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ORIGINAL ARTICLE

Vaccination with anti-idiotype antibody ganglidiomab mediates a GD_2-specific anti-neuroblastoma immune response

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Abstract
Purpose Immunotherapy targeting disialoganglioside GD_2 emerges as an important treatment option for neuroblastoma, a pediatric malignancy characterized by poor outcome. Here, we report the induction of a GD_2-specific immune response with ganglidiomab, a new anti-idiotype antibody to anti-GD_2 antibodies of the 14.18 family.
Experimental design and results Ganglidiomab was generated following immunization of Balb/c mice with 14G2a, and splenocytes were harvested to generate hybridoma cells. Clones were screened by ELISA for mouse antibody binding to hu14.18. One positive clone was selected to purify and characterize the secreted IgG protein (κ, IgG_1). This antibody bound to anti-GD_2 antibodies 14G2a, ch14.18/CHO, hu14.18, and to immunocytokines ch14.18-IL2 and hu14.18-IL2 as well as to NK-92 cells expressing scFv(ch14.18)-zeta receptor. Binding of these anti-GD_2 antibodies to the nominal antigen GD_2 as well as GD_2-specific lysis of neuroblastoma cells by NK-92-scFv(ch14.18)-zeta cells was competitively inhibited by ganglidiomab, proving GD_2 surrogate function and anti-idiotype characteristics. The dissociation constants of ganglidiomab from anti-GD_2 antibodies ranged from 10.8 ± 5.01 to 53.5 ± 1.92 nM as determined by Biacore analyses. The sequences of framework and complementarity-determining regions of ganglidiomab were identified. Finally, we demonstrated induction of a GD_2-specific humoral immune response after vaccination of mice with ganglidiomab effective in mediating GD_2-specific killing of neuroblastoma cells.
Conclusion We generated and characterized a novel anti-idiotype antibody ganglidiomab and demonstrated activity against neuroblastoma.

Keywords Pediatric oncology · Neuroblastoma · Ganglioside GD_2 · Immunotherapy · Anti-idiotype

Introduction

Neuroblastoma (NB) is one of the most common malignant solid tumors of childhood, and the overall survival rate of stage 4 disease still remains poor, despite intensive treatment regimens.

Glycolipid GD_2 is a tumor-associated antigen with high expression on neuroectodermal tumors such as neuroblastoma, melanoma, Ewing sarcoma, and low expression on normal tissues. Therefore, targeting of GD_2 by monoclonal antibodies (MABs) was pursued as a strategy to improve survival in cases of high-risk neuroblastoma. For this purpose, monoclonal antibodies against GD_2 were developed and successfully applied in clinical trials (for review, see [1, 2]). The most prominent example of this approach was recently reported by the Children's Oncology Group

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(COG) [3]. In this randomized open label phase III clinical trial, treatment with anti-GD_2 antibody ch14.18/SP2/0 in combination with interleukin 2 (IL2) and granulocyte macrophage colony-stimulating factor (GM-CSF) resulted in 20 % increased event-free and overall 3-year survival of high-risk neuroblastoma patients. Similar developments are currently pursued in Europe by the SIOPEN group facing a major challenge related to the restricted availability of ch14.18 antibody [4]. This problem was solved by establishing a safe and reliable industrial production of ch14.18 using an expression system based on CHO cells (ch14.18/CHO). However, this major change in the production process requires preclinical and clinical re-evaluation, including pharmacokinetics and pharmacodynamics of ch14.18/CHO, emphasizing the need for a ch14.18/CHO-specific anti-idiotype (Id) antibody. Even though an anti-Id antibody 1A7 generated against 14G2a was previously described [5], it is important for further clinical development to provide for an unrestricted access to an anti-Id antibody of ch14.18/CHO.

Furthermore, anti-Id MABs may open new opportunities in developing anti-Id vaccines. The use of anti-Id vaccines to stimulate anti-tumor immunity against a number of solid tumor entities has shown promising results [6], and it is in principle based on the concept that anti-Id MABs function as surrogates for the tumor-associated antigen, in our case the glycolipid GD_2. For the GD_2 antigen, the anti-Id approach is important, because active vaccination with GD_2 encounters various obstacles, including poor antigenicity and T cell independence of glycolipid antigens [7, 8] resulting occasionally in low titer antibodies against GD_2 [8]. Vaccination with peptides or proteins mimicking GD_2 could overcome these deficiencies [9] and may induce not only GD_2-specific humoral but also GD_2-specific T cell-mediated immune responses [10].

Here, we report the generation and characterization of a novel anti-GD_2 anti-idiotype antibody, ganglidiomab, which mimics GD_2, providing an important baseline for the development of vaccines against NB as well as for immune monitoring of clinical trials with anti-GD_2 antibodies of the 14.18 family. Part of this work was presented at the "XXIV Jahrestagung der Kind-Philipp-Stiftung für Leukämieforschung."

Materials and methods

Tissue culture and animal experiments

SP2/0-Ag14 cells were cultured in DMEM [4.5 g/l glucose, 2 mM glutamine, 10 % FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, (1× P/S)] (PAA, Pasching, Austria). NK-92 cells were cultured in X-VIVO-10 (LONZA, Walkersville, MD, USA) (5 % human AB serum, 100 U/ml rhIL2). The genetically engineered NK-92-scFv(ch14.18)-zeta cell line, expressing GD_2-specific chimeric ch14.18 receptor, was kindly provided by Prof. W. Wels (Georg-Speyer Haus, Frankfurt, Germany) and cultured as previously described [11]. Hybridoma cells producing ganglidiomab were cultured in DMEM (10 % FCS, 1× P/S, 1× non-essential amino acids, 50 μM β-mercaptoethanol). Human LAN-1 neuroblastoma cells were cultured in RPMI (4.5 g/l glucose, 2 mM glutamine, 10 % FCS, and 1× P/S) (PAA, Pasching, Austria). Murine NXS2 neuroblastoma cells were cultivated as previously described [12].

The generation of anti-idiotype antibody ganglidiomab and the analysis of an anti-GD_2 humoral immune response upon vaccination with ganglidiomab were performed in female Balb/c and A/J mice (6–8 weeks of age) (Charles River Laboratories, Sulzfeld, Germany), respectively. Mice were housed in standard animal laboratories (12-h light/dark cycle) with free access to water and standard laboratory chow ad libitum. Experiments were performed in compliance with the German Law for Welfare of Laboratory Animals.

Generation of anti-idiotype antibody ganglidiomab

For immunization, 2 mg of monoclonal anti-GD_2 antibody 14G2a (IgG2a, kappa) was conjugated with 2 mg ovalbumin (OVA, 4 mg/ml, 50 mM NaPi, 250 mM NaCl, pH 6.8) by addition of 50 % glutaraldehyde to a final concentration of 0.2 % (1 h, RT, in the dark). Free aldehyde groups were blocked with 0.2 M monoethanolamine-HCl (pH 8.0, 2 h, RT, in the dark), and the immunoconjugate was dialyzed three times with 50 mM NaPi/NaCl buffer. Balb/c mice were immunized four times by weekly intraperitoneal (i.p.) injections of the conjugate admixed with incomplete Freund's adjuvant (1:1, v/v, Sigma Aldrich, Steinheim, Germany). Ten days after the last immunization, anti-14G2a antibody titers in mouse sera were analyzed with a hu14.18 capture sandwich ELISA. Splenocytes of mice developing anti-14G2a antibodies were fused with SP2/0-Ag 14 cells (2:1 ratio) for 1 h at room temperature in 1 ml of polyethylene glycol 3350 (50 % (w/v); Sigma, St. Louis, MO, USA), followed by cultivation and screening of hybridoma cells (HAT medium: DMEM, 20 % FCS, 5×10^{-3} M hypoxanthine, 0.8×10^{-3} M thymidine, 2×10^{-5} M Aminopterin). Subcloning was performed by the limiting dilution technique with 1–3 cells/well.

Screening for antibodies binding to complementarity-determining regions (CDRs) of 14G2a

Screening of serum from immunized mice and hybridoma cells for CDR-specific antibodies induced by immunization with 14G2a was accomplished using hu14.18 (humanized

version of 14G2a) as a capture antibody and Fc-specific goat anti-mouse alkaline phosphatase conjugate as a detection antibody. Briefly, plates were coated with 500 ng of hu14.18 per well (50 µl of 2 µg/ml stock in 0.1 M carbonate/hydrogen carbonate buffer, pH 9.6) over night at 4 °C, washed with Tris-buffered saline (TBS: 50 mM Tris, 500 mM NaCl, pH 7.8, 0.01 % Triton-X 100) and blocked with 2 % FCS in TBS (1 h, RT). Cell supernatants and serum (1st dilution 1:100, dilution series 1:2) were incubated for 1 h (RT), washed three times with TBS, and developed with goat anti-mouse alkaline phosphatase (Sigma, St. Louis, MO, USA) and 4-nitrophenylphosphate (2 mM, 5 % diethanolamine, 1 mM $MgCl_2$). Optical density (OD) was determined at 405 nm with plate reader Synergy HT (BioTek Instruments GmbH, Bad Friedrichshall, Germany).

Binding analysis of anti-GD_2 antibodies to ganglidiomab

To examine binding of anti-GD_2 antibodies to ganglidiomab, 96-well plates were coated with 250 ng ganglidiomab per well (0.1 M carbonate/hydrogen carbonate buffer, pH 9.6, 1 h, 37 °C). After 3 wash steps (PBS, pH 7.4, 0.1 % v/v Tween-20), wells were blocked with 1 % bovine serum albumin (BSA) in PBS (pH 7.4) (1 h, 37 °C) and washed three times (PBS, pH 7.4, 0.1 % BSA). Antibodies 14G2a, ch14.18/CHO, and hu14.18 and immunocytokines ch14.18-IL2 and hu14.18-IL2 as well as rituximab and IgG isotype control (negative controls) were diluted in PBS (pH 7.4) (final concentrations: 1.0, 0.5, 0.25, 0.13, 0.06, 0.03, and 0.015 µg/ml) and incubated over night (4 °C). Wells were washed five times (PBS, pH 7.4, 0.1 % Tween-20). Two variant methods were used to visualize binding to the antibody.

In the first variant method, anti-GD_2 antibodies (ch14.18/CHO, hu14.18 ch14.18-delta-CH2, and immunocytokines ch14.18-IL2 and hu14.18-IL2) that bind to GD_2 surrogate ganglidiomab were analyzed with a horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody as a secondary antibody (50 µl/well, 1:20,000; 1 h, 37 °C) (Sigma Aldrich, Steinheim, Germany). After 5 wash steps (PBS, pH 7.4, 0.1 % BSA), 75 µl 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reagents were added by following the manufacturers guidelines (R&D Systems Inc, Minneapolis, MN, USA, Cat No. DY999). After 30 minutes, 50 µl of 2 N H_2SO_4 was added to stop the reaction. Absorption was determined in the plate reader at 405 nm.

In order to also confirm results for the native antibody 14G2a, a variant of the ELISA was established by replacing horseradish peroxidase (HRP)-conjugated goat anti-human IgG as secondary antibody with biotinylated ganglidiomab.

In the second variant method, binding of the anti-GD_2 antibodies (14G2a, ch14.18/CHO, hu14.18 ch14.18-delta-CH2 as well as immunocytokines ch14.18-IL2 and hu14.18-IL2) to GD_2 surrogate ganglidiomab was analyzed with biotinylated ganglidiomab. Biotinylation was accomplished using EZ-Link® Sulfo-NHS-LC-Biotin (Thermo Scientific, Erlangen, Germany) following the manufacturer's guidelines by using 1 mg of ganglidiomab in 1 ml PBS (pH 7.4) (30 min, RT). Free biotin was removed by dialysis with PBS (pH 7.4). Subsequently, 100 µl biotinylated ganglidiomab (0.2 µg/ml) was added to each well in order to analyze anti-GD_2 antibodies bound to GD_2. After incubation (2 h, 37 °C), plates were washed three times (PBS, pH 7.4, 0.1 % BSA). Pierce High Sensitivity NeutrAvidin-HRP (Thermo Scientific, Erlangen, Germany) was diluted 1:10,000 and 100 µl added per well, incubated for 20 min at 37 °C, and washed five times (PBS, pH 7.4, 0.1 % BSA). The ELISA was developed with TMB substrate solutions as described above.

Competitive inhibition of anti-GD_2 antibody binding to GD_2 by ganglidiomab

In order to evaluate GD_2 surrogate characteristics of ganglidiomab, that is, anti-idiotypic properties, we used it to inhibit binding of anti-GD_2 antibodies of the 14.18 family, sharing identical CDR regions, to GD_2 antigen using an ELISA. To this end, microtiter plates were coated with GD_2 (Sigma Aldrich, Steinheim, Germany) (50 ng/well; 99.9 % methanol; 1 h, 50 °C) as a capture antigen. After evaporation of methanol, wells were blocked with 1 % bovine serum albumin (BSA) in PBS (pH 7.4) (1 h, 37 °C) and washed three times (PBS, pH 7.4, 0.1 % BSA). Hybridoma cell supernatants (43 µg/ml ganglidiomab) were diluted 1:25 with PBS (1.72 µg/ml) and 1:2 serial diluted yielding 0.860 µg/ml (1:2), 0.430 µg/ml (1:4), 0.215 µg/ml (1:8), 0.108 µg/ml (1:16), 0.054 µg/ml (1:32), 0.027 µg/ml (1:64), and 0.013 µg/ml (1:128). Medium alone was used as a control.

Then, anti-GD_2 antibodies 14G2a, ch14.18/CHO, ch14.18-delta-CH2, and hu14.18 and immunocytokines ch14.18-IL2 and hu14.18-IL2 were applied to each ganglidiomab dilution and controls at a final concentration of 0.330 µg/ml, vortexed, and 200 µl of each dilution was added per well of a 96-well plate in quadruplicates. Plates were incubated (2 h, 37 °C) and washed five times (PBS, pH 7.4, 0.1 % BSA). Again, two variant methods of detection were used: In the first variant, anti-GD_2 antibodies (ch14.18/CHO, hu14.18, and ch14.18-delta-CH2 and immunocytokines ch14.18-IL2 and hu14.18-IL2) bound to GD_2 were analyzed by incubation with HRP-conjugated goat anti-human IgG antibody as a secondary antibody, developed with TMB substrate as described above.

In order to also confirm results for the native antibody 14G2a, in the second variant, goat anti-human IgG HRP was replaced by biotinylated ganglidiomab. For this purpose, 100 µl biotinylated ganglidiomab (0.2 µg/ml) was added to each well in order to analyze anti-GD_2 antibodies (14G2a, ch14.18/CHO, ch14.18-delta-CH2, and hu14.18 and immunocytokines ch14.18-IL2 and hu14.18-IL2) bound to GD_2. After incubation (2 h, 37 °C), plates were washed three times (PBS, pH 7.4, 0.1 % BSA). Incubation with NeutrAvidin-HRP and development with TMB substrate were performed as described above. ELISA signals (OD) determined with controls lacking competitive ganglidiomab were defined as 100 % binding, that is, 0 % inhibition. Percent of binding inhibition was then calculated according to the formula: binding inhibition (%) = 100 % − (experimental OD/0 % inhibition OD × 100 %).

Binding affinity of ganglidiomab and 1A7 to anti-GD_2 antibodies

Binding affinities of ganglidiomab to anti-GD_2 antibodies were determined by surface plasmon resonance analysis (25 °C, PBS: 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) using a two-channel Biacore X100 system (Biacore, Uppsala, Sweden) and compared with 1A7. Ganglidiomab and 1A7 (both mIgG1, kappa) were diluted in acetate buffer (10 mM, pH 5.5, Biacore, Uppsala, Sweden) to a final concentration of 7.5 µg/ml and immobilized on a CM5 sensor chip using an amine Coupling Kit (Biacore, Uppsala, Sweden). Mouse IgG1 (R&D Systems Inc, Minneapolis, MN, USA) was used as a negative control.

Equal volumes of 0.05 M *N*-hydroxysuccinimide and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride were injected at 5 µl/min for 8 min to activate the chip surface. Free reactive ester groups on the chip surface were blocked 8 min at 5 µl/min by ethanolamine hydrochloride buffer (1 M, pH 8.5). To calculate the dissociation constant (KD), equilibrium values were used, that is, response units (RU), after measurement of a 1:2 step dilution series of each analyte in a concentration range of 193.33–0.73 µg/ml. Data were analyzed using BiaEvaluation software, using a steady-state fit model (Biacore, Uppsala, Sweden). For regeneration of the chip surface after analysis of each analyte, it was washed four times for 30 s with glycine–HCl (10 mM glycine, pH 1.5). Affinity measurements of ganglidiomab and 1A7 for each anti-GD_2 antibody analyzed were done in triplicates.

Binding and inhibition of GD_2-specific chimeric receptor-expressing NK cells by ganglidiomab

NK-92-scFv(ch14.18)-z cells were incubated for 15 min on ice with six different concentrations (0.0625, 0.125, 0.5, 2, 4, and 5 µg) of biotinylated ganglidiomab, alone or together with 0.5 µg anti-idiotypic antibody 1A7. This step was followed by a 15-min incubation on ice with FITC-labeled streptavidin (1:400, BD Pharmingen, San Diego, CA, USA). Unstained NK-92-scFv(ch14.18)-z as well as unstained NK-92 and NK-92 stained with 0.5 µg ganglidiomab followed by incubation with FITC-labeled streptavidin were used as controls and analyzed by flow cytometry (LSRII flow cytometer, BD, Biosciences, San Jose, CA, USA). Cytotoxicity of NK-92-scFv(ch14.18)-z against the GD_2-expressing neuroblastoma cell line LAN-1 was determined in vitro with a standard ^{51}Cr release assay. The blocking effect of 1.0 or 10 µg/ml ganglidiomab on the cytotoxicity of NK-92-scFv(ch14.18)-z was evaluated and compared to a mouse IgG1 isotype control (clone X40, BD Biosciences, San Jose, CA, USA).

Amplification, cloning, and sequencing of variable heavy (VH) and light chain (VL) coding genes of ganglidiomab

Total RNA was isolated from $1x10^7$ hybridoma cells using the RNeasy® Mini Kit (QIAGEN GmbH, Hilden, Germany), and cDNA was synthesized using SuperScript® II Reverse Transcriptase (Invitrogen GmbH, Darmstadt, Germany) according to the manufacturer's protocol. Degenerate primers [13–15] (suppl. Table 1) were used for amplification of VH and VL coding regions of ganglidiomab (Eurofins MWG Operon, Ebersberg, Germany) by polymerase chain reaction (PCR: once for 2 min at 94 °C, followed by 29 cycles of 94 °C 15 s, 70 °C 15 s, 72 °C 30 s, and one final elongation at 72 °C for 30 s). PCR products were isolated from the agarose gels using a Nucleo Spin® Extract II Kit (Macherey–Nagel, Düren, Germany) and cloned with the TOPO TA® cloning system (LifeTechnologies GmbH, Darmstadt, Germany) according to the manufacturer's guidelines. Finally, plasmids containing fragments of the expected molecular size were sequenced (LGC Genomics, Berlin, Germany).

Gel electrophoresis of immunoglobulins and Western blot

The integrity of monoclonal antibodies and antibody–cytokine fusion proteins used in this study was analyzed by standard SDS–PAGE under reducing conditions. Briefly, protein concentrations were determined by the Bradford assay. For reducing conditions, 50 µl of β-mercaptoethanol (Sigma Aldrich, Steinheim, Germany) was added to 950 µl of Laemmli sample buffer (Bio-Rad Laboratories, Munich, Germany). Immunoglobulins (10 µg in 25 µl) were mixed with 25 µl sample buffer and incubated for 5 min at 95 °C. Protein separation was accomplished at 120V on a 10 %

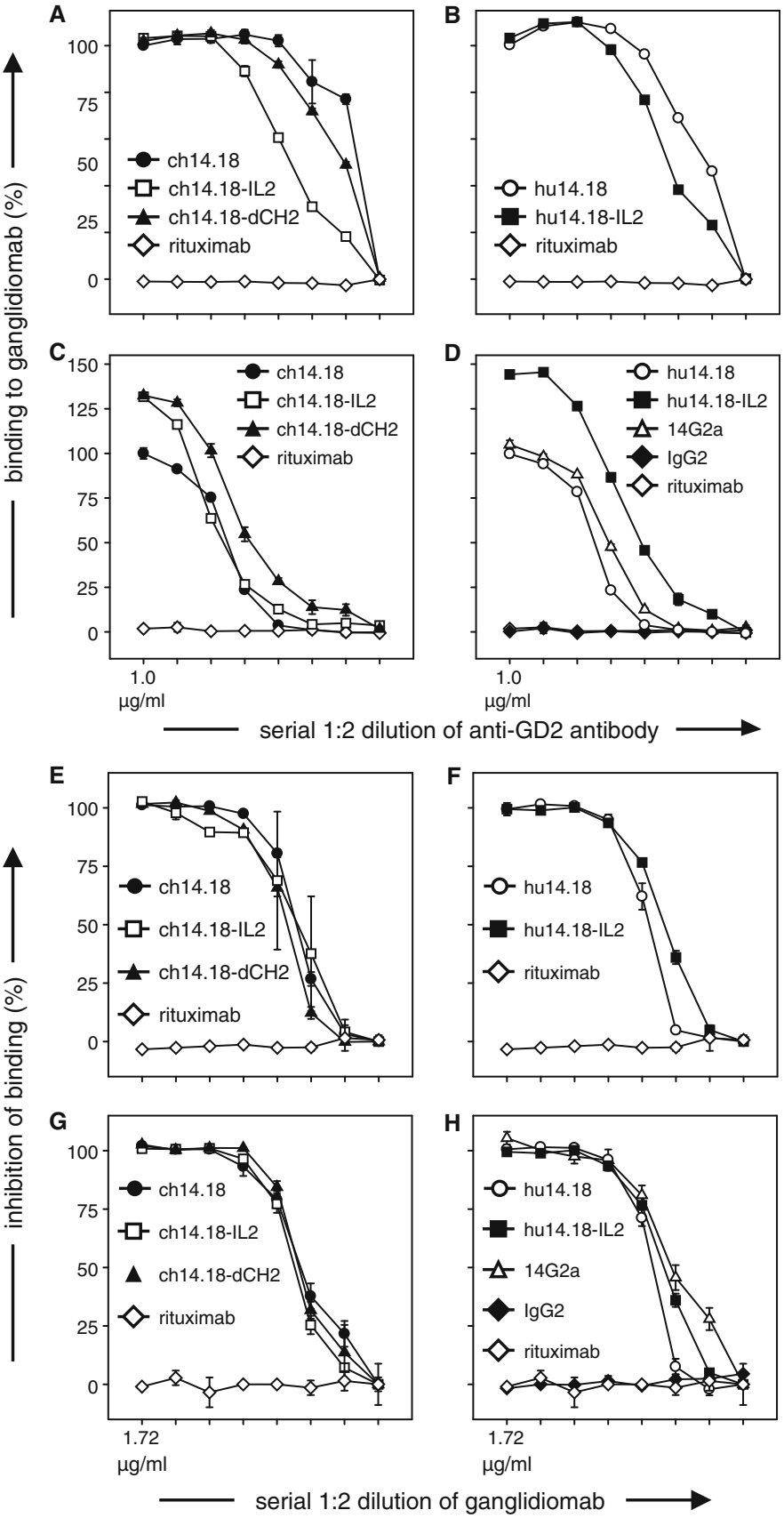


Fig. 1 Binding of anti-GD_2 antibodies to ganglidiomab and competitive binding to nominal antigen GD_2. Binding of anti-GD_2 antibodies of the 14.18 family to ganglidiomab was analyzed by two ELISA variants using anti-human HRP-conjugated IgG antibody (**a**, **b**) and biotinylated ganglidiomab (**c**, **d**) as secondary antibodies, respectively, as described in "Materials and methods." ELISA signals observed with 1 µg/ml ch14.18/CHO were defined as 100 % binding. Data are expressed relative to binding of ch14.18/CHO antibody and are expressed in mean values ± SD of experiments performed in triplicates. Differences between GD_2 antibodies and controls were statistically significant ($*p < 0.01$). Competitive binding inhibition of anti-GD_2 antibodies of the 14.18 family to GD_2 by ganglidiomab was analyzed by two ELISA variants, using anti-human HRP-conjugated IgG antibody (**e**, **f**) and biotinylated ganglidiomab (**g**, **h**) as secondary antibodies for detection, respectively, as described in "Materials and methods." Data represent percent binding inhibition and are shown in mean values ± SD of experiments performed in triplicates. Differences between GD_2 antibodies and controls were statistically significant ($*p < 0.01$)

SDS polyacrylamide gel (1 h). Protein bands were visualized by Coomassie staining for 30 min (Bio-Rad Laboratories, Munich, Germany).

Analysis of secreted protein from ganglidiomab-producing hybridoma cells was accomplished using both culture supernatants and cell lysates, respectively. After 4 days of hybridoma cultivation, culture supernatants were collected and the concentration of ganglidiomab determined with a hu14.18 solid-phase ELISA.

Hybridoma cells (1×10^6) were homogenized in 300 µl cold lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5 % Triton-X 100, 0.02 % NaN_3) containing 1 µl of a protease inhibitor cocktail and placed for 30 min on ice. The protease inhibitor cocktail stock solution consisted of 104 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 80 µM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, and 1.5 mM pepstatin A (Sigma Aldrich, Steinheim, Germany). Then, lysates were centrifuged (15 min, 10,000*g*), and protein extracts (20 µg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (8 % SDS–PAGE) and transferred onto a polyvinyldifluoride membranes (Bio-Rad Laboratories, Munich, Germany). After blocking (dry milk, 5 %, RT, 50 min), membranes were incubated over night (4 °C) with monoclonal ch14.18/CHO (1:1,000). HRP-conjugated goat anti-human IgG antibody served as a secondary antibody (1:20,000, TBS, 45 min, RT) (Sigma Aldrich, Steinheim, Germany). Visualization was achieved with Immun-Star™ HRP luminol/enhancer (6 ml) and Immun-Star™ HRP peroxide buffer (6 ml) (5 min, RT), and chemiluminescence detected by the ChemiDoc™ XRS$^+$ Molecular Imager (Bio-Rad Laboratories, Munich, Germany).

Analysis of anti-GD_2 immune response following vaccination with ganglidiomab

Mice ($n = 7$) were immunized by i.p. injection with 100 µg ganglidiomab with or without 2 mg of adjuvant aluminum hydroxide $Al(OH)_3$ (Sigma Aldrich, Steinheim, Germany). One group received $Al(OH)_3$ alone. Immunization was repeated four times at 2-week intervals. Blood samples were collected before the first and after each immunization at indicated time points and analyzed with a GD_2 solid-phase ELISA. In order to prove GD_2 specificity of the humoral response, serum probes of immunized animals were pre-incubated with ganglidiomab. Anti-neuroblastoma activity of serum from immunized mice was analyzed in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays using acetoxymethyl ester of calcein (calcein-AM) (Sigma Aldrich, Steinheim, Germany) [16].

For ADCC, effector cells were isolated from mouse spleens as described previously [17]. Murine GD_2-positive NXS2 neuroblastoma cells were used as target cells (E:T ratio 100:1). Calcein labeling of NXS2 target cells (1×10^6/ml) was accomplished by incubation of cells with 10 µM calcein-AM (30 min, 37 °C, 100 rpm). After five wash steps (37 °C), cells were added to a 96-well plate and incubated for 30 min with heat-inactivated mouse serum samples (30 min, 56 °C) and collected at the end of the immunization procedure (12.5 % serum, 37 °C). Effector cells were added for 4 h at 37 °C. Then, supernatants (50 µl) of each well were transferred to black 96-well plates (PAA, Pasching, Austria) for determination of fluorescence at 495 nm excitation and 515 nm emission wavelengths by a Synergy HT multimode microplate reader (BioTek Germany, Bad Friedrichshall, Germany). Experiments were analyzed in triplicates using at least six replicate wells for spontaneous (target cells only) and maximum release (target cells treated for 20 s with ultrasonic homogenizer) (Hielscher Ultrasonics, Teltow, Germany). Cytotoxicity mediated by ADCC in percent was calculated according to the formula: (test release − spontaneous release)/(maximum release − spontaneous release) × 100 %. For CDC, 12.5 % serum of immunized mice was incubated with calcein-AM-labeled target cells without effector cells for 4 h at 37 °C. Heat-inactivated serum (30 min, 56 °C) of the respective mouse group served as a negative control. Cytotoxicity mediated by CDC was calculated according to the formula: (test release − negative control release)/(maximum release − negative control release) × 100 %. Both ADCC and CDC data are expressed relative to respective controls.

Table 1 Dissociation constants (KD) of ganglidiomab and 1A7 for anti-GD_2 antibodies of the 14.18 family

Ligand	Ganglidiomab KD (nM)[a]	1A7 KD (nM)[a]
14G2a	53.5 ± 19.20	5.35 ± 4.72
ch14.18	13.1 ± 4.23	3.65 ± 2.39
ch14.18-IL2	49.3 ± 23.40	1.13 ± 1.06
ch14.18-delta-CH2	23.3 ± 10.20	10.9 ± 10.30
hu14.18	10.8 ± 5.01	11.8 ± 11.70
hu14.18-IL2	13.7 ± 4.95	10.8 ± 4.97

[a] Dissociation constants (KD) of ligands 14G2a, ch14.18/CHO, ch14.18-IL2, ch14.18-delta-CH2, hu14.18, and hu14.18-IL2 from ganglidiomab and 1A7 were determined by surface plasmon resonance in a Biacore X100 system followed by steady-state analysis. Values are means ± SE of 3 independent experiments

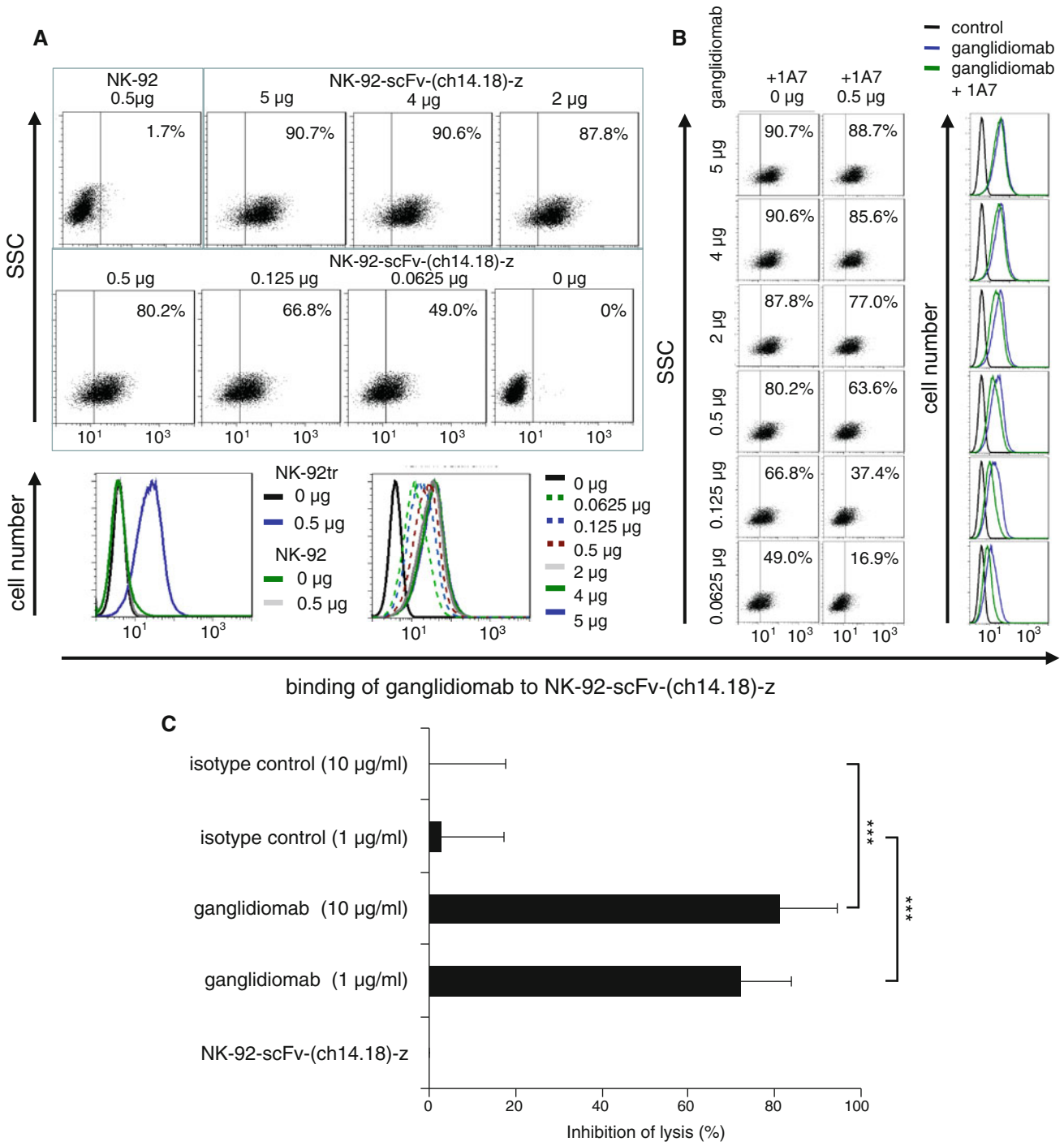


Fig. 2 Binding of ganglidiomab to NK-92-scFv(ch14.18)-z and inhibition of GD2-specific cytolysis of neuroblastoma cells. Binding of ganglidiomab to NK-92-scFv(ch14.18)-z cells (**a**), competitive binding of ganglidiomab and 1A7 (**b**) as well as inhibition of GD_2-specific NK-92-scFv(ch14.18)-z cell-mediated lysis of neuroblastoma cells by ganglidiomab (**c**) were analyzed by flow cytometry (**a**, **b**) and in a ^{51}Cr-release assays (**c**) as described. **a** NK-92-scFv(ch14.18)-z cells were incubated with increasing amounts of biotinylated ganglidiomab and FITC-labeled streptavidin. Negative controls were NK-92-scFv(ch14.18)-z incubated with isotype control and parental NK-92 incubated with ganglidiomab. **b** Competitive binding of ganglidiomab and 1A7 to NK-92-scFv(ch14.18)-z was analyzed with increasing amounts of biotinylated ganglidiomab in the presence and absence of 1A7 (0.5 µg) followed by incubation with FITC-labeled streptavidin. Cells (1×10^5) were analyzed by LSRII using FACSDiva software. Final data analysis was performed with FlowJo software. Results from one representative experiment out of three experiments are presented (**a**, **b**). **c** Inhibition of specific cytotoxicity of NK-92-scFv(ch14.18)-z toward GD_2-positive neuroblastoma cell line LAN-1 was analyzed in a standard ^{51}Cr release assay in the presence and absence of 10 and 1 µg/ml ganglidiomab and mouse IgG1 isotype controls, respectively. Results are presented as percent inhibition of lysis from three independent experiments (mean ± SD). Differences between ganglidiomab and controls were statistically significant (***$p < 0.0001$, Mann–Whitney U test)

Statistics

Differences between experimental groups of animals and controls as well as between experimental readouts and controls were analyzed by the Student's *t* test. Only *p* values <0.05 were regarded as significant.

Results

Screening of clones and identification of ganglidiomab

Immunization with 14G2a induced antibodies in the serum of mice which bound to anti-GD_2 antibodies ch14.18 and hu14.18. Splenocytes of these mice were fused with SP2/0 cells and subcloned. Subclone 17 was found to yield anti-ch14.18 and anti-hu14.18 activity in the screening ELISA (suppl. Table 2). After 5 rounds of subcloning, the resulting hybridoma cell line was used for the purification of mouse IgG and for functional and structural characterization of the antibody. The IgG subclass was identified by ELISA to be composed of murine IgG1 heavy and kappa light chains (data not shown).

Binding of anti-GD_2 antibodies to ganglidiomab and competition of binding with antigen GD_2

Binding of ganglidiomab to antibodies of the 14G2a family and competition with the nominal antigen GD_2 were analyzed by two versions of a solid-phase ELISA, and results were compared to rituximab used as negative control (Fig. 1). Binding analysis of MABs 14G2a, ch14.18, ch14.18-delta-CH2, hu14.18, and ch14.18-IL2 as well as hu14.18-IL2 immunocytokines to ganglidiomab revealed a concentration-dependent signal increase in contrast to the negative control (Fig. 1a–d). We also determined the dissociation constants of ganglidiomab from anti-GD_2 antibodies by Biacore analyses using "steady-state" conditions, ranging from 10.8 ± 5.01 to 53.5 ± 1.92 nM (Table 1). Importantly, binding of all anti-GD_2 antibodies and anti-GD_2 immunocytokines to antigen GD_2 was competitively inhibited by ganglidiomab in a concentration-dependent manner (Fig. 1e–h), thereby clearly demonstrating GD_2 surrogate activity and anti-idiotype characteristics of ganglidiomab.

Functional GD_2 surrogate activity of ganglidiomab

The analysis of functional GD_2 surrogate activity of ganglidiomab involved NK-92 cells expressing scFv(ch14.18)-zeta receptor, comprising an anti-GD_2 ch14.18 single chain Fv (scFv) antibody fusion protein with CD3 zeta chain as a signaling moiety [11]. Here, we demonstrate concentration-dependent binding of ganglidiomab to NK-92-scFv(ch14.18)-z by flow cytometry in contrast to NK-92 negative controls (Fig. 2a). Binding of ganglidiomab to NK-92-scFv(ch14.18)-z was competitively inhibited by 1A7 (Fig. 2b), another anti-idiotypic antibody of anti-GD_2 antibody 14G2a [18], supporting the GD_2 surrogate properties of ganglidiomab. This inhibition was not complete even with excess of 1A7 suggesting a

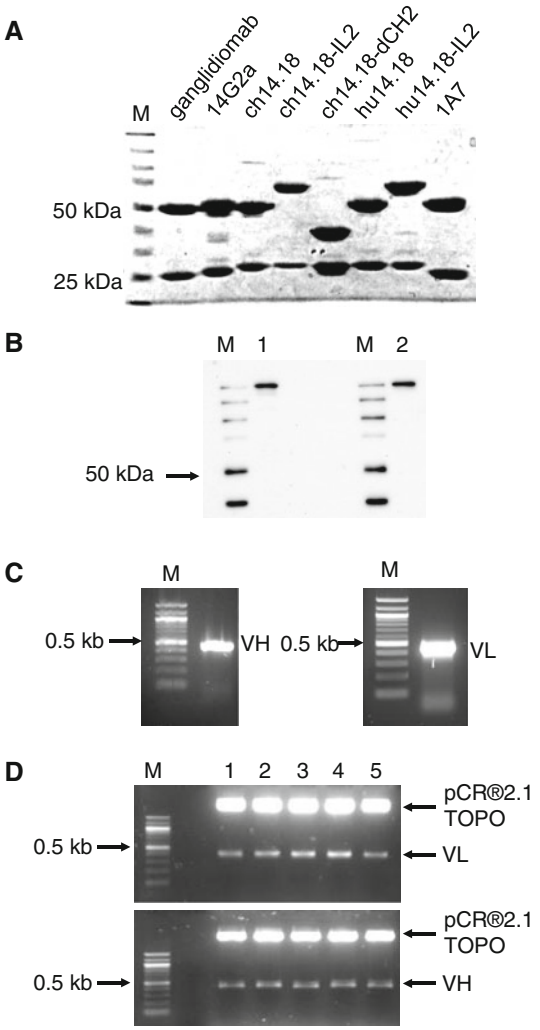


Fig. 3 Molecular characterization of ganglidiomab. **a** Integrity of immunoglobulins used in this study was analyzed by 8 % SDS polyacrylamide gel electrophoresis under reducing conditions. **b** The protein structure of ganglidiomab was analyzed by Western blot following SDS–PAGE under non-reducing conditions (**a**) from cell lysates of ganglidiomab-producing hybridoma cells (1) as well as in the supernatant (2) using anti-GD_2-antibody ch14.18/CHO as detection antibody (*M* Precision Plus Protein standard). **c** The coding sequences of variable heavy and light chains of ganglidiomab were amplified by RT-PCR as described in "Materials and methods." PCR products were analyzed by agarose gel electrophoresis. M: 100-bp ladder. **d** PCR products were cloned into pCR®2.1-TOPO plasmids and analyzed by an *Eco*RI restriction enzyme digest. *1–5* individual clones, *M* 100-bp ladder

difference in binding sites to ch14.18 variable regions between 1A7 and ganglidiomab. Interestingly, GD_2-specific lysis of LAN-1 neuroblastoma cells was inhibited up to 80 % by ganglidiomab in contrast to isotype control further supporting the anti-idiotype characteristics of ganglidiomab (Fig. 2c).

Molecular characterization of ganglidiomab

The molecular characterization of ganglidiomab first involved gel electrophoresis under denaturing reducing conditions, which revealed bands typical for heavy (~50 kDa) and light chains (~25 kDa) of an IgG molecule (Fig. 3a). Antibody–cytokine fusion proteins (hu14.18-IL2 and ch14.18-IL2) as well as ch14.18-delta-CH2 reveal the typical pattern of larger (IgG heavy chain plus IL2 equals ~65 kDa) and smaller heavy chain bands (heavy chain missing the CH2 domain equals ~38 kDa), respectively. Ganglidiomab was also analyzed by Western blot using both supernatant and lysate from ganglidiomab hybridoma cells under non-reducing conditions (Fig. 3b). The native ganglidiomab protein was probed with anti-GD_2 antibody ch14.18/CHO on the blotted membrane, revealing bands corresponding in size to immunoglobulin heavy and light chains, again supporting the anti-idiotype characteristics of ganglidiomab. In order to determine the sequence of ganglidiomab, cDNA of ganglidiomab hybridoma cells was amplified with degenerate primers [13–15] (see also suppl. Table 1) for murine VH and VL coding regions (Fig. 3c). PCR products were cloned into pCR®2.1-TOPO plasmids and analyzed by an *Eco*RI restriction enzyme digest (Fig. 3d). Clones containing an insert of the correct size were subjected to sequencing. The sequences of frameworks (FRs) and complementarity-determining regions (CDRs) of ganglidiomab VH and VL were identified by homology search in the Kabat database and

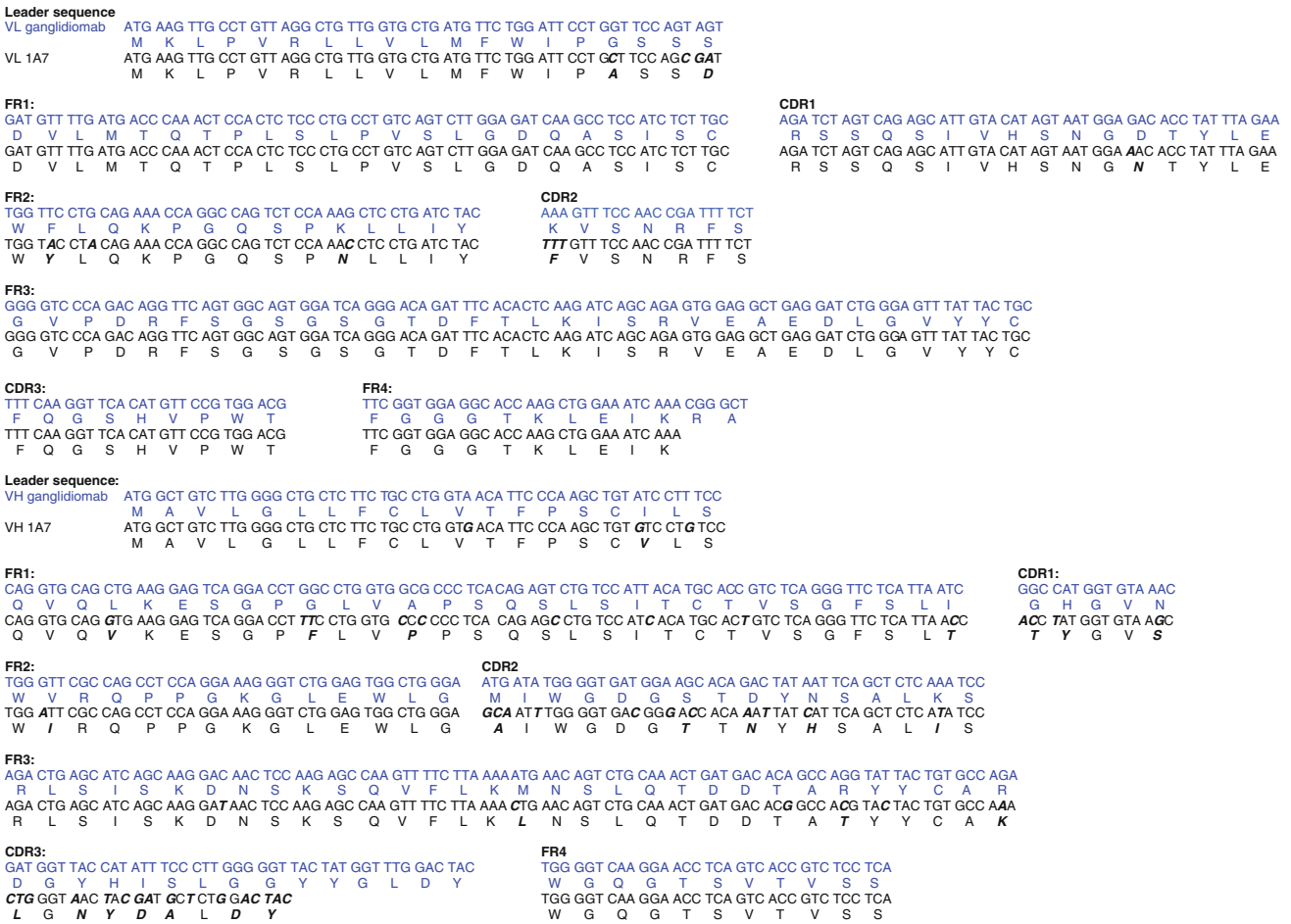
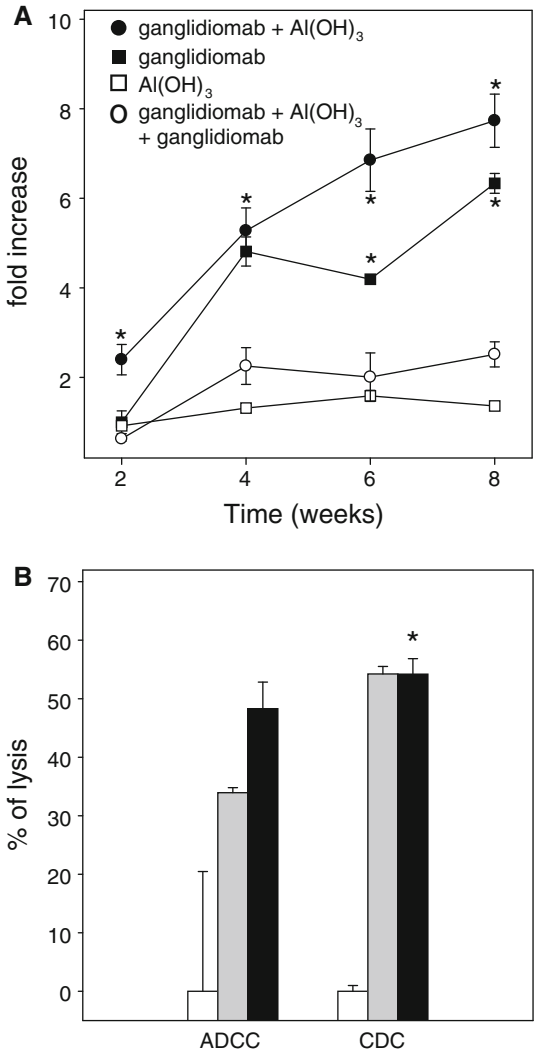


Fig. 4 Coding DNA sequence and protein sequence of variable regions of ganglidiomab. The coding DNA sequences and the protein sequences of FR 1–4 and CDR 1–3 and the leader peptide of the variable regions of heavy and light chains of ganglidiomab (*blue*) are aligned with sequences of 1A7 (*black*). Differences between the two anti-idiotypic antibodies are indicated in bold letters. The homology in coding DNA and protein sequences between ganglidiomab and 1A7 of VH are 77.46 and 71.13 % and of VL 95.74 and 93.98 %, respectively



◀ **Fig. 5** GD_2-specific humoral immune response following immunization with ganglidiomab. Groups of female A/J mice ($n = 7$) were immunized at 2-week intervals with ganglidiomab alone or in combination with $Al(OH)_3$ adjuvant and compared to $Al(OH)_3$ adjuvant controls. Serum samples were taken before the first and after each immunization. Sera sampled at each time point of the respective mouse group were pooled and analyzed by GD_2 *solid-phase* ELISA (**a**), antibody-dependent cytotoxicity (*ADCC*) and complement-dependent cytotoxicity (*CDC*) (**b**). **a** The induction of GD_2-specific antibodies following vaccination with ganglidiomab with (*closed circles*) and without $Al(OH)_3$ adjuvant (*closed squares*) compared to $Al(OH)_3$ adjuvant alone (*open squares*) was analyzed by GD_2 ELISA. Data are expressed as fold increase relative to OD values measured at the start of immunization and represent mean ± SE. In order to prove GD_2 specificity of observed responses, serum samples from mice immunized with ganglidiomab-$Al(OH)_3$ were incubated prior to the GD_2 ELISA with excess ganglidiomab. Differences between groups of mice immunized with ganglidiomab with or without $AL(OH)_3$ adjuvant and controls as well as sera of ganglidiomab-$Al(OH)_3$ group pre-treated with excess ganglidiomab (1 mg) were statistically significant (*t* test; $*p < 0.05$). **b** Cytotoxicity mediated by ADCC and CDC induced by serum of mice immunized with ganglidiomab with (*black column*) or without (*gray column*) adjuvant $Al(OH)_3$ are expressed as percent of target cell lysis relative to the control group receiving $Al(OH)_3$ adjuvant alone (*white column*). Data represent mean ± SE. Differences between sera of immunized mice and controls were statistically significant (*t* test; $*p < 0.05$)

compared to the sequence reported for 1A7 [18] (Fig. 4). The homology of coding DNA and protein sequences between ganglidiomab and 1A7 of VH were only 77.46 and 71.13 % and of VL 95.74 and 93.98 %, respectively, indicating a clear difference between the two anti-Id antibodies.

Induction of a GD_2-specific immune response following vaccination with ganglidiomab

We investigated the induction of a humoral anti-GD_2 immune response following vaccination with GD_2 surrogate ganglidiomab in vivo. For this purpose, A/J mice were immunized with ganglidiomab alone and in combination with $Al(OH)_3$ as an adjuvant and the humoral response was analyzed with a GD_2 ELISA. Mice receiving ganglidiomab immunizations clearly revealed an anti-GD_2 immune response in vivo, in contrast to the control group receiving adjuvant alone (Fig. 5a). Importantly, the ELISA signals were completely blocked when sera of immunized mice were co-incubated with an excess of ganglidiomab, proving the specificity of the humoral immune response for GD_2. The anti-neuroblastoma activity of the induced immune response was then determined with sera of immunized mice in ADCC and CDC assays and compared to controls (Fig. 5b). Only sera of mice immunized with ganglidiomab mediated a cytotoxic activity against neuroblastoma target cells in both assays, thereby again demonstrating functional anti-neuroblastoma activity following vaccination with ganglidiomab.

Discussion

Passive immunotherapy of neuroblastoma with antibody ch14.18 emerges as an established concept for the treatment of this challenging malignancy in pediatric oncology [1, 3]. The disadvantage, in most cases, is the absence of an immune memory, leading to a decrease in anti-GD_2 activity over time. Vaccines may overcome this obstacle, provided that they induce a GD_2-specific immune response in vivo. Attempts to achieve this goal with vaccines based on the GD_2 antigen revealed only marginal efficacy due to the carbohydrate nature of the antigen, which is T cell independent [19]. Therefore, the use of GD_2 surrogate protein structures may provide a promising alternative approach. Here, we describe the generation and characterization of ganglidiomab, a novel anti-idiotype antibody of the 14.18

family. We demonstrate binding of 14.18 antibody derivatives to ganglidiomab in contrast to controls (Figs. 1a–d, 2). Importantly, binding of 14.18 antibody derivatives to the nominal antigen GD_2 was competitively inhibited by ganglidiomab, thus clearly demonstrating anti-idiotype and GD_2 surrogate function of ganglidiomab (Fig. 1e, f). This finding was supported by competitive binding inhibition of ganglidiomab by another GD_2 surrogate, namely 1A7 (Fig. 2). Ganglidiomab was characterized structurally (Fig. 3), functionally (Fig. 2; Table 1), and its VL and VH chains were cloned and sequenced (Figs. 3, 4). Importantly, mice vaccinated with ganglidiomab revealed a GD_2-specific humoral immune response, which translated into GD_2-specific anti-neuroblastoma activity (Fig. 5), suggesting its usefulness as a vaccine against GD_2-expressing malignancies. A similar approach was indeed reported using murine IgG1 anti-idiotype antibody 1A7 that mimics ganglioside GD_2 to treat melanoma [20, 21] and neuroblastoma patients [22]. In the latter study, 1A7 was used alone and in combination with adjuvant Q21, $Al(OH)_3$, and GM-CSF. Interestingly, all patients developed an anti-GD_2 humoral immune response, suggesting that the xenogeneic protein vaccine provides for sufficient adjuvanticity to induce an immune response. The sera of melanoma patients mediated ADCC in all patients [20] similar to effects reported here for ganglidiomab in mice (Fig. 5). Although 1A7 was reported to have GD_2 surrogate activity, a comparison of VH and VL coding DNA and protein sequences between ganglidiomab and 1A7 revealed clear differences between these antibodies (Fig. 4). Importantly, this new anti-idiotype antibody of the ch14.18 family can be used for the monitoring of clinical trials with ch14.18/CHO antibody and its derivatives including hu14.18-IL2 [23] as well as approaches using NK- or T cell-based immunotherapies equipped with chimeric antigen receptors based on ch14.18 [11, 24]. In summary, we report here the generation and characterization of a novel anti-GD_2 anti-idiotype antibody ganglidiomab that mimics GD_2, providing an important baseline for the development of vaccines against NB as well as for immune monitoring of clinical trials with anti-GD_2 antibodies of the 14.18 family.

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Conflict of interest There is no conflict of interest to be disclosed.

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