NK Cells Recognize and Kill Human Glioblastoma Cells with Stem Cell-Like Properties[1]

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In this study, cancer cells were isolated from tumor specimens of nine glioblastoma patients. Glioblastoma cells, cultured under suitable culture conditions, displayed markers typical of neural stem cells, were capable of partial multilineage differentiation in vitro, and gave origin to infiltrating tumors when orthotopically injected in NOD/SCID mice. These cells, although resistant to freshly isolated NK cells, were highly susceptible to lysis mediated by both allogeneic and autologous IL-2 (or IL-15)-activated NK cells. Indeed, all stem cell-cultured glioblastoma cells analyzed did not express protective amounts of HLA class I molecules, while expressing various ligands of activating NK receptors that triggered optimal NK cell cytotoxicity. Importantly, glioblastoma stem cells expressed high levels of PVR and Nectin-2, the ligands of DNAM-1-activating NK receptor. *The Journal of Immunology,* **2009, 182: 3530–3539.**

Glioblastomas (GBM)[4] are highly aggressive CNS tumors with preferential localization in the cerebral hemispheres. Despite aggressive treatments combining surgery, irradiation, and chemotherapy, the prognosis remains poor, with a median survival time of 14 mo (1). The failure of conventional treatments reflects the fact that GBM infiltrate the surrounding normal brain tissues, thus frustrating attempts to perform complete surgical removal. In addition, the high frequency of molecular alterations (p53, RB, unmethylated MGMT) renders GBM cells poorly susceptible to cytotoxic therapies (2). Therefore, many efforts are being made to improve our current knowledge of GBM with the ultimate aim of improving the current therapeutic approaches. Different innovative therapies have been proposed, which include cellular immunotherapy based on the infusion of cytotoxic lymphocytes with antitumor activity (3).

Among cytolytic lymphocytes, NK cells represent, on a per cell basis, the most efficient effectors against tumors and are considered suitable candidates for adoptive immunotherapy of both hematological and nonhematological malignancies (4, 5). All NK cells from healthy donors express a large array of activating receptors that, upon recognition of specific cellular ligands, trigger the NK-mediated cytotoxicity (6). In humans, the activating receptors include NKp46, NKp30, and NKp44, collectively termed natural cytotoxicity receptors (NCR; whose expression is restricted to NK cells) and DNAM-1 and NKG2D (also present in T cell subsets). Although the cellular ligands of NCR are not fully defined, other triggering receptors recognize surface ligands that are either up-regulated or expressed de novo by tumor cells. In particular, DNAM-1 recognizes PVR and Nectin-2 (7), two members of the Nectin family that are overexpressed in tumors of different histotypes (7–9). NKG2D reacts with MICA/B and ULBPs that are MHC class I-related, stress-inducible molecules expressed by tumor or virus-infected cells (10). NK cells also express receptors that recognize self-HLA class I molecules on potential target cells (11, 12) and transduce inhibitory signals that dampen NK cell activation and function. These receptors include the CD94/NKG2A heterodimer, which recognizes HLA-E, a nonclassical HLA class I molecule, and killer Ig-like receptors (KIR; CD158), clonally distributed receptors specific for determinants shared by groups of classical HLA class I alleles (HLA-A, -B, and -C). The type and the number of receptor/ligand interactions are crucial for the NK-mediated killing of potential target cells. Thus, whereas most normal autologous cells are spared because of the expression of high levels of HLA class I molecules, tumors may be susceptible to lysis. Indeed, the lack of HLA class I molecules, or their down-regulation, which frequently occurs in cancer cells (13), is paralleled, in most instances, by the de novo expression or up-regulation of different ligands recognized by the activating NK receptors (7–9, 14, 15).

In this study, we isolated cancer cells from tumor specimens of nine different GBM patients. GBM cells were expanded in vitro in stem cell medium and analyzed for the expression of neural stem cell (NSC) markers, multilineage differentiation, and tumorigenicity in immunodeficient mice. Stem cell-cultured GBM cells were analyzed for susceptibility to lysis mediated by resting or lymphokine-activated

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[4] Abbreviations used in this paper: GBM, glioblastoma; KIR, killer Ig-like receptor; NCR, natural cytotoxicity receptor; NSC, neural stem cell; RT, room temperature.

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NK cells both in allogeneic and autologous settings. Moreover, we measured the type and number of receptor/ligand interactions involved in the NK-mediated recognition of cancer cells.

Materials and Methods

Human tumors and GBM stem cell culture

Tumor samples were obtained from patients of the Neurosurgery Department, San Martino Hospital (Genova, Italy). Informed consent was obtained for all patients as approved by the Ethics Board. Samples were processed according to Liu et al. (2) with modifications. Briefly, tissue was triturated and cells were seeded into tissue culture flasks coated with matrigel (BD Biosciences) in DMEM-F12/Neurobasal, B27 supplement (Life Technologies), human fibroblast growth factor-2 (10 ng/ml; PeproTech), and epidermal growth factor (20 ng/ml; PeproTech).

To induce differentiation, cells were seeded on matrigel-coated coverslips at 1×10^4 cells/slide and cultured for up to 21 days under differentiation conditions in DMEM-F12/Neurobasal containing B27 supplement and FCS (10% v/v; EuroClone).

Immunofluorescence

Cells were plated into matrigel-coated glass coverslips; fixed with 4% paraformaldehyde; treated with PBS, 10% FCS, and 0.5% Triton X-100; and then stained with the following Abs: mouse anti-Nestin (Novus Biologicals), mouse anti-microtubule-associated protein 2 (Chemicon International), mouse anti-glial fibrillary acidic protein (DakoCytomation), rabbit anti-SOX2 (Chemicon International), mouse anti-KI67 (Oncogene Sciences), mouse anti-Nectin-2 (CD112; IgG2a L14 mAb), and mouse anti-PVR (CD155; IgG1, M5A10 mAb), followed by FITC-conjugated (Southern Biotechnology Associates) or rhodamine-conjugated (Jackson ImmunoResearch Laboratories) appropriate secondary Abs. Nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich). Quantification of positive cells was conducted by counting all of the stained cells within 20 microscope fields per specimen, and the percentage was calculated based on the total number of nuclei counted.

Intracranial tumor assays

NOD/SCID mice were housed in pathogen-free conditions, according to the National Regulation on Animal Research Resources. For intracranial inoculation, two to four mice (6–8 wk old; Charles River Laboratories) were anesthetized with i.m. ketamine and xylazine. Thereafter, the animal was positioned into a stereotactic frame (David Kopf Instruments) and a hole was made 2 mm lateral and 1 mm anterior from the bregma. Cells (10^5) were injected using a Hamilton syringe (Sigma-Aldrich) at a depth of 3.5 mm in a vol of 2 μl. Mice were monitored for disease symptoms and sacrificed by CO_2 asphyxiation when they showed weight loss or any other sign of disease. All experiments were performed in compliance with guidelines approved by the Ethical Committee for Animal Use in Cancer Research at Istituto Nazionale Ricerca Cancro in Genova. Under our experimental conditions, the minimum number of GBM cells required to give rise to tumor in mice was 10^4.

Immunohistochemistry

For xenograft tumor analysis, brains were cryopreserved and 10-μm cryostat (Leica Microsystems) sections were cut. Sections bearing tumors were identified by H&E. Cryosections containing tumors were permeabilized in PBS containing 0.2% Triton X-100 and blocked in 5% normal FCS-PBS. After incubation with primary Abs (anti-Nestin, anti-SOX2), sections were stained with the appropriate secondary Abs and counterstained with Hoechst 33342 dye to identify all nuclei.

The expression of PVR and Nectin-2 on primary tumor has been evaluated on eight different GBM samples collected from the Department of Pathology, University of Brescia, according to the Institutional Ethical Board protocols on material submitted for diagnostic purposes. Briefly, tumor samples were snap frozen, and 5-μm-thick sections were cut on a cryostat and let to dry overnight at room temperature (RT). Sections were then fixed for 10 min in pure acetone and rehydrated, and endogenous peroxidase activity was blocked by 0.3% H_2O_2 in distilled water for 15 min. Sections were then washed in Tris-HCl buffer before preincubation in blocking buffer containing 5% normal goat serum in Tris-HCl for 5 min and incubation for 1 h at RT with primary Ab in Tris/1% BSA. Purified (1 mg/ml) anti-Nectin-2 (L14) and anti-PVR (M5A10) mAbs were used at 1/50 and 1/100 dilutions, respectively. Sections were then washed in Tris-HCl buffer and incubated for 30 min at RT with the secondary biotinylated goat anti-IgG2a or anti-IgG1 Abs (1:100; Southern Biotechnology Associates), followed by streptavidin-conjugated HRP (DakoCytomation). Signal has been revealed with diaminobenzidine and slides counterstained with H&E. Digital images were captured by a DP-70 Olympus digital camera mounted on Olympus BX60 microscope using Analysis Image Processing software (Olympus).

Lymphocytes used in the study

NK cells were purified from PBMC using the Human NK Cell Isolation kit (Miltenyi Biotec). To obtain long-term-activated NK cell populations, cells were cultured on irradiated feeder cells in the presence of 100 U/ml rIL-2 (Proleukin; Chiron) and 1.5 ng/ml PHA (Life Technologies) (8). Short-term-activated NK cells were obtained by culturing NK cells in the presence of 20 ng/ml rIL-15 (PeproTech) for 5 days. The NK cell line NKL (provided by E. Vivier, Marseille, France) was cultured in medium supplemented with human serum, in the presence of 100 U/ml rIL-2 (Proleukin; Chiron). T cell blasts were obtained by culturing PBL with 1.5 ng/ml PHA. After 24 h, cells were washed and cultured in medium supplemented with 100 U/ml rIL-2.

Monoclonal Abs

The 289 (IgG2A, anti-CD3), c218 and A6/220 (anti-CD56, IgG1, and IgM, respectively), c127 (IgG1, anti-CD16), BAB281 and KL247 (IgG1 and IgM, respectively; anti-NKp46), F252 and AZ20 (IgM and IgG1, respectively; anti-NKp30), Z231 (IgG1, anti-NKp44), ON72 (IgG1, anti-NKG2D), KRA236 (IgG1, anti-DNAM-1), MA127 (IgG1, anti-NTBA), MA344 (IgM, anti-2B4), Z270 (IgG1, anti-NKG2A), M5A10 (IgG1, anti-PVR), L14 (IgG2A, anti-Nectin-2), BAM195 (IgG1, anti-MICA), 7E22 (IgG1, anti-ICAM-1), EB6 (IgG1, anti-KI2DL1/S1), GL183 (IgG1, anti-KI2DL2/L3/S2), AZ158 (IgG2A, anti-KI3DL1/S, KIR3DL2), A6136 (IgM, anti-HLA class I-A, -B, -C, and -E), and DF200 (IgG1, anti-KIR2DL1,L2,L3/S1,S2,S5) were produced in our laboratory. Anti-ICAM-2 and anti-ICAM-3 Abs were purchased from Diaclone. The anti-CD133 mAb (AC133, IgG1) was purchased from Miltenyi Biotec. The anti-c-*kit* mAb was purchased from Immunotech. Anti-MICB (IgG2b) was purchased by R&D Systems. Anti-PVR PE was purchased by eBioscience. The IgG1 PE-negative control was purchased by AbD Serotec MorphoSys. Anti-CD45 (IgG2a) was purchased by Oxford Biomarketing. The anti-CD3 FITC/anti-CD56 PC5 mixture was purchased by Beckman Coulter. M295 (IgG1, anti-ULBP1), M310 (IgG1, anti-ULBP2), M551 (IgG1, anti-ULBP3), and M475 (IgG1, anti-ULBP4) mAbs were provided by Amgen. The 3D12 (IgG1, anti-HLA class I-E) was provided by D. Geraghty (Seattle, WA).

Flow cytofluorimetric analysis and cytolytic assays

For cytofluorimetric analysis of the various surface molecules, PBMC, purified NK cells, or GBM cell cultures were stained with the appropriate mAbs, followed by PE- and FITC-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associates). The anti-CD3 FITC/anti-CD56 PC5 mixture was used for the analysis of NK cells in PBMC. Samples were analyzed by one-, two-, or three-color flow cytometry (FACSCalibur; BD Biosciences).

For analysis of the coexpression of DNAM-1 ligands and Nestin, GBM cell cultures were permeabilized using saponin (0.1% g/ml) and stained with PE-conjugated anti-PVR mAb, anti-Nestin, and anti-Nectin-2 mAbs, followed by PE- or FITC-conjugated isotype-specific goat anti-mouse second reagent. Samples were analyzed by one- or two-color flow cytometry.

For cytofluorimetric analysis of PVR and Nectin-2 on freshly isolated GBM cells, tumor homogenates from patients at diagnosis were enriched in $CD56^+$ GBM cells by a human NK cell enrichment mixture (RosetteSep; StemCell Technologies). GBM cells were identified using three-color flow cytometry (anti-CD3 FITC/anti-CD56 PC5 mixture and anti-CD45 mAb, followed by PE-conjugated isotype-specific second reagents). All GBM cells displayed the $CD3^-$, $CD56^+$, $CD45^-$ phenotype. Thus, double fluorescence was performed using anti-CD56 mAb (followed by FITC-conjugated isotype-specific second reagents) and anti-PVR or anti-Nectin-2 mAbs (followed by PE-conjugated isotype-specific second reagents).

NK cells were tested for cytolytic activity against GBM cells or T cell blasts in a 4-h ^{51}Cr release assay, as previously described (7). To avoid nonspecific cross-linking of FcRγ, masking experiments were performed using, when available, mAbs of IgM isotype at the concentration of 10 μg/ml. The E:T ratios are indicated in the text. MA127 (IgG1, anti-NTBA) and MA344 (IgM, anti-2B4; data not shown) were used as isotype-matched controls.

Statistical analysis

The nonparametric Kruskal-Wallis test and Wilcoxon rank-sum test were applied for the analysis of the significance of the different contribution of the main activating receptors in the NK-mediated lysis of GBM.

Table I. *Immunocytochemical characteristics of stem cell-cultured GBM cells*[a]

PT[b]	Nestin	SOX2	Map2[c]	GFAP[d]	Map2/GFAP	Ki67
1						
UNDIFF[e]	95 (±2.5)	84 (±4)	75.5 (±5.5)	27 (±3)	18 (±2)	6.5 (±3.5)
DIFF	75 (±5)	45 (±5)	35 (±5)	85 (±5)	28 (±3.5)	1.5 (±0.5)
2						
UNDIFF	75 (±5)	80 (±5)	8.5 (±3.5)	4.5 (±0.5)	1 (±0.5)	47.5 (±2.5)
DIFF	20 (±4)	72.5 (±2.5)	7.5 (±2.5)	12.5 (±2.5)	5.6 (±1.2)	14.5 (±5.5)
3						
UNDIFF	97 (±2.5)	85.5 (±5.5)	7.5 (±2.5)	15 (±5)	3.5 (±1)	60 (±4)
DIFF	67.5 (±2.5)	43 (±13)	30 (±4)	80 (±10)	18 (±2.5)	6 (±4)
4						
UNDIFF	78 (±4)	82 (±0.7)	23 (±2.5)	9.5 (±1)	2.7 (±0.8)	27.6 (±1.4)
DIFF	19.5 (±2)	43 (±1.2)	10 (±2.5)	54 (±6)	1.9 (±0.9)	22 (±1.8)
5						
UNDIFF	87 (±5)	68 (±7)	10 (±1.5)	15 (±2)	1.3 (±0.6)	20 (±3.3)
DIFF	76 (±2)	28 (±2)	9.8 (±1)	40 (±3)	0.9 (±0.2)	6.5 (±0.5)
6						
UNDIFF	65 (±5)	65 (±5)	75 (±5)	7.5 (±2.5)	11.2 (±2.1)	7.5 (±2.5)
DIFF	35 (±5)	40 (±1.5)	65 (±5)	55 (±5)	45 (±2.5)	3.5 (±2.5)
7						
UNDIFF	90 (±5)	68.5 (±1.5)	12.5 (±2.5)	80 (±6)	6 (±1.3)	8.5 (±1.5)
DIFF	10 (±3)	52 (±6)	55 (±5)	85 (±5)	41 (±2.8)	3 (±1)
8						
UNDIFF	96 (±2.5)	75 (±5)	45 (±5)	7.5 (±2.5)	3.3 (±1.1)	30 (±2)
DIFF	85 (±5)	36 (±6)	25 (±5)	7.5 (±2)	4.5 (±1.9)	7.5 (±2.5)
9						
UNDIFF	24 (±4)	50 (±6)	22 (±1.5)	22 (±3)	22 (±1.5)	51 (±2.1)
DIFF	6 (±1)	9.8 (±0.2)	33 (±2.5)	68 (±2.5)	33 (±2.5)	7.9 (±0.8)

[a] Relative quantification of NSC markers on glioma cells and evaluation of their differentiation capability.
[b] PT, patient.
[c] Map2, microtubule-associated protein 2.
[d] GFAP, glial fibrillary acidic protein.
[e] UNDIFF, undifferentiated; DIFF, differentiated.

Results

Establishment of GBM stem cell cultures and differentiation capability

Only patients with typical clinical and radiological features of GBM were selected and included in this study. All patients were adults, had surgery for the first time, and did not receive chemotherapy or radiotherapy. The diagnosis of GBM has been confirmed according to the World Health Organization classification (16). Based on these criteria, 13 GBM were selected. Tumor samples were dissociated, and single-cell suspensions were plated under limiting dilution conditions in stem cell medium on matrigel-coated flasks (17). Cultures from 9 unrelated patients (7 males and 2 females) were successfully expanded in vitro for more than 10 passages and gave rise to clones within 1–2 wk. GBM cells displayed the potential of multilineage differentiation, i.e., typical features of NSC (18). Immunohistochemical analysis revealed positivity for the NCS markers Nestin (ranging between 24 and 97% of cells) and SOX2 (between 50 and 85.5%) (Table I). Western blot analysis confirmed SOX2 expression (data not shown).

Upon removal of growth factors and the addition of serum, GBM cells showed (after 3 wk) a reduced expression of stem cell markers and morphological and immunohistochemical features typical of glial or neuronal cell lineages (Table I). However, unlike normal NSCs, up to 45% of GBM cells were positive for both glial fibrillary acidic protein and neuronal microtubule-associated protein 2, suggesting that they had only partially undergone differentiation.

Expression of stem cell markers, HLA class I molecules, and tumorigenic potential of stem cell-cultured GBM cells

Stem cell-cultured GBM cells were analyzed for the expression of stem cell markers and HLA class I molecules. Cytofluorimetric analysis showed that CD133 (19) and c-Kit (20) were expressed in some, but not all, GBM cell cultures, whereas all GBM expressed both classical (HLA-A, -B, -C) and nonclassical (HLA-E) HLA class I molecules (Fig. 1*A*). GBM cells were stereotactically injected into the brain of NOD/SCID mice, and 3–6 mo later, all mice developed neurological symptoms. The symptoms-free survival time upon orthotopic implantation did not appear related to the expression of CD133, a putative stem cell marker, because mice injected with cells derived from patient 1 ($CD133^{low}$), patient 2 (CD133 undetectable), and patient 4 ($CD133^{high}$) (Fig. 1*A*) all developed neurological symptoms ~4 mo after injection. Intracranial tumors generated by GBM cells displayed various grades of infiltration into the surrounding cerebral cortex, a hallmark of human GBM (Fig. 1*B*), with cells migrating along the corpus callosum (Fig. 1*B*, *6* and *7*) or other brain structures, mimicking the behavior of human primary GBM. Cells derived from patients 1, 6, and 8 (Fig. 1*B*, *2–5*) infiltrated the white matter with the exception of the cerebellum. GBM cells from intracranial xenograft tumors were further isolated, dissociated, and cultured again under stem cell conditions. As judged by the take rate, GBM cells showed an increased tumorigenic potential when injected back into the brains of new recipient mice. All of the tumor cell populations derived from such glioma xenografts were composed exclusively of human tumor cells, with no significant presence of murine cells, as assessed by immunocytochemical analysis using anti-human and anti-mouse Nestin-specific Abs.

Stem cell-cultured GBM cells are susceptible to lysis mediated by lymphokine-activated allogeneic NK cells

Previous studies reported that GBM cells cultured under non-stem cell conditions display resistance to lysis by freshly isolated NK cell or NKL, a commonly used NK cell line, thus leading to the

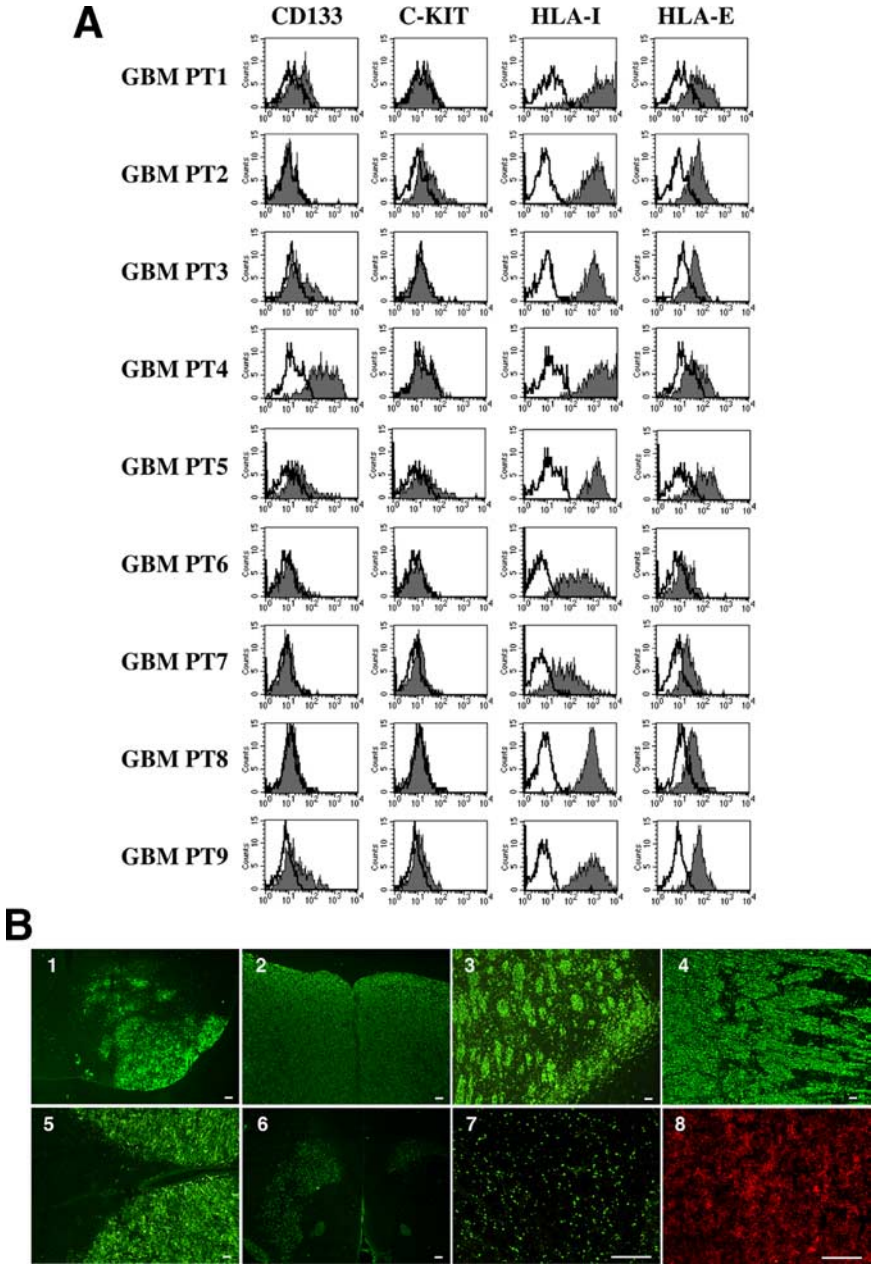

FIGURE 1. Expression of stem cell markers, HLA class I molecules, and tumorigenic potential of stem cell-cultured GBM cells. *A*, Stem cell-cultured tumor cells isolated from the tumor mass of nine GBM patients (PT1-PT9) were stained with mAbs specific for the indicated molecules. PE-conjugated goat anti-mouse isotype specific was used as second reagent. White profiles refer to cells incubated with the second reagent only. *B*, *1–5*, Immunofluorescences with anti-human Nestin Ab. *1*, Tumor mass infiltrating the surrounding parenchyma (patient 3). *2*, Diffuse infiltration of the injected and contralateral hemisphere (patient 1). *3*, Islet; *4*, palisades (patient 6). *5*, Cells migrating between the hemispheres (patient 8). *6* and *7*, Ki-67 positivity of tumor cells infiltrating the corpus callosum ipsilateral and contralateral to the side of injection (patient 2). *8*, Diffuse expression of SOX2 (patient 5). Scale bar 100 μm.

conclusion that NK cells are not suitable effectors to be used in adoptive immunotherapy in GBM patients (21–23). However, lymphokine-activated NK cells display a much higher cytolytic potential than freshly isolated NK cells. Therefore, we analyzed stem cell-cultured GBM cells for their susceptibility to lysis by IL-2-cultured polyclonal NK cell populations derived from unrelated healthy donors. These cells were characterized by the typical $CD3^-$, $CD56^+$ phenotype and expressed NK-specific markers, including NCR. Long-term-activated NK cells efficiently killed all allogeneic GBM cells analyzed. Virtually all GBM cells were killed at high E:T ratios (40:1 or 20:1), and strong cytolytic activity was detected even at low E:T ratios (Fig. 2*A*). Although GBM cells expressed HLA class I molecules, no increment of lysis was observed in the presence of the A6136 mAb, which recognizes both classical (-A, -B, -C) and nonclassical (-E) HLA class I alleles, thus indicating that the levels of HLA class I molecules are insufficient to protect GBM cells from NK-mediated lysis.

Freshly isolated (resting) NK cells and NKL cell line were analyzed for anti-GBM cytolytic activity in comparison with short-term (IL-15)- or long-term (IL-2)-cultured NK cells derived from the same donor. GBM cells were virtually resistant to both resting NK cells and NKL (notably, both effectors killed the highly NK-susceptible K562 cell line) (Fig. 2*B*). On the contrary, both short-term- and long-term-cultured polyclonal NK cell populations efficiently lysed not only K562, but also GBM cells. In all instances, mAb-mediated masking of HLA class I molecules (on target) did not result in increments of lysis (data not shown).

Altogether these data demonstrate that stem cell-cultured GBM cells analyzed are highly susceptible to killing mediated by IL-2- or IL-15-activated NK cells. On the contrary, GBM cells are virtually resistant to lysis mediated by either resting NK cells or the NKL cell line, both characterized by low cytolytic activities.

Stem cell-cultured GBM cells are susceptible to lysis mediated by lymphokine-activated autologous NK cells

We next analyzed whether stem cell-cultured GBM cells were susceptible to NK-mediated killing in an autologous setting. PBMC

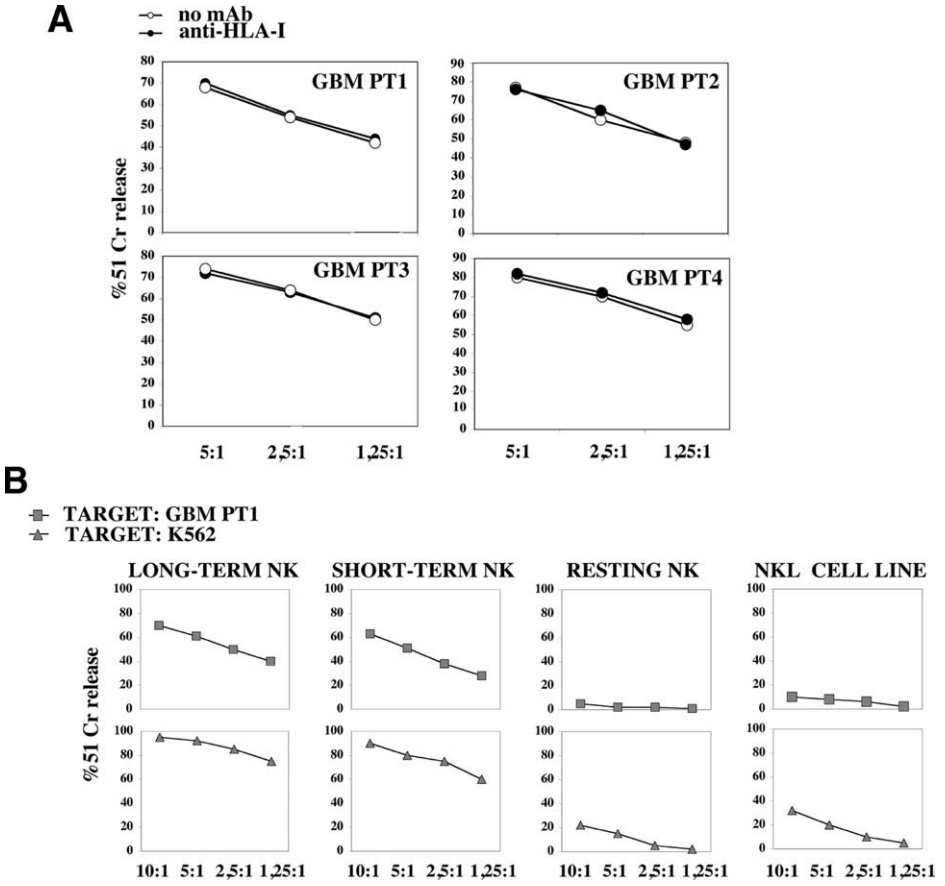

FIGURE 2. Susceptibility of stem cell-cultured GBM cells to lysis mediated by lymphokine-activated allogeneic NK cells. *A*, A representative experiment is shown in which a polyclonal, IL-2-activated NK cell population derived from an healthy donor was analyzed at different E:T ratios for cytolytic activity against stem cell-cultured GBM cells from patients 1–4 (PT1-PT4). Assays were performed either in the absence (○) or in the presence of the A6-136 mAb (IgM, anti-HLA class I) (●). *B*, Freshly isolated (resting), short-term (IL-15)-, or long-term (IL-2)-activated NK cells derived from a representative healthy donor and the NKL NK cell line were analyzed at different E:T ratios for cytolytic activity against stem cell-cultured GBM cells from patient 1 and the K562 cell line. The results are representative of triplicate experiments; the SD of the mean of the triplicates was <5%.

were isolated from blood samples of different GBM patients at diagnosis or, as control, of healthy donors. Patients' NK cells (identified within the PBMC as $CD3^-$, $CD56^+$ cells) displayed a surface phenotype similar to that of controls both in terms of percentages of positive cells and the surface densities of the molecules analyzed. These molecules included the NKp46, NKp30, DNAM-1, NKG2D-activating receptors, and the main HLA-class I-specific receptors (CD94/NKG2A and KIR2D) (supplemental Fig. 1).[5] Patients' NK cells were purified and cultured in the presence of IL-2 to obtain polyclonal activated NK cell populations. Also in this case, patients' NK cells were characterized by a surface phenotype comparable to that of activated NK cells from healthy donors. In particular, NK cells from GBM patients expressed high levels of the various activating receptors, including the NKp44 activation marker (Fig. 3*A*). These IL-2-activated NK cells were then assessed for their cytolytic activity against autologous GBM cells and, as a control, against normal PHA-activated autologous T cell blasts. As shown in Fig. 3*B*, NK cells efficiently killed autologous GBM cells. Remarkably, even in an autologous setting, the magnitude of lysis was not modified by mAb-mediated masking of HLA class I molecules. In contrast, autologous T cell blasts were poorly susceptible to lysis and, as expected, in this case, lysis was increased by mAb-mediated disruption of inhibitory receptor/HLA class I interactions (Fig. 3*B*).

Altogether these data show that GBM cells can be highly susceptible to lysis mediated not only by allogeneic, but also by autologous IL-2-activated NK cells.

[5] The online version of this article contains supplemental material.

Expression of ligands specific for NK receptors on stem cell-cultured GBM cells

The above data show that stem cell-cultured GBM cells are highly susceptible to NK-mediated lysis. Moreover, HLA class I molecules did not appear to exert a protective effect in both allogeneic and autologous combinations. To better define the molecular mechanisms involved in NK to GBM cell interactions, we analyzed the expression of ligands recognized by the inhibitory (HLA class I-specific) or activating NK receptors. Fig. 4*A* and Table II show that the surface density of HLA class I molecules in GBM cells was low (see for comparison the autologous T cell blasts). In contrast, all GBM cells expressed PVR and Nectin-2 (ligands of the DNAM-1-activating receptor). Moreover, low, but detectable amounts of at least one NKG2D-specific ligand could be detected (Fig. 4*A* and Table II). It is of note that the ICAM family of adhesion molecules was virtually absent, with the exception of ICAM-1 that was expressed by some GBM (see, for example, cells from patient 4). Unlike GBM cells, T cell blasts together with high HLA class I surface densities expressed low surface densities of PVR only, and the ligands recognized by NKG2D with the exception of ULBP3 molecules were virtually absent. The expression of the various surface molecules described above was stable over time because the same phenotype could be detected after short- or long-term culture of GBM cells.

These data suggested that the expression of PVR and Nectin-2 could represent a common feature of GBM with stem cell properties. To confirm this hypothesis, we analyzed whether their expression patterns correlated with that of SOX2 and Nestin. To this end, GBM cell cultures were costained with Abs specific for the

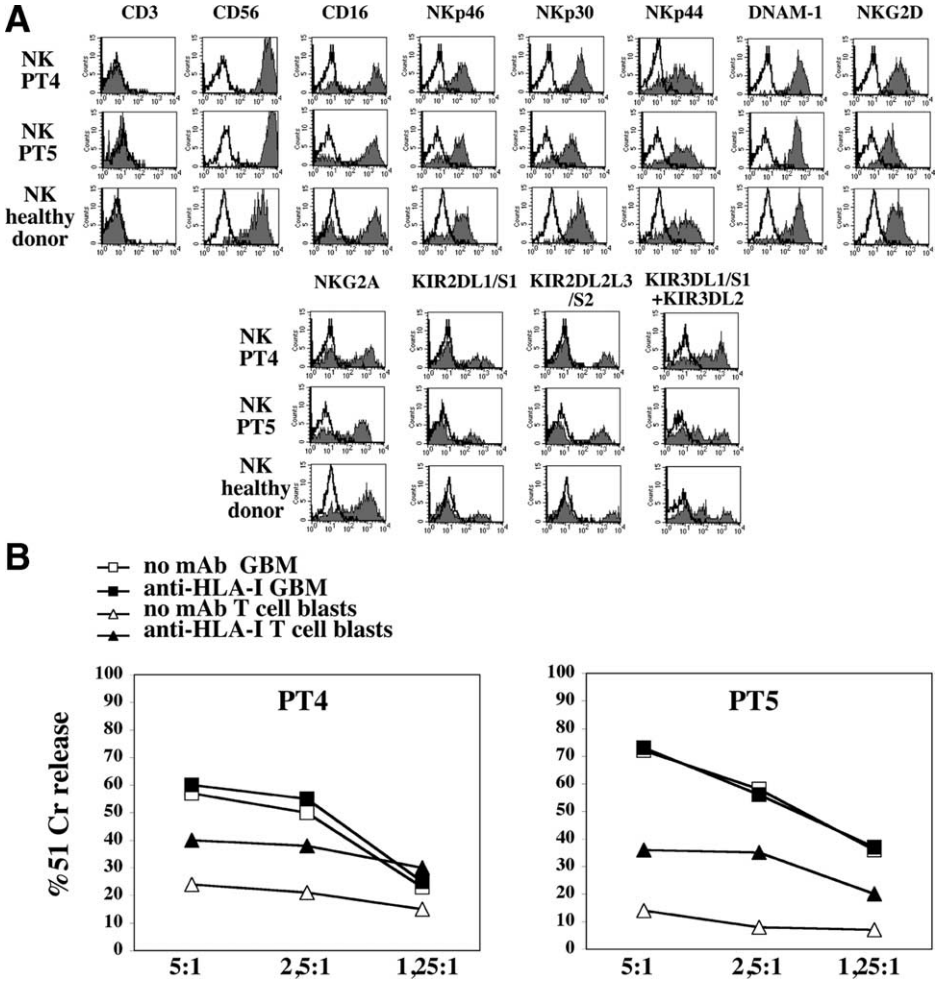

FIGURE 3. Susceptibility of stem cell-cultured GBM cells to lysis mediated by autologous IL-2-activated NK cells. *A*, Long-term (IL-2)-activated NK cell populations from patients 4 and 5 (PT4 and PT5) or from a representative healthy donor were stained with mAbs specific for the indicated molecules. A PE-conjugated goat anti-mouse isotype-specific mAb was used as second reagent. White profiles refer to cells incubated with the second reagent only. *B*, The long-term (IL-2)-activated NK cell populations from patients 4 and 5 (PT4 and PT5) were analyzed at different E:T ratios for cytolytic activity against autologous stem cell-cultured GBM cells or T cell blasts. Assays were performed either in the absence or in the presence of the A6-136 mAb (IgM, anti-HLA class I). The results are representative of triplicate experiments; the SD of the mean of the triplicates was <5%.

ligands of DNAM-1 and the NCS markers. GBM cells were analyzed either by microscope fluorescence (for the SOX2 nuclear marker) or by cytofluorimetric analysis (for the Nestin cytoplasmic marker). As shown in Fig. 4, *B* and *C*, most SOX2-positive, Nestin-positive cells coexpressed PVR and Nectin-2.

Receptor-ligand interactions involved in NK-mediated recognition of stem cell-cultured GBM cells

Overall, these data suggested that the triggering signals generated upon NK-mediated recognition of stem cell-cultured GBM cells can overcome the inhibitory signals generated by suppressive interactions between inhibitory NK receptors and HLA class I molecules. The opposite would occur in the case of interactions between NK and T cell blasts. In line with these concepts, mAb-mediated blocking of HLA class I molecules increased the NK-mediated lysis of T cell blasts, but not of GBM cells.

To assess the contribution of different activating receptor/ligand interactions in NK-mediated lysis of GBM cells, cytotoxicity experiments were performed in the presence of mAbs specific for individual triggering receptors (Fig. 5). Among NCR, NKp46 appeared to play a crucial role in lysis of GBM cells. Indeed, in both autologous and allogeneic combinations, mAb-mediated masking of NKp46 sharply inhibited cytotoxicity. Moreover, in agreement with the expression of the ligands specific for DNAM-1 on target cells, DNAM-1 receptor substantially contributed to the lysis of GBM cells. Notably, the combined mAb-mediated blocking of NKp46 and DNAM-1 receptors virtually abrogated tumor cell lysis. The addition of mAb specific for either NKp30 or NKG2D had marginal effect. However, the addition of NKp30- or NKG2D-specific mAbs had an additive effect on the inhibition of cytotoxicity mediated by anti-NKp46 or anti-DNAM-1 mAbs. The significant role played by NKp46 and DNAM-1 in killing of the GBM cells analyzed was confirmed by statistical analysis ($p < 0.001$). The above data indicate that the susceptibility of GBM cells to NK-mediated lysis depends on the imbalance between inhibitory and activating signals. Indeed, whereas GBM cells do not express protective amounts of HLA class I molecules, they display fairly high levels of multiple activating ligands capable of mediating interactions that collectively induce NK cytotoxicity.

PVR and Nectin-2 expression in primary GBM

As described above, the expression of DNAM-1 ligands was detected in all GBM cell cultures analyzed. To exclude the possibility that these molecules could be expressed de novo upon cell culture of ex vivo derived tumor cells, we checked for PVR and Nectin-2 expression on eight primary GBM. Immunohistochemical analysis (Fig. 6*A*) revealed that tumor cells expressed both molecules. PVR (*left*) showed a weaker staining with either a focal (two samples) or a diffuse pattern of expression (six samples). Nectin-2 (*right*) showed a stronger staining in the GBM cells with a predominant focal pattern of expression (five out of eight cases). Interestingly, both ligands and in particular PVR were intensely expressed also by endothelial cells of intratumoral neoplastic vessels from all GBM samples analyzed, particularly in the proliferating vessels, a feature indicating tumor malignancy.

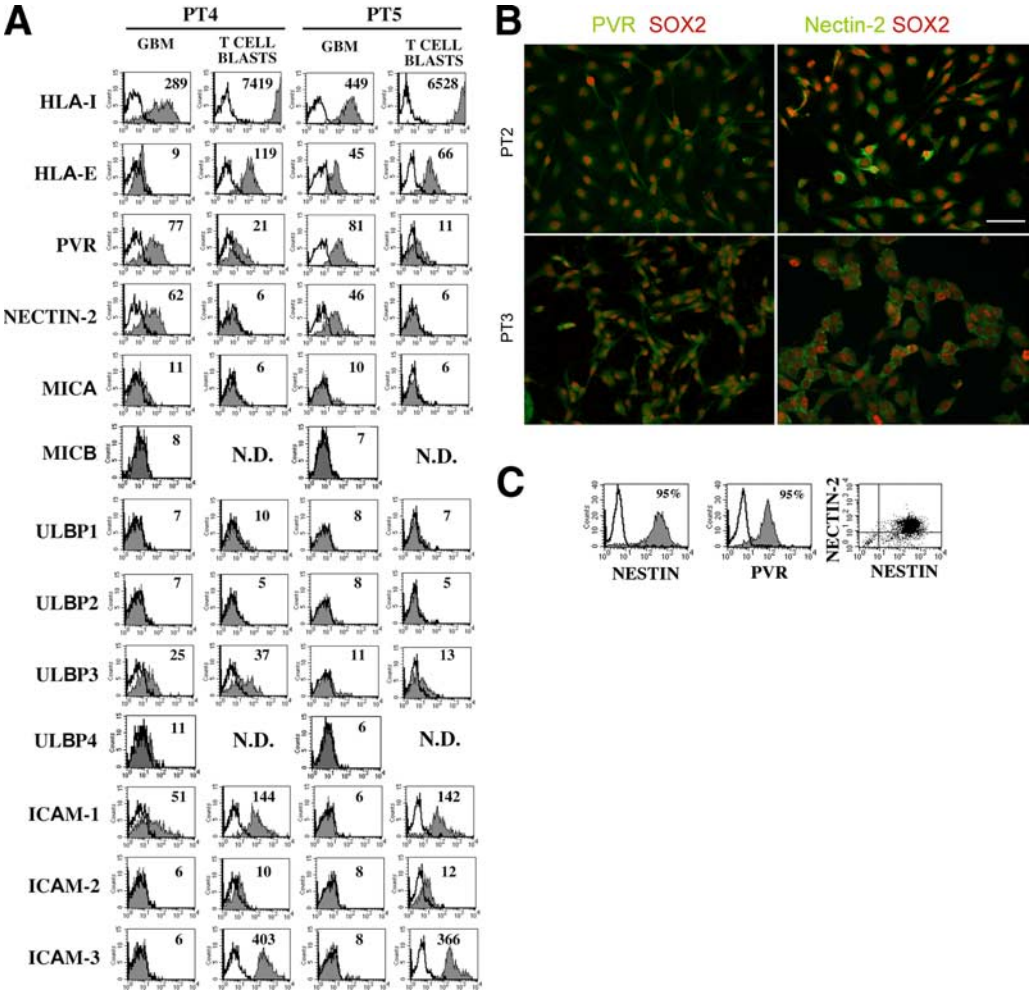

FIGURE 4. Expression of ligands for NK receptors on stem cell medium-cultured GBM cells. *A*, Stem cell-cultured GBM cells or T cell blasts derived from patients 4 and 5 (PT4 and PT5) were analyzed by one-color immunofluorescence and cytofluorimetric analysis for the expression of the indicated molecules. Isotype-specific PE-conjugated goat anti-mouse was used as second reagent. White profiles refer to cells incubated with the second reagent only. The mean fluorescence intensity is indicated. *B*, Stem cell-cultured GBM cells were double stained with anti-PVR or anti-Nectin-2 and anti-SOX2 Abs. Samples were analyzed by fluorescence microscope. Two representative experiments (GBM cells from patients 2 and 3) are shown. Scale bar 100 μm. *C*, Stem cell-cultured GBM cells were stained with PE-conjugated anti-PVR mAb, anti-Nectin-2, or anti-Nestin mAbs, followed by isotype-specific PE- or FITC-conjugated goat anti-mouse second reagents. Samples were analyzed by one- or two-color flow cytometry. A representative experiment (GBM cells from patient 2) is shown.

The expression of DNAM-1 ligands on primary GBM was further confirmed by cytofluorimetric analysis of GBM cells isolated from homogenates of tumor samples collected upon surgical intervention. As shown in Fig. 6*B*, freshly isolated GBM cells (identified as CD56-positive, CD45-negative cells) expressed high levels of PVR and Nectin-2.

Table II. *Expression on stem cell-cultured GBM cells of ligands for major activating NK receptors*[a]

PT	PVR	Nectin-2	MICA	MICB	ULBP1	ULBP2	ULBP3	ULBP4
1	489	214	8	8	10	7	36	5
2	280	240	28	8	34	19	136	7
3	764	97	11	6	6	7	55	6
6	167	119	17	5	16	6	32	7
7	233	157	17	ND	17	11	51	38
8	150	233	4	ND	5	5	8	4
9	310	264	68	ND	ND	ND	ND	ND
17	94	59	4	4	6	8	12	4

[a] The GBM cells derived from the indicated patients (PT) were analyzed by indirect immunofluorescence and FACS analysis for the expression of the indicated molecules, as in Fig. 4. The values of mean fluorescence intensity of the various molecules are indicated.

Discussion

In the present study, we provide experimental evidence that GBM cells displaying stem cell-like properties are highly susceptible to lysis by both allogeneic and autologous IL-2 (or IL-15)-activated NK cells. The molecular basis of the NK-mediated killing of GBM has been identified both in the low (non-protective) levels of HLA class I molecules and in the expression of several ligands recognized by activating NK receptors. This study offers a rationale for the use of activated NK cells in locoregional adoptive treatment of GBM, a tumor characterized by a high rate of relapses after surgery and by poor prognosis.

GBM cells were reported previously to be poorly susceptible to lysis mediated by NK cells. This was ascribed to the expression on GBM of HLA class I molecules, especially of HLA-E (i.e., the ligand of CD94/NKG2A inhibitory receptor), and to the poor expression of ligands for activating NK receptor such as NKG2D (21–23). It should be stressed, however, that, in these studies, the anti-GBM cytotoxicity was analyzed using as effectors either

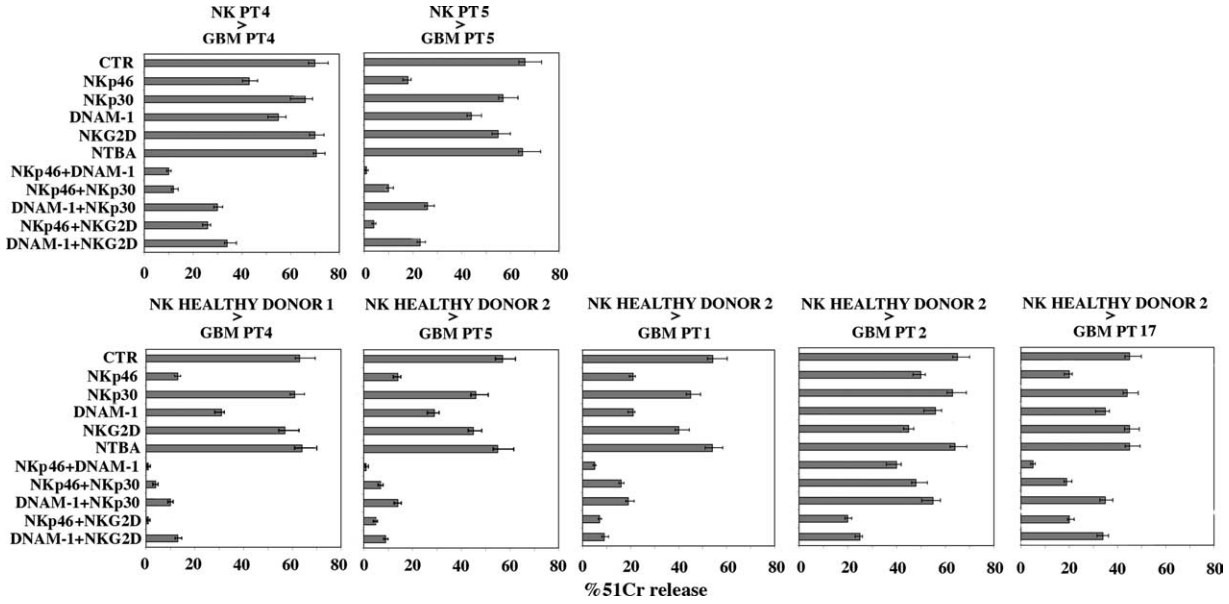

FIGURE 5. Analysis of the triggering receptors involved in NK-mediated killing of stem cell-cultured GBM cells. Stem cell-cultured GBM cells from patients 4 (PT4), 5 (PT5), 1 (PT1), 2 (PT2), and 17 (PT17) were analyzed for susceptibility to lysis mediated by autologous or allogeneic (healthy donors) long-term (IL-2)-activated NK cell populations either in the absence or in the presence of mAbs specific for the indicated molecules, used alone or in combination. The E:T ratio used was 5:1. Experiments were performed using, when available, mAbs of IgM isotype. The results are the mean of triplicate experiments; the SD is indicated.

freshly isolated (resting) NK cells or the NKL NK cell line. Both of these NK cell sources display a limited cytolytic activity, considerably lower than that of lymphokine-activated NK cells used in this study (see Fig. 2*B*). Moreover, the expression of major activating receptors such as NCR (and in particular of NKp46) is either low (resting NK cells) or virtually absent (NKL). Another major difference is that in previous studies target cells were represented by GBM cell lines cultured in medium supplemented with bovine serum. It is believed that, under these culture conditions, tumor cells accumulate various mutations and become substantially different from the original tumor (17).

In our present report, ex vivo derived GBM cells were cultured under conditions that allow propagation of brain cancer stem cells with no substantial differentiation (17). All dissociated primary GBM cultures were positive for the NSC markers Nestin and SOX2, displayed the potential of multilineage differentiation, and were tumorigenic when transplanted in immunodeficient mice. As previously shown for long-term-cultured GBM cell lines, GBM cells cultured in stem cell medium also were resistant to lysis mediated by freshly isolated resting NK cells or the NKL cell line. Importantly, however, GBM with stem cell-like properties were efficiently killed by cytokine-conditioned autologous or allogeneic NK cells. Although HLA class I molecules were present on all GBM cells analyzed, their surface density was low (see, for comparison, the levels expressed by normal T cell blasts). Thus, the levels of HLA class I molecules were inadequate to protect GBM cells from NK-mediated lysis, as demonstrated by the lack of increments of cytolytic activity in tests performed in the presence of a suitable anti-HLA class I mAb (see Figs. 2*A* and 3*B*). We also show that NK-mediated recognition and killing of GBM depend on

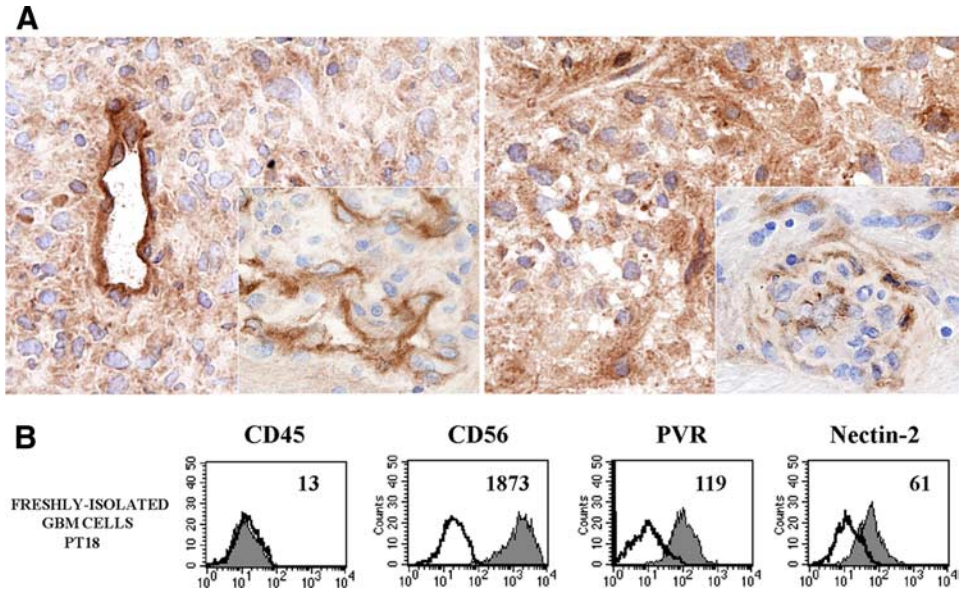

FIGURE 6. Expression of PVR and Nectin-2 on primary GBM. *A*, Frozen primary GBM tumors were analyzed by immunohistochemical staining for the expression of PVR (*left panel*) or Nectin-2 (*right panel*). *Insets*, Show expression of both markers in the intratumoral vessels. A representative experiment is shown. All images are ×40 original magnification. *B*, Freshly derived GBM cells from a representative patient (PT18) were analyzed by cytofluorimetric analysis for the expression of the indicated molecules. The mean fluorescence intensities are indicated. White profiles refer to cells incubated with the second reagent only.

the combined action of different activating NK receptors, primarily NKp46 and DNAM-1. The predominant involvement of these receptors is remarkable because their expression is not affected by TGF-β, an inhibitory cytokine secreted by various tumors, including GBM. Notably, TGF-β has been shown to induce down-regulation of the NKp30- and NKG2D-activating receptors (on NK cells) and of MICA on cancer cells (24, 25). In agreement with previous data, the analysis of the surface expression of the known ligands of activating NK receptors showed that most GBM cells express low or undetectable amounts of NKG2D ligands. Conversely, we show that all GBM cells analyzed as well as primary tumors expressed PVR and Nectin-2, i.e., the DNAM-1-specific ligands. Moreover, GBM cells also expressed the ligand(s) for NKp46, as demonstrated by the strong inhibition of lysis observed upon mAb-mediated masking of the receptor on NK cells.

In most instances, conventional therapies fail to cure patients affected by GBM, whereas they may only allow a minor delay of death (2). Hence, the development of more effective therapies is urgently needed (3). Cell-based, adoptive immunotherapeutic strategies have been proposed to treat patients affected by hematological or solid malignances (4, 5). CTL or NK cells may be infused locoregionally, possibly after surgical resection of the primary tumor mass, or may be attracted at the tumor site (26). In this context, we have showed that, in a murine model of GBM, recipient mice could be cured by a cytotoxic response (involving NK cells) elicited by intratumor injection of IL-21 (27). Moreover, preclinical studies in immunodeficient mice suggested that NK cells could contribute to the eradication of human tumor cells in vivo (28–31). Little is known, however, about the ability of NK cells to reach tumors located in privileged sites, such as the CNS. In this context, by cytofluorimetric analysis we failed to detect NK lymphocytes in GBM tumor homogenates from primary tumors (data not shown). Further studies on the adoptive transfer of activated NK cells in human GBM-bearing NOD/SCID mice will be necessary to analyze the effectiveness of activated NK cells administrated either systemically or at the tumor site.

An obvious requirement for the success of these therapeutic approaches is that tumor cells are susceptible to the T- or NK-mediated cytotoxicity. Our present data show that human GBM cells may indeed represent targets suitable for NK cell-based therapies. Because GBM cells do not express protective amounts of self-HLA class I molecules and lymphokine-activated NK cells from patients at diagnosis (who never received chemo- or radiotherapy) do not display receptor down-regulation and functional impairment, as it may occur in other malignancies (32, 33), autologous lymphokine-activated NK cells may be used. Importantly, our data suggest that lymphokine-activated NK cells can be able to kill cancer cells displaying stem cell-like properties, which are considered the ultimate target of both conventional and innovative therapies (34, 35). Unfortunately, at present there are no reliable markers that unequivocally identify the stem cell population. In this context, it is noteworthy that, according to a recent report (36), even the reliability of CD133 should be questioned, because the tumorigenic potential of GBM cells used in that study did not seem to correlate with the expression of CD133. Our own data seem to confirm this finding; indeed, some, but not all, GBM cell cultures expressed CD133 (and c-Kit) surface markers (see, for example, GBM cells from patients 1, 2, and 4).

An unwanted effect of immunotherapy, however, may be the immune selective pressure. In this context, the NK or T cell-mediated killing of susceptible cancer cells might combine with random mutational events, leading to the selection of tumor cell variants lacking surface molecules crucial for the immune-mediated recognition (37). Down-regulation of HLA class I and MICA molecules represents classical examples of tumor escape from T and NK cell-mediated recognition, respectively (13). Tumor variants, however, must preserve the expression of molecules that are essential for their survival. Interestingly, PVR (DNAM-1 ligand) has been detected in most cancer cell lines or fresh tumors, including not only GBM, but also tumors of different histotype, such as neuroblastomas, medulloblastomas, carcinomas, and leukemias (8, 9, 15, 38). PVR could then represent a molecule essential for tumor survival and/or maintenance of the malignant properties. Accordingly, PVR expression/up-regulation has been shown to correlate with the invasive and migratory capabilities of GBM cells (38, 39).

In conclusion, our present study provides relevant information on ex vivo derived GBM cells with stem cell properties and important clues that justify novel therapeutic approaches based on the use of activated NK cells in an attempt to eradicate tumor cells residual after surgery. Indeed, all GBM analyzed display features that render them susceptible to NK-mediated cytotoxicity.

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Disclosures

The authors have no financial conflict of interest.

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