Costimulatory Protein 4IgB7H3 Drives the Malignant Phenotype of Glioblastoma by Mediating Immune Escape and Invasiveness

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Abstract

Purpose: Recent work points out a role of B7H3, a member of the B7-family of costimulatory proteins, in conveying immunosuppression and enforced invasiveness in a variety of tumor entities. Glioblastoma is armed with effective immunosuppressive properties resulting in an impaired recognition and ineffective attack of tumor cells by the immune system. In addition, extensive and diffuse invasion of tumor cells into the surrounding brain tissue limits the efficacy of local therapies. Here, 4IgB7H3 is assessed as diagnostic and therapeutic target for glioblastoma.

Experimental Design: To characterize B7H3 in glioblastoma, we conduct analyses not only in glioma cell lines and glioma-initiating cells but also in human glioma tissue specimens.

Results: B7H3 expression by tumor and endothelial cells correlates with the grade of malignancy in gliomas and with poor survival. Both soluble 4IgB7H3 in the supernatant of glioma cells and cell-bound 4IgB7H3 are functional and suppress natural killer cell–mediated tumor cell lysis. Gene silencing showed that membrane and soluble 4IgB7H3 convey a proinvasive phenotype in glioma cells and glioma-initiating cells in vitro. These proinvasive and immunosuppressive properties were confirmed in vivo by xenografted 4IgB7H3 gene silenced glioma-initiating cells, which invaded significantly less into the surrounding brain tissue in an orthotopic model and by subcutaneously injected LN-229 cells, which were more susceptible to natural killer cell–mediated cytotoxicity than unsilenced control cells.

Conclusions: Because of its immunosuppressive and proinvasive function, 4IgB7H3 may serve as a therapeutic target in the treatment of glioblastoma.

Introduction

One of the key biologic features of glioblastoma is its ability to suppress the immune system resulting in an impaired recognition and attack of the tumor cells by the immune system. A number of factors have been identified within the last 2 decades that are held responsible for the immunosuppressive nature of glioblastomas (1-8). TGF-β turned out to induce apoptosis in T cells, to downregulate costimulatory proteins on cytotoxic T cells, natural killer (NK) cells, and glioma cells, as well as to upregulate immunosuppressive ligands on glioblastoma cells. In addition to the immunosuppressive properties, invasion of tumor cells into the surrounding brain tissue is another hallmark of human glioblastoma. This invasive behavior limits the feasibility of local treatment such as surgical tumor resection or involved-field radiotherapy. Glioblastoma cells usually invade as single cells migrating along white matter tracts implicating the involvement of integrin-mediated signaling pathways and the degradation of components of the extracellular matrix by matrix metalloproteinases (MMP). Recently, two isoforms of a novel member of the B7-family of costimulatory proteins, 2IgB7H3 and 4IgB7H3, have been identified (9, 10). The latter was more widely expressed in human maturing dendritic cells, T cells, and many human tumor cell lines including glioblastoma (11). 4IgB7H3 was initially supposed to convey T-cell activation and to induce IFN-γ production, but more recent evidence suggests that 4IgB7H3 expressed by different human malignancies suppresses NK cells and cytotoxic T cells. In this regard, NK-mediated lysis of neuroblastoma cell lines was...
Cells and cell culture

LN-229 glioma cells were kindly provided by Dr. N. de Tribolet (Department of Neurosurgery, University Hospital, Lausanne, Switzerland). The cell line was maintained in Dulbecco’s Modified Eagle’s Medium containing 10% fetal calf serum and penicillin (100 IU/mL)/streptomycin (100 mg/mL; ref. 24). For the generation of GIC cultures, tumor samples were obtained from adult patients diagnosed with glioblastoma after informed consent. The tumor and the GIC culture methods were modified from the study of Svendsen and colleagues (25) as previously described (26). Glioma characteristic chromosomal abnormalities have been verified by array comparative genome hybridization (B. Radlwimmer, Department of Molecular genetics, German Cancer Research Center Heidelberg, Heidelberg, Germany). Lentiviral knockdown of 4lgB7H3 and control knockdown cells were produced with lentiviral short hairpin (sh) 4lgB7H3 and control particles from Santa Cruz Biotechnology (Cat. no.: sc-45444-V and sc-108080). The unselected 4lgB7H3 knockdown cells were named sh 4lgB7H3 pool cells. From these cells, a clonal knockdown was selected with a higher knockdown rate, which was termed sh 4lgB7H3 clone19. Human cerebral microvascular endothelial cells (HCMEC) and human brain vascular pericytes were purchased from Sciencell.

Lysates for immunoblots and cell culture supernatants were prepared as described previously (27, 28). Briefly, supernatants were generated for 72 hours after plating 3 × 10^6 cells in serum-free medium. Serum-free supernatants were concentrated with the Centriplus centrifugal filter device YM-3 (3,000-Da cutoff point; Millipore). Afterward, a Bradford assay was conducted to assure that equal amounts of supernatant-derived protein were used for the NK cell lysis assays or immunoblot analysis. To unravel the mechanisms of how 4lgB7H3 is cleaved by glioblastoma cells and how 4lgB7H3 is regulated, 1.25 × 10^5 LN-229 cells were seeded in serum-free medium and the following compounds and treatments were used:

1. To evaluate hypoxia, cells were incubated for 48 hours in serum-free medium under hypoxic conditions at 1% O_2 and compared with cells that were kept at 21% O_2.

2. To evaluate the influence of protein kinase C activation, cells were incubated with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) diluted in...
dimethyl sulfoxide (DMSO) at a final concentration of 100 nmol/L for 48 hours (29).

3. The influence of TGF-β inhibition was tested with LY2157299, a TGF-β receptor kinase I inhibitor at 25 nmol/L (Axon Medchem BV; ref. 29) or the synthetic furin inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk; Bachem), which was diluted in methanol. Cells were incubated at 15 μmol/L. New furin inhibitor was added every 12 hours (30).

4. To examine whether 4IgB7H3 is cleaved by MMP, the MMP inhibitor ilomastat (22) was used at concentrations of 5 to 10 μmol/L (Chemicon International). Respective DMSO controls were included.

Afterward, cells were collected to carry out quantitative real-time PCR (qRT-PCR), immunoblotting of supernatants and lysates, and flow cytometric analysis. All compounds used were shown to be functional in other assays.

**Immunoblot analysis**

Cells were lysed in 50 mmol/L Tris-HCl (pH 8) containing 120 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Nonidet P-40, 2 μg/ml aprotinin, 10 μg/ml leupeptin (Sigma-Aldrich), and 100 μg/ml phenylmethylsulfonylfluoride. Protein levels were analyzed by immunoblotting using 4IgB7H3 at 30 μg of protein per lane with the respective antibodies in the concentrations recommended by the manufacturer (28). The antibodies used were goat polyclonal anti-human B7H3 (R&D) and rabbit anti-human anti-B7H3 antibody HPA 017139 (Atlas Antibodies). Protein bands were visualized using horseradish peroxidase–coupled secondary antibodies (Sigma-Aldrich). Equal protein loading was ascertained by Ponceau S staining as well as α-tubulin stainings with mouse anti-α-tubulin antibody (Sigma-Aldrich) and rabbit anti β-actin antibody (Cell Signalling).

**Exosome isolation**

Serum-free conditioned medium from human glioblastoma cells was collected after 48 hours. Microvesicles were purified by differential centrifugation steps (300 × g for 10 minutes; 2,000 × g for 20 minutes; and 10,000 × g for 30 minutes), pelleted by ultracentrifugation at 175,000 × g for 60 minutes, and washed in PBS. Exosomes were identified by immunoblotting using the exosomal marker protein CD9 (mouse anti-human CD9; 1:1,000; Chemicon Temecula).

**Flow cytometry**

For flow cytometry, cells were dissociated with accutase, washed and stained with goat polyclonal anti-human B7H3 1:100 (R&D) or respective isotype control. As secondary antibody, donkey Alexa 488 anti-goat antibody was used (Invitrogen), and fluorescence in a total of 10,000 events per condition was detected. Cells were analyzed with a BD-FACS Canto II flow cytometer (BD Biosciences), final data were processed with the help of FlowJo flow cytometry analysis software (Tree Star). Specific fluorescence intensity (SFI) was calculated by using the mean fluorescence signal of B7H3 divided by the mean fluorescence isotype signal.

**qRT-PCR**

Total RNA was extracted using a RNA purification system (Qiagen) and treated with RNase-free DNase I to remove genomic DNA (Roche). The cDNA was prepared from 5 mg of total RNA using Superscript RNase H-Reverse Transcriptase (Invitrogen) and random hexamers (Sigma-Aldrich). For qRT-PCR, gene expression was measured in an ABI Prism 7000 sequence detection system (Applied Biosystems) with SYBR Green Master Mix (Eurogentec) and primers at optimized concentrations (31). Primers (Sigma-Aldrich) were selected to span exon–exon junctions. The sequence for human 4IgB7H3 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: 4IgB7H3 forward primer 5′-CATCACACCCCCAGAGGCC-3′, reverse primer 5′-AGAGGGCCGTG CGGTTGGCA-3′, GAPDH primers have been described previously (32). Standard curves were generated for each gene and the amplification was 90% to 100% efficient. Relative quantification of gene expression was determined by comparison of threshold values. All results were normalized to GAPDH.

**Mass spectrometry analysis**

**Probe preparation.** The band corresponding to 4IgB7H3-immunostained areas on the immunoblot were excised from SDS-PAGE. Gel pieces were consecutively washed with water and 50% acetonitrile, reduced with 10 mmol/L dithiotherol (DTT) at 56°C for 1 hour and alkylated with 55 mmol/L iodoacetamide (Sigma-Aldrich) at 25°C for 30 minutes in the dark. After alkylation, gel plugs were repeatedly washed with water and 50% acetonitrile, dehydrated with 100% acetonitrile, and air dried. The dried gel plugs were reswollen in 40 mmol/L ammonium bicarbonate containing 17 ng/μl sequencing grade–modified trypsin (Promega). Following enzymatic digestion overnight at 37°C, peptides were repeatedly extracted with 0.1% trifluoroacetic acid (TFA) and acetonitrile/0.1% TFA 50:50 (v/v). The combined solutions were dried in a speed-vac for 2 hours at 37°C. Peptides were redisolved in 5 μl 0.1% TFA by sonication for 10 minutes and applied for electrospray-tandem mass spectrometry (ESI-MS/MS) analysis.

**Orbitrap mass spectrometric analysis.** NanoLC-MS/MS analysis was conducted using the nanoACQUITY (Waters) coupled to a nanoESI-LTQ-Orbitrap mass spectrometer (Thermo Scientific) using a stepped linear acetonitrile/water gradient ranging from 5% to 90% within 45 minutes. For enhancing the number of detected peptides, a targeted proteomics approach was used for the identification of 4IgB7H3 (gi67188443, CD276 antigen isoform a, Homo sapiens). Only peptide masses obtained from an in silico trypsin digestion of 4IgB7H3 using an MS-Digest tool from the online ProteinProspector v5.7.2 software (UCSF, San Francisco, CA) were isolated and fragmented by Orbitrap. Processed data were searched against the NCBI nr database using the Mascot algorithm version v2.2.0 (Matrix Science).
Peripheral blood lymphocytes (PBL) were isolated from whole blood samples of healthy donors by Ficoll-Hypaque density gradient centrifugation. NK cells were then isolated from PBLs by depletion of non-NK cells using an NK cell isolation kit (Miltenyi Biotec). Isolated CD56+CD3− NK cells were maintained in RPMI-1640 medium (PAA Laboratories) containing 10% FBS (Perbio) and 1,000 U/mL interleukin-2 (Immunotools) for 5 days at 37°C/5%CO₂.

NK cell cytotoxicity was assessed using ⁵¹chromium (⁵¹Cr)-release assay (3). Briefly, labeled glioma cells (5 × 10⁴ per well) were seeded in triplicates into a U-shaped 96-well microtiter plate and incubated in triplicates with NK effector cells with effective target-to-effector ratios of 1:30, 1:10, and 1:3. Minimum and maximum ⁵¹Cr release was determined by using target cells incubated in medium alone or 10% Triton X-100 (Applichem). After incubation at 37°C/5% CO₂ for 4 hours, supernatants were collected from each well and counted in a gamma counter (Packard). Specific NK lysis in percent was calculated as follows: (experimental ⁵¹Cr release − minimum release)/(maximum release − minimum release) × 100.

Matrigel invasion assay

The invasive properties of glioma cells were assessed in Boyden chamber assays (BD Biosciences), where a porous membrane (8-μm pore size) coated with Matrigel matrix separates upper and lower wells. Glioma cells were harvested in enzyme-free cell dissociation buffer (Cibico Life Technologies), and a total of 4 × 10⁴ cells in culture medium were added in triplicates to the upper chamber (33). NIH 3T3-conditioned medium (0.5 mL) was used as a chemoattractant in the bottom well. Cell invasion was evaluated by counting the number of cells that had migrated across the membrane in 5 independent fields and expressed as percentage of invasiveness relative to control.

Spheroid invasion assay

A total of 1 × 10⁶ GICs were seeded in neural sphere cell medium (NSCM; Invitrogen) and kept in culture until spheroids had formed. Extracellular matrix gel was prepared as described previously (28). Glioma cell spheroids were seeded into the collagen gel solution in a 24-well plate in triplicates. After gelation, the gel was covered with medium and cultured at 37°C/5% CO₂. Microscopic images of the area covered by each spheroid were taken at 0, 24, 48, and 72 hours after implantation. For quantification, the mean area which was covered by invaded glioma cells at an indicated time point was measured in intervals of 24 hours and compared with the area at 0 hours.

Animal experiments

Animal work was approved by the governmental authorities (Regierungspräsidium Karlsruhe, Germany) and supervised by institutional animal protection officials in accordance with the NIH guidelines Guide for the Care and Use of Laboratory Animals.

Orthotopic brain tumor model

A total of 1 × 10⁵ human T269 4lgB7H3 knockdown or T269 control cells were stereotactically implanted into the right striatum of five 6- to 12-week-old athymic mice (CD1 nu/nu; Charles River Laboratories), respectively. Ten weeks after implantation, animals were sacrificed, brains removed and cryosectioned. To access infiltration, immunostainings were conducted with rabbit anti-human nestin antibody (Millipore) after fixation of cryosections with acetone. As secondary antibody, Alexa 488 anti-rabbit antibody (Invitrogen) was used.

Subcutaneous tumor model

Flank injection of human glioma cells and systemic depletion of NK cells were conducted as described previously (34). Briefly, 1 × 10⁶ human LN-229 B7H3 knockdown cells or LN-229 sh-Ctrl. cells were injected subcutaneously into the right flank of CD1 nu/nu mice after mixing with an equal volume of liquid Matrigel (BD Matrigel Basement Membrane Mix; BD Biosciences). NK cell depletion was conducted by biweekly intraperitoneal injection of 1.5 μg/mL of rabbit anti-asialo GM1 antibody (Wako Chemicals) starting 2 days before tumor cell injection. Controls were injected with rabbit IgG (Calbiochem). Tumor growth was regularly monitored using metric calipers accordingly. Flow cytometric analysis of spleen cells with rat anti-mouse DX5 (Ly49B) antibody from Caltag confirmed NK cell depletion. After 20 days, the mice were sacrificed, tumors were excised, and weighed.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue of human diffuse astrocytomas (WHO grade II, n = 3), anaplastic astrocytomas (WHO grade III, n = 7), and glioblastoma (WHO grade IV, n = 13) were provided by the Department of Neuropathology, Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany. Sections cut to 3 μm were processed using a Ventana Benchmark XT immunostainer (Ventana Medical Systems). Staining procedure included a pretreatment with cell conditioner 1 (pH 8) for 60 minutes, followed by incubation with either goat anti-human B7H3 antibody (1:200; R&D) or mouse anti-human CD8 (1:50; Dako) at 37°C for 32 minutes and for detection of B7H3, application of rabbit anti-goat immunoglobulins (P0446; DAKO) for 32 minutes and for detection of CD8, application of goat anti-mouse immunoglobulins (PO446; DAKO) for 32 minutes at room temperature. Incubation was followed by Ventana standard signal amplification, UltraWash, counterstaining with one drop of hematoxylin for 4 minutes and one drop of bluing reagent for 4 minutes. For visualization, ultraView Universal DAB Detection Kit (Ventana Medical Systems) was used. For quantitative analysis of the B7H3 staining pattern, the immunoreactive score (IRS) was applied. IRS was calculated as product of staining intensity and percentage of positive cells, determined as follows: staining intensity was subdivided into 4 groups: 0 (negative), 1 (weak), 2 (moderate),
and 3 (strong). Percentage of positive cells was regarded as 0 (none), 1 (<10%), 2 (10%–50%), 3 (51%–80%), and 4 (>80% positive tumor cells). Infiltration of CD8-positive cells was assessed in 4 glioblastoma with high (IRS > 8) and 4 glioblastoma with low (IRS < 8) B7H3 immunoreactivity. Of each case, four 200× magnification fields were analyzed. Presence of respective cells was scored as 0 (no positive cells), 1 (single positive cells), 2 (single groups of positive cells), or 3 (several groups of >3 positive cells).

Fluorescent immunohistochemistry

The B7H3 colocalization studies on cryosections of human glioblastoma tissue samples were conducted after acetone fixation and staining with a goat polyclonal anti-human B7H3 antibody (R&D), a mouse anti-human CD31 antibody (Dako), a mouse anti-human α-smooth muscle actin (SMA) antibody (Sigma-Aldrich), and a rabbit anti-human nestin (Millipore). As secondary antibodies, a donkey anti-mouse Cy3 antibody (Dianova) and an Alexa 750 goat anti-rabbit antibody (Invitrogen) were used. Finally, sections were counterstained with 4’,6-diamidino-2-phenylindol (DAPI) and analyzed with a Zeiss Axio Observer Z1 immunofluorescence microscope (Zeiss).

Clinical survival data

Queries of the Repository of Molecular Brain Neoplasia Data (REMBRANDT, National Cancer Institute, Bethesda, MD) for CD276 were conducted online in 2011 following the webpage’s instructions.

Statistical analysis

Statistical significance was assessed by the Student t test (Excel, Microsoft). All in vitro experiments reported here were carried out at least 3 times in triplicate or more. For in vivo experiments, 5 animals per group were operated. Survival data were plotted by the Kaplan–Meier method and analyzed by the log-rank test.

Results

B7H3 is expressed in human glioma tissue specimens and cultured glioma cells

B7H3 is detected in specimens of freshly dissected human glioma tissue by immunohistochemistry and is markedly upregulated in glioma tissue compared with the surrounding brain tissue at the infiltration zone (Fig. 1A). Here, strong B7H3 expression is found in close proximity to blood vessels. The degree of B7H3 expression correlated with the grade of malignancy of different gliomas (Fig. 1A). All of 18 glioblastoma samples, tested by immunohistochemistry (13 samples) and immunofluorescence analysis (5 samples), were positive for B7H3 (Figs. 1A and C and 4D). Queries of the NIH’s REMBRANDT brain tumor database, based on Affymetrix gene expression data and survival data, indicated in addition a correlation between decreased survival and increased gene expression of B7H3 in anaplastic astrocytomas (WHO grade III). In glioblastoma, this correlation is just under the level of significance (Fig. 1B).

To further characterize the localization of the focally enhanced B7H3 expression, costainings with CD31 for endothelial cells, SMA for pericytes, or nestin for glioma cells were conducted (Fig. 1C). These colocalization studies revealed that B7H3 is expressed by endothelial cells and also weakly by SMA-positive cells but in particular by the primary glioma cells surrounding the vessels [Fig. 1C (‡)]. On the cellular level, B7H3 protein was expressed in the cytoplasm and on cell membranes.

Moreover, 4 GIC cultures (Supplementary Fig. S1), which were established from human glioblastoma tissue and kept under stem cell conditions, expressed B7H3 mRNA, as did 5 of 5 glioma cell lines. To rule out that expression of 4IgB7H3 in vessels of brain tumor tissue (Fig. 1) is due to soluble B7H3 from the glioma cells themselves, we conducted expression studies of B7H3 in cultivated human pericytes and HCMCs. The expression detected in HCMCs and pericytes was weaker than in tumor cells (Fig. 2A). B7H3 protein of the predicted size of approximately 100 kDa was detectable in all tested glioma cultures (Fig. 2A, top), but the 45-kDa 2IgB7H3 could not be detected in the glioma samples. Finally, B7H3 was detected on the surface of glioma cell lines and GIC cultures as assessed by flow cytometry (Fig. 2A). There was no significant difference in B7H3 expression between GIC cultures and cell lines (Fig. 2A).

In an attempt to detect the recently published (22) soluble 16.5-kDa fragment of B7H3 in cell culture supernatants, we used 2 different antibodies: one directed against a peptide sequence in the first N-terminal immunoglobulin-like extracellular domain and the other one detecting the entire extracellular protein moiety of 2IgB7H3 and in addition due to sequence homology also 4IgB7H3 (Supplementary Fig. S3). The existence of a 16.5-kDa fragment could be substantiated neither in supernatant of glioma cell line cultures nor GIC cultures with these commercial antibodies (Fig. 2B).

To further analyze the mechanism of soluble 4IgB7H3 secretion, we first examined whether 4IgB7H3 could be detected in the exosomal compartment of LN-229 cells. Indeed, 4IgB7H3 colocalized with the exosomal marker CD9 (34) after exosome preparation but was not detected in the unconcentrated supernatant fraction (Fig. 2C). Comparing 4IgB7H3 originating from cell lysates directly with its secreted form obtained from cell culture supernatants, a size difference of about 5 to 7 kDa was detected in immunoblot analyses (Fig. 2D). In nanoLC ESI-MS/MS analyses, this size difference was confirmed, due to a missing peptide sequence of about 7 kDa in the supernatant probe. This part represents the intracellular and transmembrane fragment of 4IgB7H3 (Fig. 2D), which is not present in the concentrated supernatant fraction analyzed by two different antibodies. From this, we assume that after exosomal release of 4IgB7H3 by glioma cells, a 93-kDa fragment is processed and can be detected in concentrated supernatant. 4IgB7H3 appears to be the major isoform of B7H3 expressed in glioblastoma.
4IgB7H3 is released into the supernatant and inhibits NK-mediated lysis of glioma cells in vitro and in vivo

We next evaluated the functional activity of 4IgB7H3 expressed by glioma cells. 4IgB7H3 was silenced in the glioma cell line LN-229 with a lentiviral system. The initial knockdown has been about 80% effective; after clonal selection, the knockdown reached 93% in clone 19 on the mRNA level. Immunoblot and flow cytometry reveal a clear reduction of 4IgB7H3 protein levels in lysates, supernatant, and on the surface of the knockdown cells (Fig. 3A). Of note, the transfectants and control cells do not differ in morphology, generation time, or clonogenicity (data not shown). LN-229 sh-4IgB7H3 cells were more susceptible to NK cell–mediated lysis. The clonal knockdown was lysed best with around 60% specific lysis at a target-to-effector cell ratio of 1:30, the less efficient B7H3-silenced pool transfectants showed an intermediate lysis at 40% whereas the controls were at 25% (Fig. 3B, left). Given that 4IgB7H3 is released into the supernatant, we also analyzed whether soluble glioma cell–derived 4IgB7H3 suppresses NK cell–mediated lysis. Hence, we conducted NK lysis assays with LN-229 sh-4IgB7H3 clone 19 cells, which were susceptible to lysis, and supplemented supernatant of LN-229 sh-4IgB7H3 clone 19 or control cells. Compared with the specific NK-mediated lysis of LN-229 sh-4IgB7H3 clone 19 cells of around 60%, the specific lysis of these cells was reduced to 20% by coincubation with concentrated supernatant of control cells. This reduction of specific lysis was weaker after diluting the control supernatant 1:100. The supernatant of LN-229 sh-4IgB7H3 clone 19 cells does not reduce the lysis of LN-229 sh-4IgB7H3 clone 19 cells (Fig. 3B, right). We also generated 4IgB7H3-knockdown cells of the GIC culture T269, with a knockdown of 80% on mRNA level and a significant reduction on protein level measured by immunoblot and flow cytometry (Fig. 3C). T269 sh-4IgB7H3 cells, too, have been significantly more susceptible to NK cell–mediated lysis (Fig. 3D). Taken together, these data indicate that 4IgB7H3 and its secreted form suppress NK cell–mediated lysis of glioma cells and GICs in vitro.
To further characterize the impact of 4IgB7H3 expression in vivo, subcutaneous tumors were generated with LN-229 sh-4IgB7H3 and control cells. Tumor growth was monitored in LN-229 sh-4IgB7H3 and control cells in NK cell–depleted compared with control IgG antibody–treated mice (35). Here, a significant difference in tumor growth and tumor weight at the end of the experiment was only detected between LN-229 sh-4IgB7H3–derived tumors in NK cell–depleted versus nondepleted animals. In NK cell–retaining animals, LN-229 sh-4IgB7H3–derived tumors grew significantly smaller and were lighter at the end of the experiment. In LN-229 sh-control–derived tumors, NK cell depletion had no significant impact on tumor growth and weight (Fig. 4A and B) showing that silencing of 4IgB7H3 made glioma cells susceptible to NK cell–dependent cytotoxicity. Moreover, human glioblastoma samples showed a highly significant inverse correlation between B7H3 expression and invasion of CD8-positive immune cells. Costaining of B7H3 and CD8 revealed that the influx of CD8-positive cells is significantly higher in B7H3-low–expressing tumors than in B7H3-high–expressing tumors (Fig. 4C and D)

Regulation and cleavage of 4IgB7H3 in glioma cells differ from other tumor entities and immune cells

To evaluate how 4IgB7H3 expression is regulated and how soluble 4IgB7H3 is cleaved from the cell surface, we assessed the influence of protein kinase C activation PMA, inhibition of MMP by ilomastat (22), inhibition of TGF-β with LY2157299 or a furin inhibitor, and hypoxia on 4IgB7H3 levels. All compounds are sufficiently active in control assays (data not shown). 4IgB7H3 was neither induced by PMA on mRNA level nor on the protein level in cell lysates or with regard to the 93-kDa soluble form in the supernatant analyzed by nanoLC-ESI-MS/MS (right) as well as illustrate sketch visualizing hypothetical position of soluble 4IgB7H3 processing (interrupted line). CT, cytoplasmatic tail; TM, transmembrane.
Moreover, cleavage of 4IgB7H3 from the surface of the glioma cell line LN-229 (Supplementary Fig. S2B) and GIC cultures T325 (Supplementary Fig. S2A and S2B) was not influenced by inhibition of MMPs with ilomastat as shown before for monocytes, dendritic cells, activated T cells, and various carcinoma cells. Inhibition of TGF-β, which is known to suppress NK cells and to convey immune escape of gliomas in various manners (36), did not result in downregulation of 4IgB7H3, nor did hypoxia influence its expression (Supplementary Fig. S2A). Further experiments evaluating the regulation of 4IgB7H3 in glioma cells did not show a significant upregulation of 4IgB7H3 on the mRNA level, nor in the supernatant of LN-229 cells following irradiation, IFN-γ, dexamethasone, or H2O2 treatment (Supplementary Fig. S2C).

4IgB7H3 modulates the invasive phenotype in glioma cells in vitro and in vivo

Mounting evidence suggests that 4IgB7H3 is involved in tumor cell migration and invasiveness. Therefore,
Boyden chamber Matrigel and spheroid invasion assays were conducted with LN-229 cells as well as with the GIC culture T269. LN-229 sh-4IgB7H3 cells displayed reduced transmigration compared with control cells in these assays (Fig. 5A). Interestingly, the invasive phenotype was partly restored in LN-229 sh-4IgB7H3 cells when invasion assays were conducted with supplementation of concentrated supernatant of LN-229 control cells (Fig. 5B). There was also a reduction in the invasiveness of LN-229 control cells when the cells were incubated with supernatant from LN-229 sh-4IgB7H3 cells instead of supernatant from control cells during the invasion experiments (Fig. 5B), indicating that glioma supernatant may have an anti-invasive property, which can be partly overcome by the proinvasive effect of soluble 4IgB7H3.

To verify this proinvasive effect of 4IgB7H3 in primary glioma cells, primary 4IgB7H3–silenced T269 GICs were further used in a functional invasion assay. This spheroid invasion assay showed a significant reduction of the invasive phenotype in T269 cells after 4IgB7H3 gene silencing (Fig. 5C).

Aiming at confirming the *in vitro* data in an *in vivo* model, T269 sh-4IgB7H3 knockdown or control cells were orthotopically implanted into the brains of CD1 *nu/nu* mice. Ten weeks later, animals were sacrificed and tumor invasion was assessed by staining of the tumor cells with anti-human nestin antibody. Brain sections displayed a highly infiltrative tumor growth pattern with tumor cells reaching brain regions in the ipsilateral and contralateral hemisphere far off the implantation site in T269 control xenograft animals (Fig. 6, top). In contrast, clearly defined bulky tumors were found in T269 sh-4IgB7H3–xenografted brains without detectable tumor cells in ipsilateral or contralateral brain regions distant from the implantation site (Fig. 6, bottom).
Glioblastoma is a paradigmatic tumor for tumor-associated immunosuppression. B7H3 is a novel member of the B7-family of costimulatory proteins. As parts of our efforts to characterize and understand the immune phenotype of glioblastoma, we here report that B7H3 is expressed in human glioblastoma tissue by both glioblastoma and endothelial cells (Figs. 1 and 2A). Glioma cells that surround the blood vessels preferentially express B7H3. In addition to that, expression correlates significantly with increasing tumor grade and is associated with poor survival within WHO grade III gliomas.

By mass spectrometry and immunoblot analysis, we identified that 4IgB7H3 but not the smaller isoform 2IgB7H3 was preferentially expressed by different GIC cultures and glioma cell lines what was not determined in immunohistochemical analysis because of the lack of isoform-specific antibodies (Supplementary Fig. S3). Furthermore, it was showed that 4lgB7H3 is secreted in exosomes into the supernatant of glioma cells and finally processed to a soluble form of about 93 kDa. Interestingly, this soluble 4lgB7H3 is not the 16.5-kDa soluble form of B7H3 postulated in nonmalignant and malignant cells before (22). Although two different antibodies were used, the one detecting whole extracellular protein and the other one being the first immunoglobulin-like domain of the extracellular N-Terminus, we could not detect the published fragment of about 16.5 kDa in the supernatant of glioma cells. Here, the soluble fragment was about 7 kDa smaller than 4IgB7H3 originating from cell lysates (Fig. 2). In an attempt to further characterize...
the regulation and cleavage mechanism of B7H3 in glioblastoma, the cells were incubated with PMA, which was published to induce B7H3 on immune and tumor cells (9, 37). This regimen had no effect on B7H3 expression in glioblastoma cells on mRNA or protein level, neither did the inhibition of MMP, which have been suggested to cleave soluble B7H3 from the surface of tumor cells (22). We finally evaluated whether blockade of TGF-β activation with a furin inhibitor in the glioblastoma cell line LN-229 influenced 4IgB7H3 expression because TGF-β is known to be responsible for a variety of immunosuppressive and proinvasive effects (5, 6, 8, 20). However, TGF-β inhibition did neither result in the downregulation of 4IgB7H3 nor its soluble form (Supplementary Fig. S2).

Importantly, 4IgB7H3 was functional in the glioblastoma cell line LN-229 and the GIC culture T269 and suppressed NK cell–mediated tumor lysis in vitro (Fig. 2) as well as in vivo (Fig. 3). In attempt to elucidate the immunosuppressive function of B7H3 in human glioblastoma, we conducted colocalization studies of B7H3 expression and CD8-positive cells. Here, a significant higher influx of CD8-positive cells in B7H3-low–expressing tumors was detected than B7H3-high–expressing tumors. As CD8 is a marker for cytotoxic T cells which were also published to be suppressed by B7H3 (20) and NK cells, these data provide further evidence for an immunosuppressive function of B7H3. Furthermore, 4IgB7H3 exerted a proinvasive effect in glioblastoma cells in vitro (Fig. 5). We could additionally show the proinvasive effect of 4IgB7H3 in the GIC compartment in vivo: The highly invasive phenotype of the GIC culture T269 was significantly reduced by 4IgB7H3 gene silencing (Fig. 6).

Interestingly, native glioblastoma-derived soluble 4IgB7H3 suppresses NK lysis and enhances glioblastoma cell invasiveness in vitro. Insofar, our data let us to postulate that NK cell suppression and the proinvasive effect attributed to 4IgB7H3 expression in different tumor entities (37) are in fact exerted by membrane-bound and soluble B7H3, which was until now only evaluated for its diagnostic and prognostic capacity (22, 23, 38). Finally, as soluble 4IgB7H3 was sufficient to restore the invasiveness of 4IgB7H3-silenced LN-229 cells, the presence of a yet unknown counter-receptor must be postulated on glioblastoma cells, too.

To summarize, our data provide new insights on the role of B7H3 expression in glioblastoma. There is an immunosuppressive function of 4IgB7H3 in glioblastoma similar to other tumor entities. This is also true for a proinvasive phenotype. These effects can be exerted by secreted soluble 4IgB7H3 on its own leading to the assumption that the yet unidentified counter-receptor is expressed on immune and glioblastoma cells. Glioblastoma unlike nonmalignant cells do not generate and release the 16.5-kDa B7H3 fragment into the supernatant analyzed by commercially available antibodies. Our data support a fragment of about 93 kDa to exert that effect rather than a small 16.5-kDa fragment.

These data provide a therapeutic rationale to ameliorate treatment of glioblastoma by blockage of 4IgB7H3. First attempts to use 4IgB7H3 as a therapeutic target have been made recently with an antibody against 4IgB7H3 used to track neuroblastoma cells without intending to block 4IgB7H3 function itself (39) and a pseudomonas immunotoxin-coupled anti-B7H3 antibody to track glioblastoma xenograft tumors (40). Further experiments are needed to
evaluate 4IgB7H3 as a target in the treatment of 4IgB7H3-expressing tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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