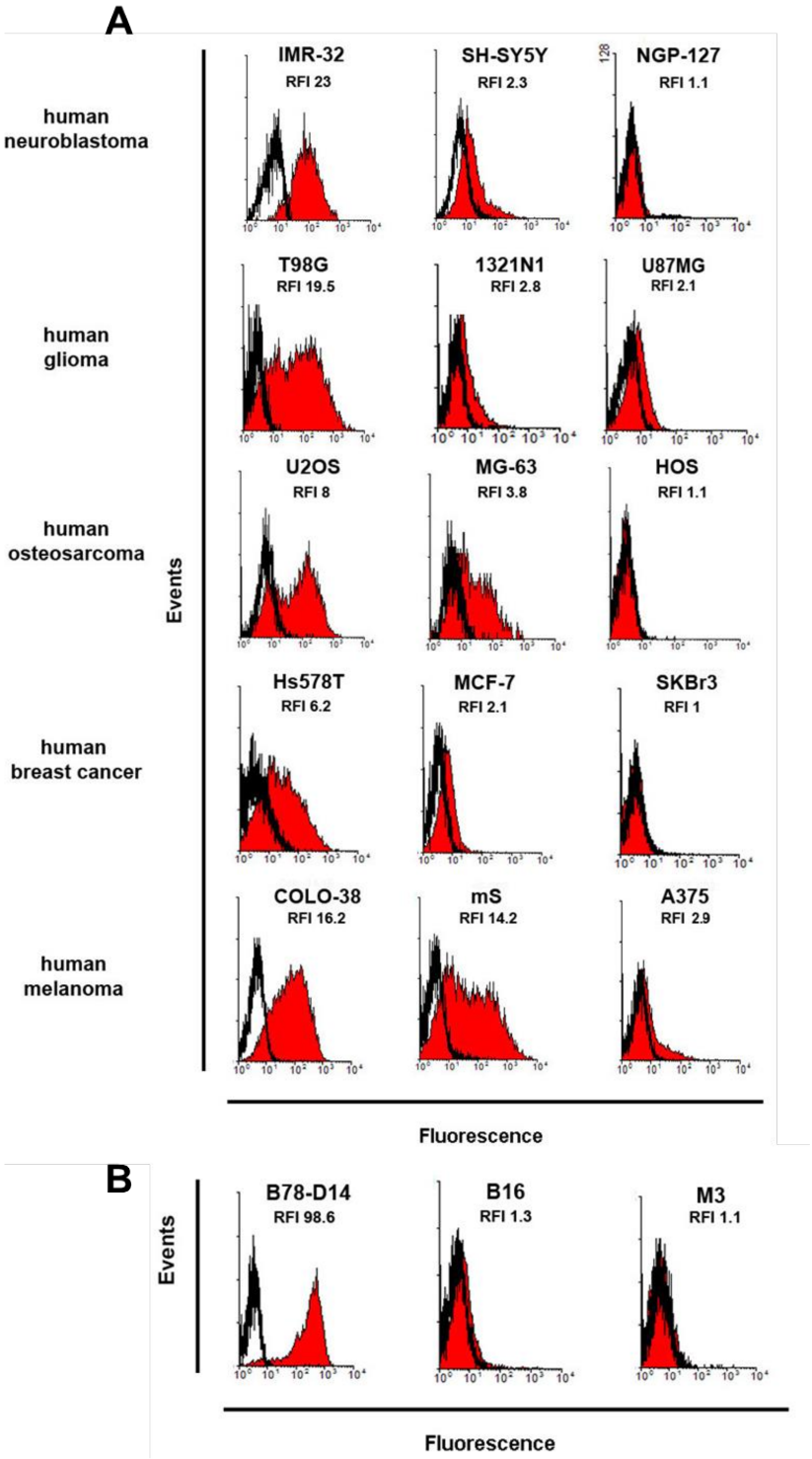

Figure S3.
Flow cytometry analysis of ganglioside GD2 expression in a panel of tumor cell lines. **A**, Staining of human cell lines with ch14.18-FAM. **B**, Staining of murine cell lines with ch14.18-FAM. Red histograms represent staining with ch14.18-FAM, empty histograms represent autofluorescence of unstained cells. RFI – ratio of specific fluorescence of cell staining with fluorescently labeled antibodies ch14.18 and autofluorescence of control unstained cells.

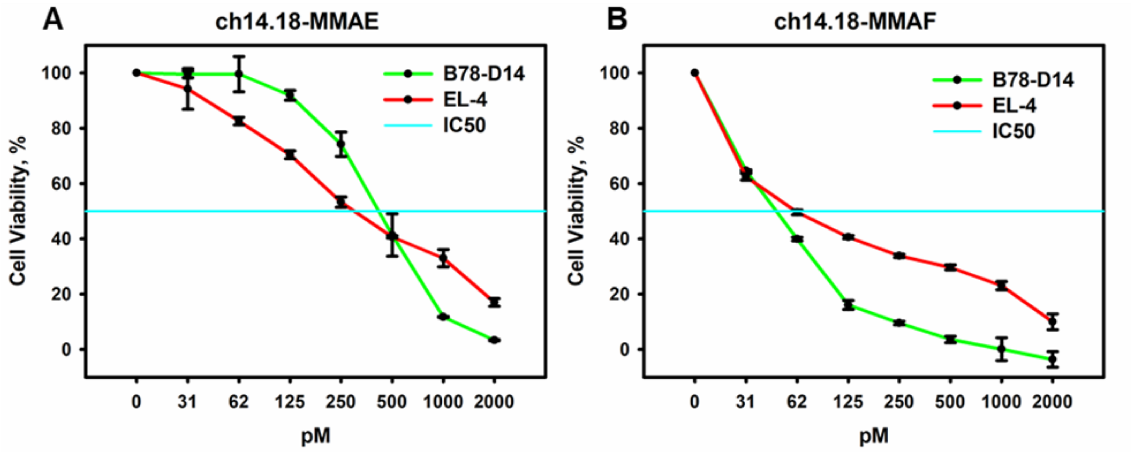

Figure S4.

Viability of murine cell lines with different expression of ganglioside GD2 analyzed by MTT assay following 72 h incubation. **A**, – ch14.18-MMAE, **B**, – ch14.18-MMAF.

Table S2.

Classification of human and murine cell lines by GD2 expression and cell viability following incubation with ch14.18-MMAE and ch14.18-MMAF. RFI calculated by flow cytometry using the ch14.18-FAM conjugate.

Tumor type	Cell lines	Origin	RFI	GD2 level*	IC50%, nM		IC20% nM	
					Ch14.18-MMAE	Ch14.18-MMAF	Ch14.18-MMAE	Ch14.18-MMAF
NBL	IMR-32	H	23±4	+++	0.3±0.1	0.81±0.2	0.15±0.1	0.25±0.1
	SH-SY5Y	H	2.3±0.5	+	2.48±1.2	>40	0.51±0.2	5.3±0.5
	NGP-127	H	1.1±0.3	-	>40	>40	>20	>20
glioma	T98G	H	19.5±3	+++	0.68±0.2	1.84±0.5	0.23±0.1	0.19±0.1
	1321N1	H	2.8±0.6	+	4.28±1.2	8±1.6	0.48±0.2	1±0.3
	U87MG	H	2.1±0.7	+	>40	>40	8.47±2.2	12.4±3.6
sarcoma	U2OS	H	8±2	++	0.685±0.3	3.3±1.5	0.28±0.1	0.3±0.2
	MG-63	H	3,8±1.1	+	13.1±2.8	>20	2.98±1.3	7.3±2.1
	HOS	H	1.1±0.5	-	>40	>40	>20	>20
breast cancer	Hs578T	H	6.2±2.1	++	0.93±0.5	6.8±1.1	0.38±0.2	0.48±0.3
	MCF-7	H	2.1±1.1	+	9.4±1.6	18.5±2.8	3±0.7	5.3±0.8
	SKBr3	H	1±0.5	-	>40	>40	>20	>20
melanoma	COLO-38	H	16.2±4	+++	0.4±0.1	1.27±0.7	0.14±0.1	0.3±0.1
	mS	H	14.2±3	++	0.51±0.2	3.7±0.9	0.2±0.2	0.3±0.1
	A375	H	2.2±1.2	+	7.8±1.4	>40	2.7±0.5	5.3±1.4
	B16	M	1.3±0.3	-	>40	>40	17±2.7	>20
	B78-D14	M	98.6±4	++++	0.442±0.2	0.048±0.01	0.217±0.2	0.024±0.02
	M3	M	1.1±0.4	-	>40	>40	>20	>20
lymphoma	EL-4	M	59±8	++++	0.292±0.1	0.062±0.01	0.086	0.024±0.01

* GD2 level: negative (-), low (+), medium (++), high (+++), overexpression (++++); NBL – neuroblastoma, H – human, M – mouse.

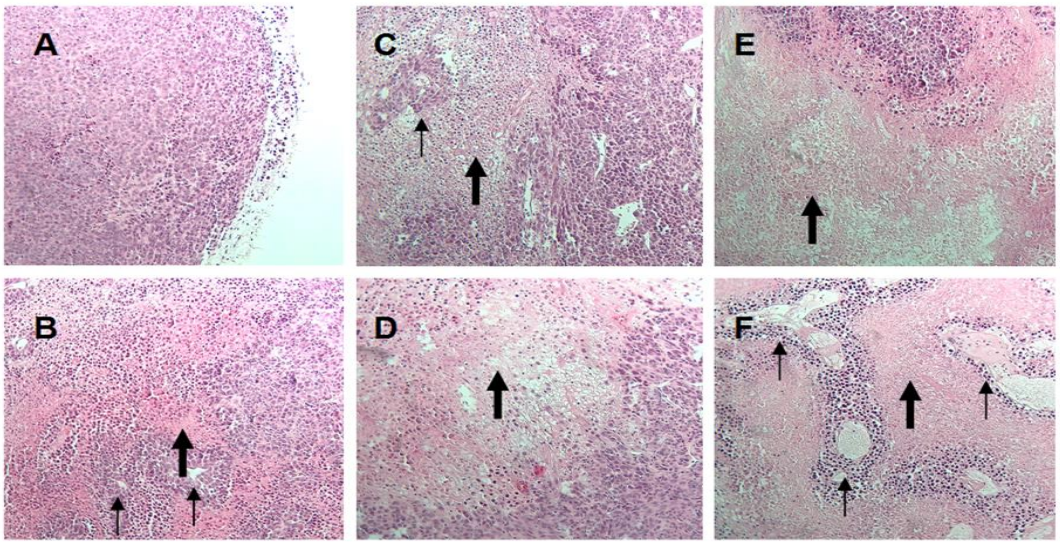

Figure S5.

Histology of control untreated B78-D14 melanoma tumors (**A-B**) and tumors treated with ch14.18-MMAE (**C-D**) or ch14.18-MMAF (**E-F**). Thin arrows indicate blood vessels, thick arrows indicate necrosis zones. Staining with H&E. The B78-D14 melanoma represents a solid tumor (A) with small areas of necrosis (B, thick arrow). Treatment of the tumor with ch14.18-MMAE or ch14.18-MMAF results in the formation of large necrotic zones around blood vessels (C-F). Effect of ch14.18-MMAF is more evident due to larger necrotic zones.

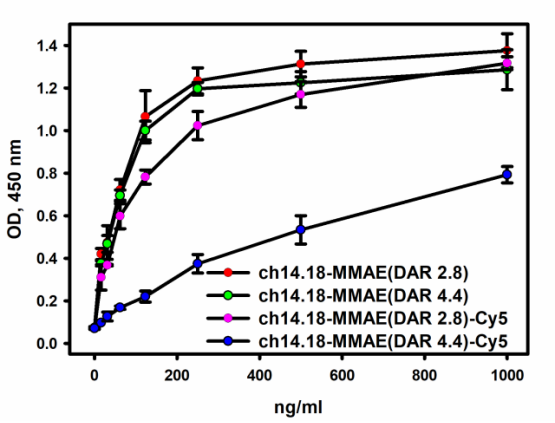

Figure S6.

Analysis of the antigen-binding properties of ch14.18-MMAE and ch14.18-MMAE-Cy5 with DAR 2.8 and DAR 4.4 by direct ELISA; ganglioside GD2 was adsorbed on the plate, serial dilutions of the analytes were added to the wells.

Table S3. Tissue distribution following intravenous administration of ch14.18-MMAE (DAR 2.8) or its parent antibody ch14.18 in C57BL/6 mice at 24 h and 48 h post-injection. Data are presented as % of injected dose per gram of tissue type, and values represent the mean ± S.E.M derived from groups of three animals.

Tissue	ch14.18 24h	ch14.18-MMAE 24h	ch14.18 48h	ch14.18-MMAE 48h
	% ID/g tissue type ± S.E.M.			
Blood	20.03 ± 2.42	19.67 ± 3.53	9.81 ± 1.62	13.40 ± 2.50
Tumor	3.24 ± 0.72	3.57 ± 0.78	6.38 ± 1.06	7.65 ± 0.78
Muscle	0.25 ± 0.02	0.29 ± 0.06	0.24 ± 0.06	0.19 ± 0.07
Spleen	1.25 ± 0.20	1.60 ± 0.24	1.10 ± 0.10	1.21 ± 0.14
Kidney	1.25 ± 0.11	0.96 ± 0.08	0.93 ± 0.26	0.78 ± 0.12
Liver	3.92 ± 0.82	3.55 ± 0.53	1.98 ± 0.55	2.56 ± 0.56
Lung	0.96 ± 0.20	1.01 ± 0.14	0.70 ± 0.17	0.82 ± 0.14
Heart	0.75 ± 0.07	0.57 ± 0.07	0.55 ± 0.13	0.79 ± 0.13
Brain	0.02 ± 0.02	0.08 ± 0.03	0.07 ± 0.02	0.03 ± 0.02