

## Expression of MHC I and NK ligands on human CD133<sup>+</sup> glioma cells: possible targets of immunotherapy

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**Abstract** Mounting evidence suggests that gliomas are comprised of differentiated tumor cells and brain tumor stem cells (BTSCs). BTSCs account for a fraction of total tumor cells, yet are apparently the sole cells capable of tumor initiation and tumor renewal. BTSCs have been identified as the CD133-positive fraction of human glioma, whereas their CD133-negative daughter cells have limited proliferative ability and are not tumorigenic. It is well established that the bulk tumor mass escapes immune surveillance by multiple mechanisms, yet little is known about the immunogenicity of the CD133-positive fraction of the tumor mass. We investigated the immunogenicity of CD133-

positive cells in two human astrocytoma and two glioblastoma multiforme samples. Flow cytometry analyses revealed that the majority of CD133-positive cells do not express detectable MHC I or natural killer (NK) cell activating ligands, which may render them resistant to adaptive and innate immune surveillance. Incubating CD133-positive cells in interferon gamma (INF- $\gamma$ ) significantly increased the percentage of CD133-positive cells that expressed MHC I and NK cell ligands. Furthermore, pretreatment of CD133-positive cells with INF- $\gamma$  rendered them sensitive to NK cell-mediated lysis in vitro. There were no consistent differences in immunogenicity between the CD133-positive and CD133-negative cells in these experiments. We conclude that CD133-positive and CD133-negative glioma cells may be similarly resistant to immune surveillance, but that INF- $\gamma$  may partially restore their immunogenicity and potentiate their lysis by NK cells.

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### Introduction

Glioblastoma multiforme (GBM) represents one of the most common primary brain tumors of children and adults. Despite advances in treatment such as surgery, radiotherapy, and chemotherapy, the median survival of patients with GBM is only one year [1]. One reason attributed to the poor prognosis of this incurable neoplasm is the highly infiltrative nature of the GBM cells. GBM will invariably reoccur from a limited number of residual tumor cells even after very aggressive surgical procedures. Due to the highly

invasive and heterogeneous nature of high-grade gliomas, there is a need to develop therapies capable of eliminating migratory tumor cells that may have different genetic mutations and diverse phenotypic characteristics [2].

There has been intense interest in the use of immunotherapy to treat glioma, including antibody-based therapy, immunotoxins, dendritic cell vaccines, genetically engineered tumor cells, and immunogene therapy (reviewed in [3–5]). The theoretical advantage of cellular immunotherapy is that tumor-specific T cells can seek and kill invasive GBM cells that remain after surgical resection and are resistant to conventional therapy. Expansion of tumor-specific cytotoxic T lymphocytes (CTLs) has been observed in GBM patients treated with an autologous dendritic cell vaccine [6–8]. However, many patients receiving the vaccine eventually died due to tumor progression, despite the presence of tumor-specific CTLs in peripheral blood and/or marked infiltration of CTLs in the tumor [6–8]. In order for immunotherapy to successfully treat glioma, the cells responsible for tumor renewal must be targeted and eliminated.

Recently, so called “cancer stem cells” have been discovered in a variety of malignant tumors including leukemia [9], prostate [10], breast [11], and lung carcinoma [12]. Cancer stem cells are characterized by their ability to self-renew, extensive proliferative ability, capacity to give rise to differentiated daughter cells, and by the expression of progenitor cell markers that are also found on normal stem cells [13]. CD133 is a 120 kDa cell surface glycoprotein that is expressed on hematopoietic stem cells and neural stem cells [13, 14]. Singh et al. [15] isolated a population of CD133-positive cells from low- and high-grade human brain tumors, then demonstrated their ability to self-renew and to differentiate into heterogeneous daughter cells. As few as 100 CD133-positive human glioma cells were capable of tumor initiation in immunodeficient mice, whereas up to 100,000 CD133-negative tumor cells isolated from the same bulk tumor mass engrafted but did not form tumors [16]. CD133-positive brain tumor cells have been termed “brain tumor stem cells” (BTSCs), because they have similar properties to normal neural stem cells and appear to be responsible for tumor initiation and tumor renewal [15–19].

Further investigation into the mechanisms of immune evasion that GBM cells utilize is likely to provide novel strategies to target glioma by immunotherapy. The expression of major histocompatibility complex one (MHC I) is critical for efficient antigen presentation to CTLs and subsequent tumor cell lysis [20]. It is well known that gliomas express low levels of MHC I

and this may be one mechanism of immune evasion [4]. In addition, it has been documented that glioma cells secrete high levels of transforming growth factor beta-2, which results in downregulation of activating ligands for natural killer (NK) cells on tumor cells, and downregulation of NKG2D, a potent activating receptor expressed on NK cells and CTLs [21]. NK cells play a major role in tumor immune surveillance and their killing activity is regulated by several NK activating receptors, which include natural cytotoxic receptors (NCRs), and NKG2D [22, 23]. A previous study showed involvement of these receptors in recognition and killing of neural tumors [24]. Lack of MHC I expression, and upregulation of activating ligands promotes NK cell-mediated tumor cell lysis [25].

Although there is considerable knowledge regarding the immunology of the bulk tumor mass, no study has delineated the immunogenicity of the CD133-positive fraction of glioma, which appears to be the cells that ultimately contribute to tumor renewal, disease progression, and death. In order to investigate the potential mechanisms CD133-positive cells use for immune evasion, we examined the expression of MHC I and ligands for NK cell-activating receptors in primary GBM and astrocytoma cells. The response of CD133-positive and CD133-negative cells to interferon gamma (INF- $\gamma$ ), a potent immunostimulatory cytokine, was examined using flow cytometry (FCM) and in vitro cytotoxicity assays. This study represents the first investigation into the immunogenicity of CD133-positive cells and has implications for tumor immunology and immunotherapy.

## Material and methods

### Cell culture

Two GBM samples and two astrocytoma samples were obtained from surgically resected tumor tissue and the diagnoses were verified by a certified pathologist. Tumor tissues were washed with PBS, cut into very small pieces, and incubated in a 2% Trypsin solution at 37°C for 10 min. The supernatant containing liberated cells was collected, washed with PBS, and plated in 75 cm<sup>2</sup> tissue culture flasks in DMEM with 20% FBS, 100  $\mu$ g/ml penicillin, and 100  $\mu$ g/ml streptomycin. One day later the adherent cells were detached by treatment with a trypsin/EDTA solution and seeded at a concentration of 10<sup>5</sup> cells/ml into 10 cm polyHEMA-coated tissue culture plates. Recombinant human epidermal growth factor (EGF; 20 ng/ml, sigma, St. Louis, MO) and recombinant human basic fibroblast

growth factor (bFGF; 20 ng/ml, Sigma, St. Louis, MO) were added to the media, and the cultures were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator. K562 NK target cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 with 10% FBS.

### Immunofluorescence

For immunostaining, undifferentiated tumor spheres were plated onto Neuromix proliferation media-coated chamber slides (Nalge Nunc international, Rochester, NY) and cultured in Neuromix stem cell proliferation media (Neuromix, Minneapolis, MN) for 12 h. Cells were then fixed with 4% paraformaldehyde, permeabilized with Triton X, and incubated in primary antibody against CD133 (mouse anti-human CD133/2, Miltenyi Biotec, Auburn, CA; 1:100) for 24 h at 4°C. Cy3-conjugated secondary antibody (donkey anti-mouse, Jackson Immunoresearch Lab. Inc, West Grove, PA, 1:200) was added and incubated for 2 h at RT, after washing with PBS. Dapi (Sigma, St. Louis, MO) was used for counterstaining cell nuclei.

For immunostaining of differentiated tumor cells, isolated tumor spheres were plated in commitment media (Neuromix, Minneapolis, MN) in chamber slides. Seven days later cells were fixed and permeabilized as described above. Primary antibodies against GFAP (polyclonal rabbit anti-GFAP, Dakocytomation, Denmark; 1:100), Tuj1 (purified rabbit anti-beta-tubulin, Covance Research Products, Inc, Denver, PA; 1:1000) were added and incubated for 24 h at 4°C. Cy3-conjugated secondary antibody (donkey anti-rabbit, Jackson Immunoresearch Lab Inc., West Grove, PA; 1:200) was then applied for 2 h at RT. Cells were also counterstained with Dapi, and all the stained samples were mounted on slides and analyzed using a Nikon ECLIPSE 600 system (Nikon, Tokyo, Japan) attached to a CCD camera. For negative controls, slides were incubated in blocking serum alone at the primary antibody staining step, and stained with secondary antibody identically to samples that received primary antibody (Fig. 1F, H); or as a negative control for CD133 staining, an isotype antibody was used instead of anti-CD133 primary antibody (mouse IgG2b control, Chemicon, Temecula, CA; 1:100) for 24 h at 4°C (Fig. 1D).

### Cytokine treatment

Lyophilized recombinant human IFN- $\gamma$  (R&D Systems, Minneapolis, MN) was reconstituted (with PBS/

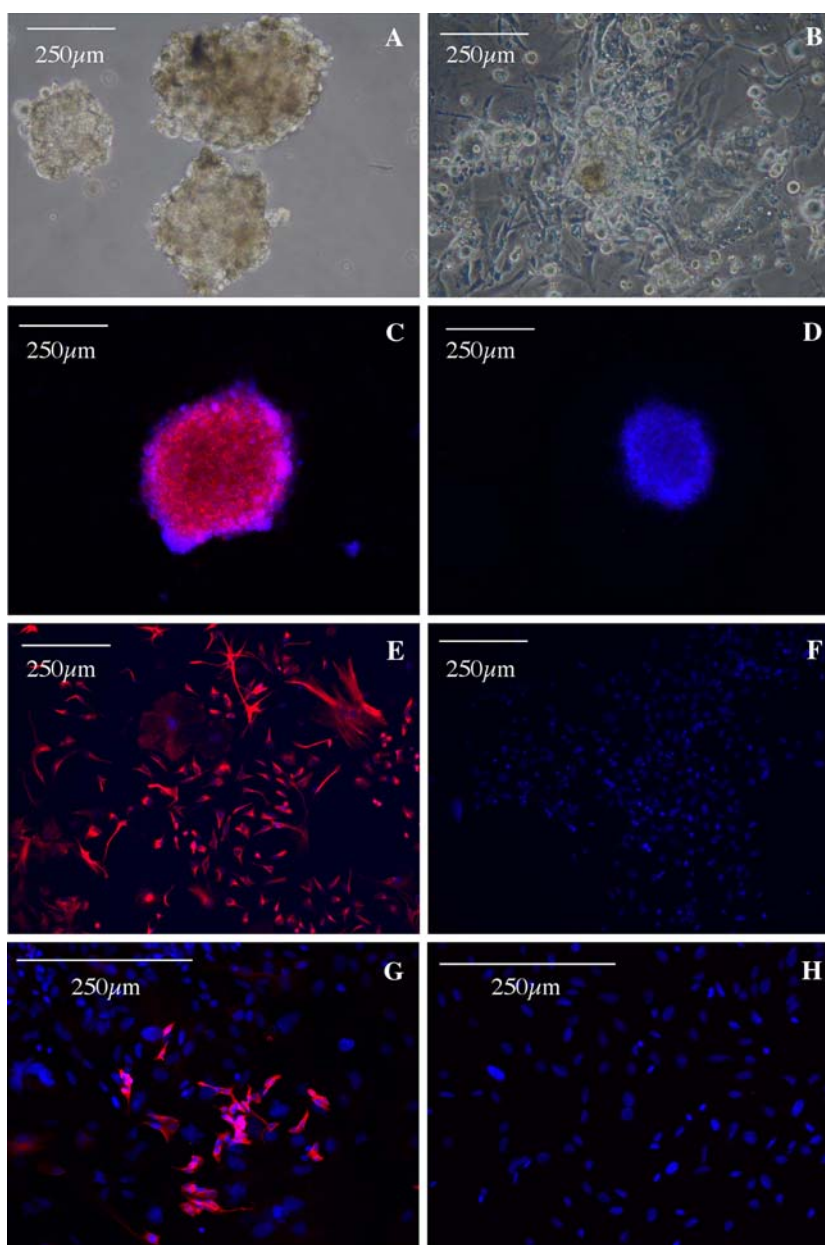
0.1%BSA) at a final concentration of 10  $\mu$ g/ml, aliquoted, and stored at -20°C. IFN- $\gamma$  was added to the growth media starting five days after tumor dissociation at a final concentration of 100 ng/ml. Expression of MHC I and NK ligands was evaluated 24 or 48 h later.

### Flow cytometry analysis

After six to seven days in culture, brain tumor cells, treated or untreated with IFN- $\gamma$ , were analyzed by FCM to assess the expression of MHC I and NK ligands. For MHC I expression, cells for analysis were dissociated with a narrowed, fire polished Pasteur pipette and resuspended in FCM buffer. One hundred microliter aliquots of 10<sup>5</sup> cells were placed in FCM tubes (Becton Dickinson Immunocytometry Systems, San Jose, CA) and incubated on ice for 30 min with APC-conjugated anti-CD133 antibody (Miltenyi Biotec, Auburn, CA; 1:10) and PE-conjugated anti-MHC I (BD Pharmingen, CA, USA; 1:10), or with isotype control antibodies (BD Pharmingen, CA, USA; 1:10). Cells were washed twice with FCM buffer, and then resuspended in 0.5 ml of a 1% formalin/PBS solution. Analysis was performed on a FACScalibur system (Becton Dickinson Immunocytometry Systems, San Jose, CA). Two thousand to ten thousand cells were collected and analyzed with CELLQUEST software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Forward and side scatter plots were used to exclude debris/dead cells from further analysis. Every sample was analyzed in triplicate.

To investigate the expression of NK ligands, we used recombinant human NKp30-IgGfc, NKp44-IgGfc, NKp46-IgGfc, NKG2D-IgGfc fusion proteins (R & D Systems, Minneapolis, MN); the receptor portion of the fusion protein binds the corresponding ligand(s) on the tumor cell to allow detection of the ligand. The lyophilized protein was reconstituted in PBS with 0.1% BSA at a final concentration of 100  $\mu$ g/ml. Staining of NK ligands was performed by the addition of 10  $\mu$ l of reconstituted IgG-fusion proteins to 2  $\times$  10<sup>4</sup> cells in a 100  $\mu$ l volume, and incubated for 2 h on ice without blocking. After washing with PBS, cells were incubated with FITC-conjugated mouse anti-human IgGfc and APC-conjugated anti-CD133 antibody (Miltenyi Biotec, Auburn, CA). Ten thousand events were collected and analyzed using Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). K562, a well-known NK target cell line was used as a positive control [26]. All the FCM evaluations were performed in triplicate.

**Fig. 1** Isolation and differentiation of CD133-positive cells in vitro **(A)** Tumor spheres formed three days after the primary tumor (GBM) was cultured in stem cell media. **(B)** Tumor spheres that were cultured in commitment media became adherent and morphologically resembled a more differentiated cell. **(C)** Immunostaining of tumor spheres for CD133 (red) and cell nuclei with Dapi (blue). **(D)** Tumor spheres stained with secondary antibody and Dapi, but with isotype primary antibody (control). **(E)** Immunostaining of differentiated tumor cells for glial cell marker (GFAP, red) and cell nuclei with Dapi (blue). **(F)** Adherent tumor cells stained with secondary antibody and Dapi, but without anti-GFAP primary antibody (control). **(G)** Immunostaining of differentiated tumor cells for early neuronal marker (TuJ1, red) and cell nuclei with Dapi (blue). **(H)** Adherent tumor cells stained with secondary antibody and Dapi, but without anti-beta-tubulin primary antibody (control)



#### Isolation of NK Cells and CD133-positive glioma Cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy blood donors (Memorial Blood Centers of Minnesota, Minneapolis, MN) by Ficoll-Paque density gradient centrifugation. NK cells were purified from human PBMCs by using a NK cell isolation kit (Miltenyi Biotec, Auburn, CA). The degree of purity (93% CD56-positive CD3-negative) was measured with FCM using antibodies against CD56 and CD3. Isolated NK cells were cultured in RPMI 1640 with 10% FBS supplemented with

500 IU/ml recombinant human IL-2 (R&D Systems, Minneapolis, MN), and used as effector cells at seven days after tumor dissociation.

CD133-positive cells were isolated using the Miltenyi Biotec CD133 isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. Briefly, after a seven-day culture in stem cell media, cells from the tumor sample were collected, dissociated with a narrowed, fire-polished Pasteur pipette, labeled with microbeads, and isolated by using magnetic sorting. CD133-sorted cells were evaluated for purity (about 94% CD133-positive; 99% CD133-negative) by FCM. Aliquots of CD133-positive cells were resuspended

in stem cell media with EGF (20 ng/ml, Sigma, St. Louis, MO) and bFGF (20 ng/ml, Sigma, St. Louis, MO) and seeded in six well plates; CD133-positive cells were also incubated with IFN- $\gamma$  (100 ng/ml). After two days in culture, the IFN- $\gamma$ -treated and -untreated CD133-positive or CD133-negative cells were used as target cells in an in vitro NK killing assay.

### NK cytotoxic assay

The susceptibility of tumor cells to be lysed by IL-2-activated peripheral NK cells was tested using FCM-based method as previously described [27]. Briefly, target cells were labeled with the green-fluorescent membrane dye DIOC18 (Sigma, St. Louis, MO). Effector cells were then mixed with the labeled target cells at (NK effector/tumor target) E/T ratios of 100:1, 50:1, 25:1, 10:1, 5:1 and 1:1. Following the addition of propidium iodide (labels dead cells), the cell suspension was centrifuged briefly and incubated at 37°C for 4 h. To achieve maximum lysis, cells were incubated with saponin at 0.3 mg/ml; spontaneous cell death was determined by incubating the effector cells and target cells in 4°C. Samples were run on a flow cytometer (BD FACScalibur system, Becton Dickinson) and analyzed with Cell Quest software. Cytotoxicity was calculated as previously described [27]. For the NK blocking experiment,  $2 \times 10^5$  target cells were stained with DIOC-18, after washing, the cells were resuspended in culture media and incubated with 10  $\mu$ g/ml each NK fusion receptor (NKp30, NKp44, NKp46, NKG2D) at 37°C, 5% CO<sub>2</sub> for 30 min; the cytotoxicity assay was then carried out identically as above.

## Results

### Expression of MHC I in CD133-positive glioma Cells

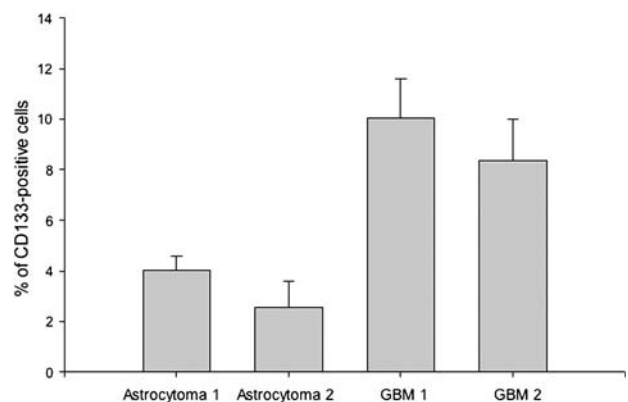
To obtain human CD133-positive cells, we cultured freshly isolated glioma samples in vitro in stem cell media as described in the methods; under these culture conditions we observed the formation of tumor spheres in all the cultures (tumor sphere formation is a hallmark of BTSCs [15–19]). Immunofluorescent staining revealed that the tumor spheres were comprised of CD133-positive cells (Fig. 1C). After plating in commitment media to cause adherence and differentiation, tumor spheres differentiated into adherent GFAP-positive (astrocyte marker) and Tuj1-positive (neuronal marker) cells (Fig. 1E, G), which is inconsistent with

results of other studies [15, 28] and confirms the existence of multipotent CD133-positive cells in our samples.

After a week of culture in stem cell media, all cells in each culture (including the formed tumor spheres and non-sphere cells) were dissociated into a single cell suspension, stained with antibodies against CD133 and MHC I, and evaluated using FCM. CD133/MHC I double-staining was performed to evaluate the expression of MHC I in different grades of glioma (astrocytoma and GBM). FCM analysis revealed a low percentage of CD133-positive cells in low-grade astrocytomas (2–4% CD133-positive), but a relatively higher percentage of CD133-positive cells in GBM (8–12% CD133-positive; Fig. 2). This data is consistent with the data reported by other investigators, which found a positive correlation between the abundance of CD133-positive cells and tumor grade [15]. FCM analysis indicated that CD133-positive and CD133-negative cells from all samples expressed low levels of MHC I. Less than a third of the cells in all tumor samples from either population expressed detectable MHC I, with the exception of the CD133-negative cells from astrocytoma-1 (60% expressed MHC I; Fig. 4).

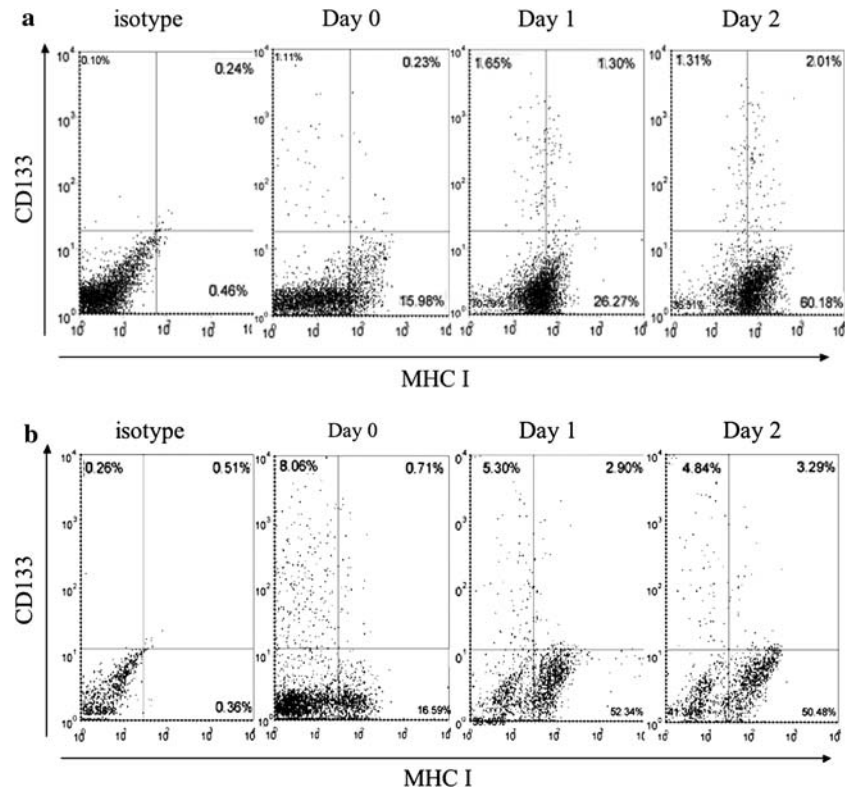
### Effects of IFN- $\gamma$ on CD133-positive glioma cells

MHC I molecules are polymorphic proteins that are critical for cellular immune responses, but the CD133-positive cells in our cultures expressed low levels of MHC I on their cell surface (Figs. 3, 4). Cytokines, such as IFN- $\gamma$ , have the ability to upregulate the expression of MHC I on tumor cells [29–32]. In order to investigate the responsiveness of CD133-positive



**Fig. 2** Percentage of CD133-positive tumor cells in glioma cultures. Surgically resected glioma tissue was cultured for one week and analyzed for CD133 expression using flow cytometry. Three analyses were performed for each sample and the error bars represent  $\pm$  standard deviation

**Fig. 3** Flow cytometry analysis of MHC I expression on CD133-positive and CD133-negative glioma cells. Representative example of FCM analysis for MHC I and CD133 expression on tumor cells from astrocytoma (**A**), and GBM (**B**). Time points shown are before (day 0), or after (days 1, 2), incubation in 100 ng/ml IFN- $\gamma$ . Each sample was analyzed in triplicate with similar results



cells to IFN- $\gamma$ , we added IFN- $\gamma$  to the glioma cultures five days after surgical resection and isolation. The expression of MHC I on CD133-positive and CD133-negative cells cultured in stem cell media was then measured by FCM. IFN- $\gamma$  treatment appreciably up-regulated expression of MHC I on CD133-positive and CD133-negative cells (Figs. 3, 4).

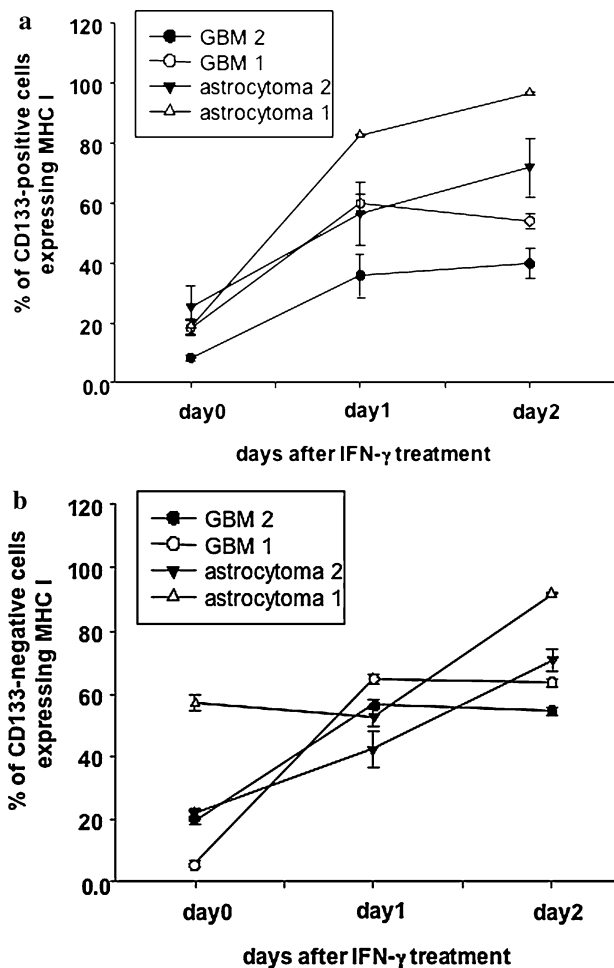
For the astrocytoma-1 sample, more than 90% of CD133-positive and CD133-negative cells expressed MHC I after exposure to IFN- $\gamma$  (Fig. 4). For the other samples, high percentages of CD133-positive cells (about 30% for astrocytoma-2, 50% for GBM-1, and 60% for GBM-2) did not express MHC I even after 48 h of IFN- $\gamma$  treatment; similar trends were apparent in the CD133-negative fraction (Figs. 3, 4).

#### CD133-positive GBM Cells are killed by NK Cells in response to IFN- $\gamma$

Given that no information is available about the ligands for NCRs and NKG2D expressed on CD133-positive cells, we investigated the expression of these ligands on CD133-positive glioma cells using FCM. Since several of the ligands that bind NCRs have not been identified, we used recombinant fusion proteins composed of the NK cell receptor and IgGfc: NKp30-IgGfc, NKp44IgGfc, NKp46-IgGfc and NKG2D-IgGfc to evaluate the corresponding NK ligands. To

determine the specificity of these reagents, the fusions were first used to stain K562 cells, a cell line which is sensitive NK-mediated lysis [26]. Ligands for NCRs and NKG2D were highly expressed on the control K562 cells (over 90% of cells), but expressed at a low level on CD133-positive tumor cells (less than 30% of cells; Figs. 5, 6). IFN- $\gamma$  has the ability to upregulate the expression of NK ligands [33], so we next tested whether IFN- $\gamma$  treatment could increase the expression of these ligands. Expression of ligands for NKp30, NKp44 and NKp46 and NKG2D was induced in CD133-positive cells in response to IFN- $\gamma$ . Expression of NK ligands increased from less than 20% of cells expressing any particular ligand, to 50–85% of cells expressing ligand; the same trend was apparent in the CD133-negative cells. (Figs. 5, 6).

The finding that IFN- $\gamma$ -treated CD133-positive cells expressed ligands for activating NK receptors, together with the low expression of MHC I, suggests the possibility that CD133-positive cells could be susceptible to NK cell-mediated lysis. To test this hypothesis, we next determined the susceptibility of IFN- $\gamma$ -treated CD133-positive cells to NK cell-mediated cytotoxic activity using cells isolated from GBM-1. NK cells efficiently killed the control K562 cells, did not kill CD133-positive cells even at an E/T ratio of 100, which may be due to the lack of expression of NK ligands. A significant increase in the killing of CD133-positive cells by NK



**Fig. 4** The effect of IFN- $\gamma$  on MHC I expression on CD133-positive and CD133-negative glioma cells. **(A)** The percentage of CD133-positive cells expressing MHC I was determined by flow cytometry before and after incubation in 100 ng/ml IFN- $\gamma$ . **(B)** The percentage of CD133-negative cells expressing MHC I was determined by FCM before and after incubation in 100 ng/ml IFN- $\gamma$ . The values were calculated by dividing the number of MHC I/CD133-double positive cells by total CD133-positive cells (in A) or MHC I-positive, CD133-negative cells by total CD133-negative cells (in B). Each sample was analyzed in triplicate and the error bars represent  $\pm$  standard deviation

cells was observed when tumor cells were pre-treated with IFN- $\gamma$ ; no killing increased to about 40% tumor lysis at an E/T of 100 (Fig. 7A). A very similar trend was observed in the CD133-negative fraction; approximately 20% of CD133-negative cells were lysed by NK cells at an E/T ratio of 100 without IFN- $\gamma$  treatment, and this lysis increased to 60% with IFN- $\gamma$  pre-treatment (Fig. 7B).

In order to determine if NK-mediated lysis was dependent on receptor/ligand interaction between the NK cell and tumor cell, we conducted a blocking experiment by incubating the tumor cells with recombinant NK receptors (NKp30, NKp44 and NKp46

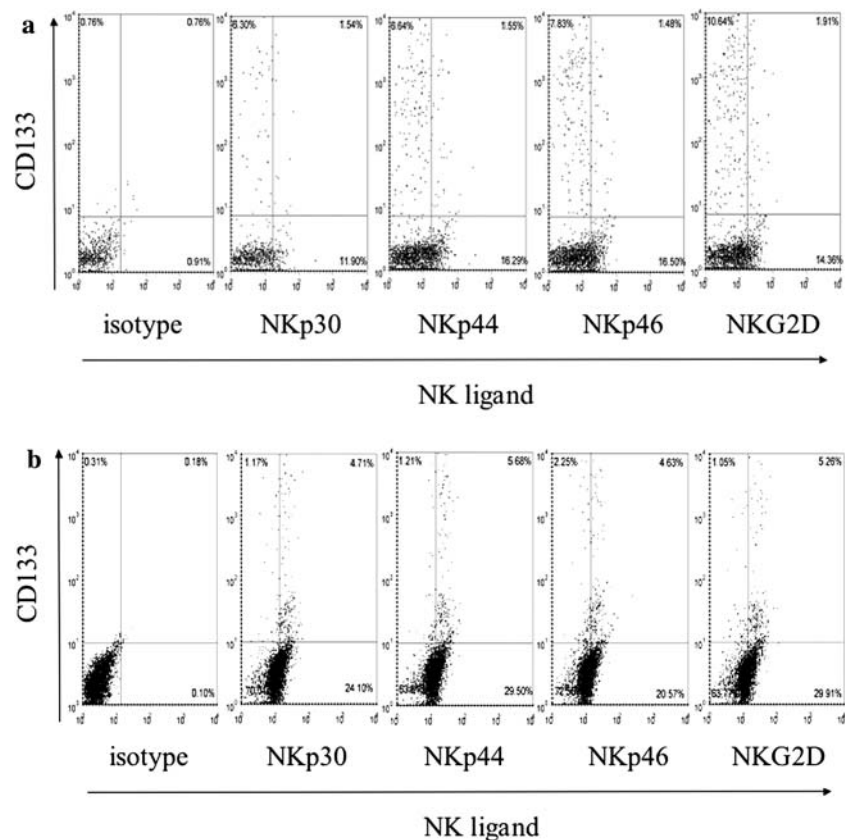
and NKG2D) prior to incubation with NK cells. Blocking reduced the cytotoxicity of NK cells on CD133-positive and CD133-negative glioma cells by over 50% at an E/T ratio of 100 (Fig. 7). Taken together these data demonstrate that glioma cells can be lysed by NK cells in a NK receptor/ligand-dependent manner, but that exposure to IFN- $\gamma$  was required to allow lysis of CD133-positive cells.

## Discussion

The BTSC theory suggests that disease progression and the high incidence of recurrence after treatment is mainly caused by CD133-positive cells [2, 16, 18]. The CD133-negative cells, although presumably bearing similar genetic mutations as the CD133-positive parental cells, appear to lose the self-renewal and tumor initiating capabilities [16, 19]. It is therefore critical to study the CD133-positive glioma cells in all areas of brain tumor research, rather than viewing the tumor as a bulk mass of cells that can be considered biologically and functionally similar. The discovery of BTSCs suggests that studies conducted to bulk tumor should be revisited, and the differences (or similarities) between the CD133-positive and CD133-negative cells should be delineated in order to develop therapies capable eliminating both populations.

Escape from immune surveillance is believed to be a primary feature of malignant disease in humans. It is well known that glioma cells can downregulate the expression of MHC I [4], and may therefore decrease their susceptibility to being lysed by CTLs. Therefore, we first investigated the expression of MHC I on CD133-positive glioma cells. The majority (~80%) of CD133-positive cells isolated from glioma patients did not express MHC I at levels detectable by FCM (Figs. 3, 4). The current study demonstrates that exposure to IFN- $\gamma$  is capable upregulating the cell surface expression of MHC I on CD133-positive cells. In one low-grade astrocytoma we analyzed, most of the CD133-positive cells upregulated expression of MHC I after treatment with IFN- $\gamma$  (up to 90% became MHC I-positive); this may indicate a relatively normal response to IFN- $\gamma$ , normal MHC I expression pathway, and less heterogeneous nature. However, three of the four tumors we analyzed contained a large percentage of CD133-positive cells that did not express MHC class I even after treatment with IFN- $\gamma$ . This may indicate a loss responsiveness to IFN- $\gamma$ , or non-functional MHC expression pathway, which occurred in CD133-positive cells and is therefore present in their CD133-daughter tumor cells. The apparent lack of ability to express

**Fig. 5** Flow cytometry analysis of NK ligand expression on CD133-positive and CD133-negative glioma cells. Representative example of FCM analysis of the expression of NK ligands on CD133-positive and CD133-negative GBM-2 cells before (A) or one day after (B) incubation in 100 ng/ml IFN- $\gamma$ . Each sample was analyzed in triplicate with similar results



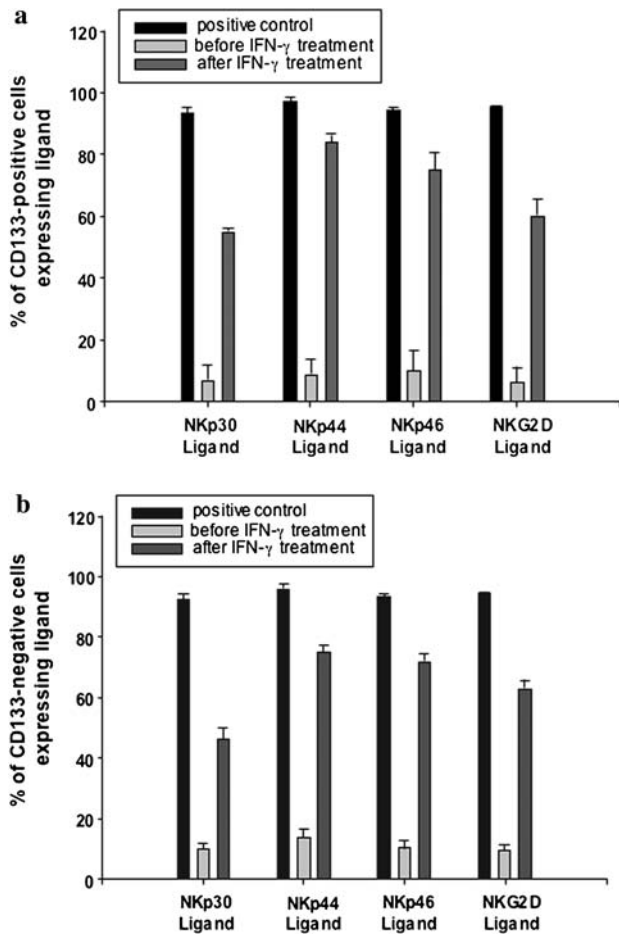
MHC I in response to IFN- $\gamma$  suggests that MHC I-independent immunotherapy may be a beneficial adjuvant to treatment of glioma by immunotherapy.

Glioma cells that do not express MHC I may be more susceptible to lysis by NK cells, which recognize cells lacking MHC I expression [34]. NK cells are potent cytotoxic effector cells of the innate immune system that play a major role in early host defense against tumors [35]. They are characterized by the cell surface expression of CD56 and absence of CD3 [36]. Their activity is regulated by inhibitory (MHC I) and stimulatory (NK activating ligands) signals sensed by receptors that constantly probe nearby cells [34]. The MHC I molecules at the cell surface can interact with killer inhibitory (KIR) receptors and inhibit NK cell activation [37]. Loss of MHC I expression can result in a lack of inhibitory signals and lead to NK cell activation [34]. The NKG2D receptor, and NCRs (NKp30, NKp44, NKp46) represent crucial receptors for NK cell activation [23]. The susceptibility of tumors to NK lysis relies on the expression of ligands for these activation receptors [23].

Low expression of NK ligands has been hypothesized as a mechanism for tumor cells to escape NK cell recognition [38]. In the current study we found that, without treatment with IFN- $\gamma$ , CD133-positive cells

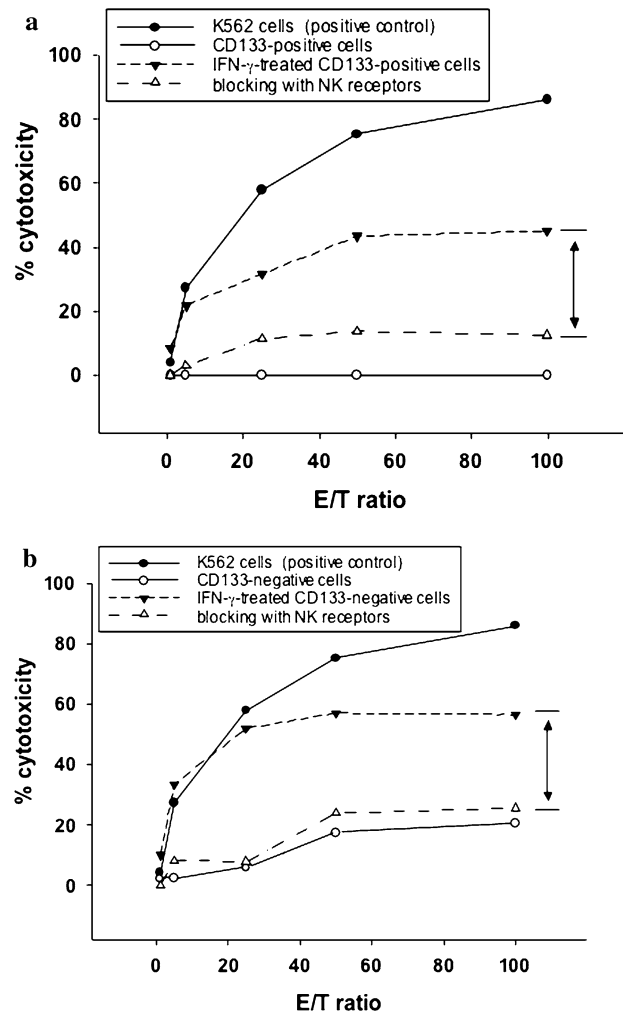
express low levels of NK ligands (Fig. 6) and CD133-positive cells were not killed by NK cells (Fig. 7). In spite of this, after exposure to IFN- $\gamma$ , CD133-positive cells significantly increased the expression of NK ligands and became susceptible to lysis by NK cells (Fig. 6, 7). This indicates that under non-inflammatory conditions, CD133-positive cells may not be recognized by NK cells. Nevertheless, in an inflammatory environment, or under conditions that induce IFN- $\gamma$  secretion, NK cells may be capable of lysing CD133-positive cells in vivo. It is important to consider that even though an E/T ratio of 100 used in the aforementioned experiment may not be physiologically relevant, that NK cells interact with antigen presenting cells to modulate and potentiate a robust adaptive immune response [39, 40]. Therefore, minimal NK activity in a glioma may be capable of initiating and/or amplifying an adaptive anti-tumor immune response.

Adoptive transfer of ex vivo-expanded, IL-2-activated NK cells might be a suitable approach. Activated NK cells could potentially pass through the tumor stroma and target CD133-positive cells. Alternatively, any vaccine or immunogene therapy approach that can effectively recruit endogenous NK cells into the tumor stroma may be beneficial to kill cells that downregulate MHC I. However, our results suggest that IFN- $\gamma$



**Fig. 6** NK ligand expression on CD133-positive and CD133-negative glioma cells was upregulated by IFN- $\gamma$ . FCM analysis of the expression of NK ligands on CD133-positive (A), and CD133-negative (B), GBM-1 cells before (day 0) or one day after incubation in 100 ng/ml IFN- $\gamma$ . The average values were calculated by dividing the number of NK ligand/CD133-double positive cells by total CD133-positive cells (in A) or by dividing the number of NK ligand-positive, CD133-negative cells by total CD133-negative cells (in B). Each sample was analyzed in triplicate and the error bars represent  $\pm$  standard deviation

adjuvant therapy, or another approach to upregulate MHC I and NK ligands, may be required to achieve a robust immune reaction. Intratumoral INF- $\gamma$  infusion, as protein or by gene transfer, may also increase the ability of CTLs to recognize and kill CD133-positive cells by upregulating MHC I. Systemic administration of INF- $\gamma$  has shown modest clinical efficacy in patients with metastatic melanoma (5–13% responded), but has been combined with chemotherapy to achieve response rates in up to 66% of patients [41]. Intratumoral INF- $\gamma$  gene therapy has been investigated in intracranial rodent models of glioblastoma with encouraging results. Using retroviral a vector, Saleh et al. [42] showed curative effects of intratumoral INF- $\gamma$  gene transfer into C6 glioma cells implanted into the rat



**Fig. 7** Effect of IFN- $\gamma$  on the susceptibility of CD133-positive and CD133-negative glioma cells to NK-mediated lysis. Representative data from NK cytotoxicity experiment using untreated (no IFN- $\gamma$ ) or IFN- $\gamma$ -treated (100 ng /ml for 48 h) tumor cells. NK cells were isolated from human PBMC (93% CD56-positive). CD133-positive and CD133-negative cells were isolated from cultured GBM-1 cells and the purity was determined by flow cytometry (94% pure CD133-positive and 99% pure CD133-negative). IL-2-activated NK cells were incubated with CD133-positive (A), or CD133-negative (B), tumor cells for 4 h at effector (NK) to target (tumor cell) ratios indicated. In addition, blocking of NK ligands was done by adding soluble NK receptors as described in methods. The arrow marks the difference in cytotoxicity with and without blocking. Cytotoxicity analysis was performed using flow cytometry as previously described [27]. Determinations were performed in triplicate with similar results

brain. Using a GL26 syngeneic intracranial model, Ehtesham et al. [43] used intratumoral adenoviral-mediated gene transfer of INF- $\gamma$ ; they demonstrated an increase in animal survival that was accompanied by increased MHC I expression and recruitment of CD4<sup>+</sup> T cells and CTLs cells into the tumor. Taken together, the current in vitro study and previous in vivo studies

indicate that intratumoral INF- $\gamma$  gene transfer combined with a dendritic cell vaccine [44] or NK cell transplant may be capable increasing the immunogenicity of CD133-positive cells and thereby enhance the efficiency of immunotherapy. However, even after INF- $\gamma$  treatment, a significant fraction of CD133-positive cells still did not express detectable MHC I or NK ligands (Figs. 3–6). Therefore, additional treatment modalities should be used in combination with immunotherapy to achieve maximum anti-tumor efficacy.

Our study is the first to illustrate the immunological properties of human CD133-positive glioma cells. Based on data obtained from GBM and astrocytoma samples, it appears that a significant fraction of CD133-positive cells may not be recognized by T cells or NK cells. Interestingly, we did not find any appreciable difference in the expression of MHC I and NK ligands on CD133-positive and CD133-negative cells in these studies, which suggests that both cell populations may utilize a similar mechanism to avoid immune surveillance. Whether or not this finding is true in vivo remains to be determined. Nonetheless, these data may account for tumor progression in GBM patients, despite an expansion of tumor-specific CTLs after administration of a dendritic cell vaccine [6–8], because the tumor-renewing BTSCs may not be visible to the immune system. Treatment with IFN- $\gamma$  causes a fraction of CD133-positive cells to express MHC I and NK ligands, thereby enhancing the possibility of their recognition and destruction. The concentration and duration of IFN- $\gamma$  exposure required to increase the immunogenicity of CD133-positive cells in vivo is an important unknown that should be addressed. Further study of CD133-positive glioma cells may reveal unique epitopes that could be targeted by immunotherapy and will improve on our basic understanding of brain tumor immunology and enhance future treatment approaches.

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