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Resistance to cytotoxicity and sustained release of interleukin-6 and interleukin-8 in the presence of decreased interferon-γ after differentiation of glioblastoma by human natural killer cells

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Abstract

Natural killer (NK) cells are functionally suppressed in the glioblastoma multiforme (GBM) tumor microenvironment. We have recently shown that survival and differentiation of cancer stem-like cells (CSCs)/poorly differentiated tumors are controlled through two distinct phenotypes of cytotoxic and non-cytotoxic/split anergized NK cells, respectively. In this paper, we studied the function of NK cells against brain CSCs/poorly differentiated GBM and their NK cell-differentiated counterparts. Brain CSCs/poorly differentiated GBM, differentiated by split anergized NK supernatants (supernatants from NK cells treated with IL-2 + anti-CD16mAb) expressed higher levels of CD54, B7H1 and MHC-I and were killed less by the NK cells, whereas their CSCs/poorly differentiated counterparts were highly susceptible to NK cell lysis. Resistance to NK cells and differentiation of brain CSCs/poorly differentiated GBM by split anergized NK cells were mediated by interferon (IFN)-γ and tumor necrosis factor (TNF)-α. Brain CSCs/poorly differentiated GBM expressed low levels of TNFRs and IFN-γRs, and when differentiated and cultured with IL-2-treated NK cells, they induced increased secretion of pro-inflammatory cytokine interleukin (IL)-6 and chemokine IL-8 in the presence of decreased IFN-γ secretion. NK-induced differentiation of brain CSCs/poorly differentiated GBM cells was independent of the function of IL-6 and/or IL-8. The inability of NK cells to lyse GBM tumors and the presence of a sustained release of pro-inflammatory cytokines IL-6 and chemokine IL-8 in the presence of a

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

decreased IFN-γ secretion may lead to the inadequacy of NK cells to differentiate GBM CSCs/poorly differentiated tumors, thus failing to control tumor growth.

Keywords

IFN-γ; NK cells; GBM; Brain cancer stem cells; Immunotherapy

Introduction

Patients diagnosed with glioblastoma multiforme (GBM) have a poor prognosis and therefore, there is an urgent need for the development of novel immunotherapeutics to combat this disease. GBM contains stem-like populations, known as cancer stem-like cells (CSCs), which are capable of self-renewal, and sustains tumor growth. CSCs and poorly differentiated GBMs have developed mechanisms to resist chemo- and radiotherapy by increasing DNA mismatch repair and multidrug resistance genes [1, 2]. Several studies have also demonstrated detection of IL-6 and IL-8 in the GBM microenvironment [3–9]. A correlation between the differentiation status of tumor CSCs and their therapeutic outcome has been shown previously [10].

Recently, T cell-based immunotherapy failed to reveal a sufficient clinical benefit, perhaps because of a decreased MHC-I expression on GBM cells [11, 12]. NK cells are prime candidates for targeting and lysing tumor cells with no or lower MHC-I expression. It has been demonstrated that NK cells are present in the human brain and central nervous system of patients suffering from multiple sclerosis [13, 14] and also in non-pathological brain conditions [15], and have significant influence in maintaining the balance in immune regulation. Furthermore, NK cell administration as an adjuvant therapy was shown to reduce GBM growth resulting in increased survival in preclinical animal models [16]. Consequently, NK cell-based immunotherapy may become a promising strategy for GBM patients.

Immunosuppression and escape from immune surveillance are key factors contributing to tumor progression [17–20]. Thus, the precise mechanism of NK cell inactivation in the GBM microenvironment must be fully elucidated in order to develop effective therapeutic strategies.

Castriconi et al. [21, 22] have shown that GBM cells derived from patients are poorly differentiated and have stem cell properties. Our previous studies have demonstrated that NK-sensitive tumor cells induce “split anergy” in NK cells, a term coined by our laboratory [23–29]. We observed that IL-2-activated NK cells lose the ability to mediate cytotoxicity and down-modulate CD16 receptor expression, but secrete IFN-γ when triggered by NK cell-sensitive tumors [23–29]. In contrast, NK-resistant tumor cells neither induce CD16 receptor down-modulation nor cause split anergy in NK cells [25, 26]. Decreased NK cell cytotoxicity and down-modulation of CD16 receptors on the surface of NK cells have been observed in patients with cancer [30, 31]. Our previous research has also demonstrated that split anergy can be induced in NK cells treated with IL-2 and anti-CD16mAb [24, 28, 29]. In addition, we have shown that increased NK cell cytotoxicity and augmented secretion of

IFN-γ were observed against oral squamous carcinoma stem cells (OSCSCs) which displayed CSC properties, including high CD44 and low MHC-I, CD54 and B7 homolog 1 (B7H1) expression [32].

Based on our data obtained from a number of tumor types, we have established that cytotoxic NK cells target and kill stem-like cancer cells, while split anergized NK cells promote tumor cell differentiation. Tumor differentiation by split anergized NK cells inhibits tumor growth, increases MHC-I, CD54 and B7H1 surface expression and results in the resistance of differentiated tumors to NK cell-mediated cytotoxicity [10]. We have also demonstrated that NK cells were able to lyse healthy stem cells significantly more than their differentiated counterparts [32].

In this study, we analyzed the interaction of NK cells with GBM CSCs/poorly differentiated tumors. Overall, our data indicate that NK cells target and differentiate GBM CSCs/poorly differentiated tumors. However, sustained release of pro-inflammatory cytokines IL-6 and IL-8 by GBM in the presence of decreased IFN-γ secretion from interacting NK cells and inactivation of NK cell cytotoxic function may lead to GBM CSC survival and suboptimal tumor differentiation, resulting in sustained inflammation and progression of cancer.

Materials and methods

Cell lines, reagents and antibodies

RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) (Gemini Bio-Product) was used for human NK cells, monocytes and OSCSC cultures. OSCSCs were isolated from oral cancer patients with tongue tumors at UCLA. U87 glioma cell line was cultured in DMEM medium supplemented with 10 % FBS, 1 % nonessential amino acids, 1 % sodium pyruvate, 1 % antibiotic/antimycotic and 1 % L-glutamine. X01GB and X02GB GBM CSCs were isolated from freshly resected human tumor tissues [33, 34] and were cultured under conditions that allow propagation of brain CSCs as described previously [21, 35]. Human recombinant IL-2 was obtained from NIH-BRB. Human recombinant tumor necrosis factor alpha (hrTNF-α) and interferon gamma (hrIFN-γ) were purchased from Biolegend. Monoclonal antibodies to TNF-α were purified in our laboratory from ascites of mice injected with TNF-α hybridomas. Polyclonal IFN-γ, IL-6 and IL-8 antibodies were prepared in rabbits and purified. Specificity of the antibodies was determined with ELISA and functional assays against hrTNF-α, hrIFN-γ, IL-6 and IL-8. 1:100 dilutions of anti-TNF-α, anti-IFN-γ, anti-IL-6 and anti-IL-8 antibodies were found to be the optimal concentration to block the cytokine function. Human recombinant IL-6 and IL-8 were purchased from Biolegend and PeproTech, respectively. PE-conjugated antibodies for CD44, CD54, B7H1, CD16, TNF-αR, MHC class I polypeptide-related sequence A (MICA), killer cell lectin-like receptor subfamily G member 1 (KLRG1) and IFN-γR were purchased from Biolegend. Anti-MHC class I antibody was prepared in our laboratory and used at 1:100 dilution and purified mouse anti-human UL16-binding protein (ULBP) 1–6 antibodies were purchased from R&D Systems. Secondary PE-conjugated goat anti-mouse antibody was used (Biolegend). Propidium iodide (PI) was purchased from Sigma-Aldrich.

Purification of NK cells and monocytes

NK cells and monocytes were purified from healthy donors using negative isolation kits from stem cell technologies as described previously [25]. Monocytes were irradiated at 20 Gy. Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from the blood donors, and all procedures were approved by the UCLA-IRB.

Preparation of split anergized NK supernatants and differentiation of OSCSCs, X01GB and X02GB cells

Freshly purified NK cells were treated with the combination of anti-CD16mAb (3 μg/mL) and IL-2 (1000 units/mL) for 18–24 h, then split anergized NK cell supernatants were harvested, and IFN-γ concentration in supernatants was determined using IFN-γ ELISA (Biolegend). Poorly differentiated tumor cells were differentiated using increasing amounts of split anergized NK cell supernatants added on a daily basis. Split anergized NK cell supernatants containing 1000 pg of IFN-γ were required to induce differentiation of 1×10^6 OSCSCs while 35,000 pg of IFN-γ was needed to differentiate 1×10^6 X01GB and X02GB over the course of 5–7 days. Differentiation status of each cell type was evaluated based on their susceptibility to NK cell-mediated cytotoxicity and surface expression of CD54, B7H1, MHC-I and CD44.

ELISA

IFN-γ, IL-6 and IL-8 (Biolegend) measurements were performed using single ELISAs as described previously [25].

Surface staining and cell death assays

As previously described, cells were stained with fluoro-chrome-conjugated antibodies or PI, followed by analysis using EPICS XL cytometer and FlowJo software (Tree Star) [24, 25, 36].

^{51}Cr release cytotoxicity assay

The ^{51}Cr release assay was performed as described previously [37], and the (lytic unit) LU $30/10^6$ was calculated by using the inverse of the number of effector cells needed to lyse 30 % of target cells X100.

Statistical analysis

An unpaired, two-tailed student *t*-test was performed for the statistical analysis.

Results

NK cells were cytotoxic and secreted IFN-γ when cultured with X02GB CSCs/ undifferentiated tumors

X02GB tumors were found to form neuronal sphere-like aggregates and were immunoreactive with the neural progenitor markers, nestin and musashi-1 [34, 38]. X02GB also expressed common cancer stem cell marker CD133 [35, 38, 39]. These cells formed tumors in the brain of immunodeficient mice with high proliferative capacity [34,35]. U87

cell line was chosen as the control based on the previous characterization [35]. U87 cells were found to have low ability to generate spheroids, colony formation and migration [40]. Although U87 expressed CD44, unlike X02GB, no expression of CD133, nestin, SRY (sex determining region Y)-box 2 (SOX-2) and musashi-1 could be detected [40].

IL-2-activated NK cells were able to lyse X02GB significantly more than U87 GBM cells (Fig. 1a), and they were also able to induce significant secretion of IFN-γ (Fig. 1b). Interestingly, even untreated NK cells were able to kill X02GB more than U87; however, no secretion of IFN-γ was observed in either culture. A significant decrease in cytotoxicity could be observed when NK cells were treated with the combination of IL-2 and anti-CD16mAb (this condition induces split anergy in NK cells), as compared to IL-2-treated NK cells cultured with X02GB and U87 (Fig. 1a). In contrast, the level of IFN-γ secretion was significantly higher by split anergized NK cells when compared to IL-2-induced secretion of IFN-γ by NK cells co-cultured with and without X02GB and U87 (Fig. 1b). Anti-CD16mAb-treated NK cells lost significant cytotoxicity and were unable to trigger IFN-γ secretion (Fig. 1). IL-2-treated NK cells in the absence of brain tumors also secreted IFN-γ; however, the levels were lower than those secreted by IL-2-treated NK cells cultured with either U87 or X02GB (Fig. 1b). Split anergized NK cells secreted significantly higher levels of IFN-γ in the absence of tumors and the levels increased even more when cultured with either U87 or X02GB CSCs (Fig. 1b).

X02GB tumors treated with supernatants from split anergized NK cells were killed less by NK cells

When X02GB cells were differentiated with split anergized NK cell supernatants and then tested against freshly isolated NK cells, a significantly decreased susceptibility to NK cell-mediated cytotoxicity was observed ($P < 0.05$) (Fig. 2a). Supernatants from IL-2-treated NK cells could also induce resistance of X02GB to NK cell-mediated lysis, but the decrease was significantly less when compared to that induced with split anergized NK cell supernatants (data not shown). Supernatants from untreated NK cells had no effect (Fig. 2a).

To examine the mechanisms by which X02GB cells became resistant by split anergized NK supernatants, we measured NK cell cytotoxicity when X02GB cells were treated with supernatants from split anergized NK cells, with and without anti-IFN-γ and/or anti-TNF-α antibodies. Treatment with anti-TNF-α antibody was not able to restore cytotoxicity, whereas addition of anti-IFN-γ antibody restored NK cell cytotoxicity to the levels obtained by non-differentiated X02GB. The combination of anti-IFN-γ and anti-TNF-α blocked NK cell-mediated differentiation of X02GB's significantly and increased their sensitivity to NK cell cytotoxicity (Fig. 2a). The restoration of cytotoxicity against X02GB after treatment with split anergized NK supernatants in the presence of anti-IFN-γ antibody alone or the combination of anti-IFN-γ and anti-TNF-α antibodies could be observed when IL-2-treated NK cells were used to determine cytotoxicity (Fig. 2a). Treatment of X02GB with the combination of anti-TNF-α and anti-IFN-γ in the absence of NK cells supernatants had no effect on NK cell cytotoxicity (Fig. 2a).

Increased expression of CD54 and MHC-I on X02GB was observed after treatment with supernatants collected from split anergized NK cells

We then assessed the expression of key cell surface receptors on X02GB after differentiation with supernatants from split anergized NK cells. As shown in Fig. 2b, the expression of CD54 and MHC-I increased substantially on X02GB after the addition of supernatants from split anergized NK cells and CD44, CD54 and MHC-I expression levels were found to significantly correlate with the resistance of NK supernatant-differentiated X02GB (Fig. 2b). Untreated NK cell supernatants did not change the surface expression on X02GB (Fig. 2b). The combination of anti-TNF-α and anti-IFN-γ antibodies prevented the upregulation of CD54 and MHC-I on X02GB to X02GB before treatment with split anergized NK supernatants (Fig. 2b). Anti-TNF-α was able to inhibit CD54 and MHC-I increase partially on split-anergized NK supernatant-treated X02GB, whereas anti-IFN-γ was able to block the increase in CD54 and MHC-I surface receptors completely (Fig. 2b).

We have previously shown that monocytes are able to induce split anergy in NK cells; therefore, we determined the expression of CD54, B7H1 and MHC-I on X02GB treated with supernatants from split anergized NK cells, cultured with irradiated monocytes (Fig. 3a). The addition of supernatants from irradiated monocytes in the absence of NK cells did not change the CD54, B7H1 and MHC-I surface expression on GBM (Fig. 3a) and OSCSCs (data not shown). However, when supernatants from monocytes cultured with split anergized NK cells were added to X02GB (Fig. 3a), or OSCSCs [10], a synergistic increase in the expression of all three receptors was observed. Accordingly, OSCSCs lost sensitivity and acquired resistance to NK cell-mediated cytotoxicity (Supplementary Fig. S2A) and [10].

We next determined cell numbers of X02GB after treatment with NK supernatants by microscopic evaluation (Fig. 3b, left) and the levels of cell death by staining with PI followed by flow cytometric analysis (Fig. 3b, right). As shown in Fig. 3b, a decrease in the numbers of X02GB was observed after their culture with split anergized NK cell supernatants when compared to untreated X02GB or those treated with untreated NK cell supernatants (Fig. 3b, left). The highest decrease was observed when X02GB was treated with the supernatants from split anergized NK cells cultured with monocytes (data not shown). The addition of anti-TNF-α antibody in the presence of split anergized NK supernatants increased the numbers of X02GB compared to X02GB treated with split anergized NK cells, and anti-IFN-γ or the combination of anti-TNF-α and anti-IFN-γ antibodies restored the numbers of cells to the levels observed with untreated X02GB (Fig. 3b, left). In addition, low/no cell death could be observed in X02GB treated with the supernatants from split anergized NK cells in the absence or presence of irradiated monocytes (Fig. 3b, right).

Although treatment of X02GB with supernatants from split anergized NK cells was able to inhibit NK cell cytotoxicity, it increased IL-6 and IL-8 secretion by GBM while inhibiting IFN-γ secretion in co-cultures of NK cells with tumor cells

We have previously shown that OSCSCs treated with supernatants from split anergized NK cells inhibited NK cell cytotoxicity, increased CD54 and MHC-I and blocked secretion of cytokines and chemokines significantly [10] (Supplementary Figs. S1, S2). Although split

anergized NK cell supernatant-differentiated X02GB cultured with IL-2-treated NK cells had significant inhibitory effect on IFN-γ secretion (Fig. 4a), it resulted in a significant increase in IL-6 and IL-8 secretion when compared to either untreated X02GB, split anergized NK cell supernatant-differentiated X02GB treated with anti-IFN-γ and anti-TNF-α or X02GB treated with supernatants from untreated NK cells (Fig. 4a).

Therefore, increase in IL-8 and IL-6 after differentiation of X02GB with split anergized NK supernatants is mediated by either TNF-α or IFN-γ since blocking of either cytokine or both together inhibited increased secretion of IL-6 and IL-8 (Fig. 4a). In addition, increased secretion of IL-8 and IL-6 could be seen in split anergized NK cell supernatant-differentiated X02GB regardless of whether they were cultured with or without untreated or IL-2-treated NK cells. Pre-treatment of X02GB with untreated NK supernatants slightly inhibited IFN-γ, IL-6 and IL-8 secretion (Fig. 4a). Thus, differentiation of X02GB with split anergized NK cell supernatants had a significant inhibitory effect on IFN-γ secretion by IL-2-treated NK cells, while it upregulated IL-6 and IL-8 secretion by glioma tumors in the presence or absence of their co-cultures with NK cells (Fig. 4a).

We then assessed the levels of TNFR I and II and IFN-γRα and IFN-γRβ on X02GB and compared the levels to those expressed on OSCSCs. We aimed to determine whether the magnitude of functional differences observed in differentiation could relate to the lower expression of these receptors on X02GB. As shown in Fig. 4b, receptor levels were lower on X02GB than on OSCSCs (Fig. 4b, Supplementary Fig. S3). Differentiation with split anergized NK cell supernatants decreased the levels of TNFR I, largely, TNFR II and IFN-γRβ moderately on OSCSCs (Supplementary Fig. S3A), but not on X02GB cells (Fig. 4b). Similarly, addition of both rTNF-α and rIFN-γ to OSCSCs decreased the levels of TNFR I and IFN-γRβ largely, and TNFR II moderately, but not IFN-γRα (Supplementary Fig. S3B).

Differentiation of GBM by split anergized NK cell supernatants is independent of the effects mediated by IL-6 and IL-8

To determine whether secreted IL-6 or IL-8 by split anergized NK supernatant-differentiated tumors had any contributory effect on tumor differentiation, antibodies to each cytokine alone or to both cytokines together were added during differentiation, and the effect on NK cell-mediated cytotoxicity and expression of differentiation antigens on glioma cells were determined. As shown in Fig. 5, unlike anti-IFN-γ/anti-TNF-α, addition of anti-IL-6 and/or anti-IL-8 antibodies neither changed the resistance of differentiated X02GB (Fig. 5a) or X01GB (Fig. 5b) to NK cell-mediated cytotoxicity nor modulated MHC-I and CD54 differentiation antigen levels expressed on these tumor cells (Fig. 5c, d, respectively). X01GB, another GBM CSCs/undifferentiated tumor, previously characterized by surface marker expression and ability to form tumors in mouse brain [35, 39], was found to be less susceptible to NK cell-mediated cytotoxicity (Fig. 5b) when compared to X02GB (Fig. 5a). In contrast to anti-IFN-γ/anti-TNF-α antibodies that blocked upregulation of differentiation antigen expression on glioma cells during differentiation with split anergized NK supernatants (Fig. 2b), no significant change of MHC class I and CD54 expression was observed in X02GB differentiated with split anergized NK super-natants with or without

anti-IL-6 and anti-IL-8 antibodies (Fig. 5d). Furthermore, no correlation was observed with the levels of NK cell cytotoxicity (Fig. 5a, d). The levels of CD54 fluctuated on X01GB depending on the specific antibody treatment (Fig. 5c), and no correlation could be found with the levels of NK cell cytotoxicity (Fig. 5b). The addition of hrIL-6 and/or hrIL-8 to untreated X02GB did not significantly affect cytotoxicity (Fig. 5e), and no correlation could be found with the levels of differentiation antigens on X02GB and X01GB (Fig. 5f). Thus, unlike IFN-γ and TNF-α, which drive split anergized NK supernatant-mediated differentiation of CSCs/poorly differentiated GBM cells, IL-6 and IL-8 did not contribute to the differentiation process.

Lack of MICA, ULBP (1–6) and KLRG1 NK cell ligand modulation on differentiated X02GB

We determined the levels of NK cell ligands on untreated and split anergized NK supernatant-differentiated X02GB. Although both CD54 and MHC class I were significantly increased on NK-differentiated X02GB, no or a very slight decrease in MICA, ULBP (1–6) and KLRG1 could be observed on these tumors (Fig. 6).

Discussion

The stage of differentiation of many tumors including GBM is predictive of their sensitivity to NK cell lysis since CSCs/poorly differentiated GBM tumors are targets of NK cells, whereas those differentiated with split anergized NK cell supernatants display resistance to NK cell-mediated cytotoxicity [32, 41–46]. We have shown that NK cells play a significant role in differentiation of tumors by providing critical signals via secreted IFN-γ and TNF-α, as well as direct cell–cell contact [10, 35]. Furthermore, tumor differentiation is likely supported by signals from other immune effectors, such as monocytes and macrophages within the tumor stroma to induce split anergy in NK cells [41, 43–46]. Interestingly, significantly higher amounts of NK supernatant containing IFN-γ were required to differentiate X02GB in comparison with OSCSCs resulting in tumor growth inhibition, upregulation of CD54, B7H1 and MHC-I expression and resistance to NK cell-mediated cytotoxicity. Moreover, tumor differentiation by split anergized NK cell supernatants also inhibited cytokine and chemokine secretion in the cultures of NK cells with OSCSCs. In contrast, treatment of X02GB with split anergized NK supernatants significantly inhibited NK cell cytotoxicity and decreased IFN-γ secretion, whereas it had the opposite effect on IL-6 and IL-8 production. Additionally, although high amounts of split anergized NK cell supernatants triggered cell death in OSCSCs [10], supernatants containing higher levels of IFN-γ were unable to induce cell death in X02GB. These experiments revealed distinct responses of GBM and oral tumors to NK cell-mediated differentiation, which could likely be one of the underlying mechanisms for GBM's aggressive behavior and poor prognosis in patients. GBM tumor cells may survive because of (1) the inability of NK cells to lyse the tumors through granzyme/perforin pathway, and (2) they may resist cell death induced by death inducing cytokines such as TNF-α and Fas ligand.

GBM may remain persistently inflammatory since upon differentiation with split anergized NK cell supernatants they increase, rather than decrease, inflammatory cytokine and chemokine IL-6 and IL-8. IL-6 is known to favor the survival of CSCs [47]. Lack of cell

death and decreased ability to control the release of inflammatory IL-6 and IL-8 in X02GB differentiated with split anergized NK supernatants correlate with the lower surface expression and function of TNF-α and IFN-γ receptors. OSCSCs express much higher levels of TNFRI and IFN-γRα and IFN-γRβ when compared to X02GB. Accordingly, upon differentiation with split anergized NK supernatants or treatment with rTNF-α and/or rIFN-γ, OSCSCs down-modulate both TNFRI and IFN-γRβ. This suggests an increased receptor signaling and function, whereas no such changes are observed for X02GB. Therefore, strategies to augment both TNF-α and IFN-γ receptor levels on GBM cells may be effective in controlling these tumors.

GBM patients are likely to benefit from NK cell adoptive therapy since NK cells are capable of eliminating CSCs. In this regard, depletion of NK split anergizing effectors, such as monocytes, in patients before NK cell transplantation should provide such a strategy for NK cells to target GBM CSCs. However, this approach may also decrease the ability of NK cells to secrete cytokines and chemokines due to the removal of the synergizing effect of monocytes with NK cells for the secretion of TNF-α and IFN-γ [42]. Interestingly, secretion of TNF-α and IFN-γ by split anergized NK cells is responsible for the increased induction and secretion of inflammatory cytokines and chemokines by NK-differentiated X02GB. This mechanism differs remarkably from NK cell-differentiated oral, lung and pancreatic tumor cells in which a significant reduction rather than upregulation of inflammatory cytokines by those tumors was observed (Supplementary Fig. S1 and data not shown). Therefore, this effect appears to be very specific to GBM. Indeed, in previous reports it was suggested that glioma cells may increase the expression of immunosuppressive cytokines in response to an increased lymphocyte infiltration in the tumor stroma, which is in agreement with our findings [48].

Tumor differentiation by split anergized NK cell supernatants containing TNF-α and IFN-γ was not only responsible for the decrease in inflammatory cytokines and chemokines in oral and pancreatic tumor cells, but it also inhibited tumor invasion and metastasis in animals (Supplementary Fig. S2B and manuscript in preparation). However, since GBM differentiation with split anergized NK cell supernatants elevated the inflammatory cytokine IL-6 and chemokine IL-8, it may pose a challenge for tumor therapy. It is unclear, why GBM behaves differently from other tumors with regard to their responses to differentiation by NK cell supernatants. However, considering the elevated inflammatory index in patients with GBM and its possible underlying role in cancer progression, strategies for immunotherapeutics should be designed to retain the tumor killing function as well as IFN-γ secretion by the NK cells, while suppressing tumor-induced IL-6 and IL-8 secretion. Moreover, most studies in GBM patients reveal an immunosuppressive tumor micro-environment [49, 50]. Since NK supernatant-differentiated GBM cells decreased IFN-γ secretion by the NK cells while increasing IL-6 and IL-8 secretion by the tumor cells, these studies closely resemble those obtained from the clinical data of GBM patients [49, 50] and thus can provide an important preclinical model to study the mechanisms for immunosuppression in GBM patients.

Increased IL-8 induction in glioma also occurs in response to hypoxia, Fas ligation, death receptor activation, cytosolic Ca^{2+} and cytokines [5]. Thus, we tested the ability of secreted

IL-6 and/or IL-8 from NK-differentiated GBM cells to modulate differentiation of CSCs/poorly differentiated GBM. Unlike antibodies to IFN-γ and/or TNF-α, which blocked differentiation of tumors with supernatants from split anergized NK cells, addition of anti-IL-6 and/or anti-IL-8 during differentiation neither changed resistance of X02GB or X01GB against NK cell cytotoxicity, nor significantly modified expression of differentiation antigens, indicating the lack of contribution of IL-6 and/or IL-8 to differentiation of GBM by NK cell supernatants. Furthermore, unlike rIFN-γ and/or rTNF-α, which significantly upregulated the expression of differentiation antigens CD54 or MHC-I on OSCSCs [10], the addition of rIL-6 and/or rIL-8 to X02GB or X01GB did not cause significant changes in the expression of differentiation antigens.

No significant differences could be seen in the expression of MICA, ULBP (1–6) and KLRG1 ligands on NK-differentiated X02GB when compared to untreated tumors. These results indicated that these ligands might not play a role in the loss of NK cytotoxicity observed when tumors are differentiated with supernatants from split anergized NK cells.

Emerging data suggest that differentiated GBM cells may revert to their stem-like stage and thus supply the pool of stem cells for the continuous growth of the GBM tumors (Dr. Inder Verma, Salk Institute; personal communication). The induction of pro-differentiation cytokines such as IFN-γ and TNF-α by the effector NK cells may likely keep the GBM tumors in a differentiated state, thereby limiting its growth. However, if NK cells within the tumor micro-environment lose their ability to secrete IFN-γ, GBM cells may revert to their stem-like stage and fuel tumor growth. Indeed, within 2 weeks of removal of NK cell supernatants from differentiated OSCSCs, all tumor cells were found to revert to their stem-like stage [10].

Considering that NK-differentiated tumors are highly susceptible to chemotherapy, whereas their stem-like tumors are highly resistant, the combination of NK cell immunotherapy and chemotherapy may be effective in the treatment of GBM patients [10, 51, 52]. The most troubling outcome of GBM to overcome when considering therapies is the ability to deplete cytotoxic NK cells due to their immunosuppressive effect. In this case, the expansion of CSCs is not kept under control by the NK cells, resulting in their invasion, metastasis and the progression of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

B7H1	B7 homolog 1
CSCs	Cancer stem-like cells

GBM	Glioblastoma multiforme
hr	Human recombinant
IFN-γR	Interferon-γ receptor
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LIF	Leukemia inhibitory factor
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
NK	Natural killer
OSCSCs	Oral squamous carcinoma stem cells
TNFR	Tumor necrosis factor receptor
ULBP	UL16-binding protein

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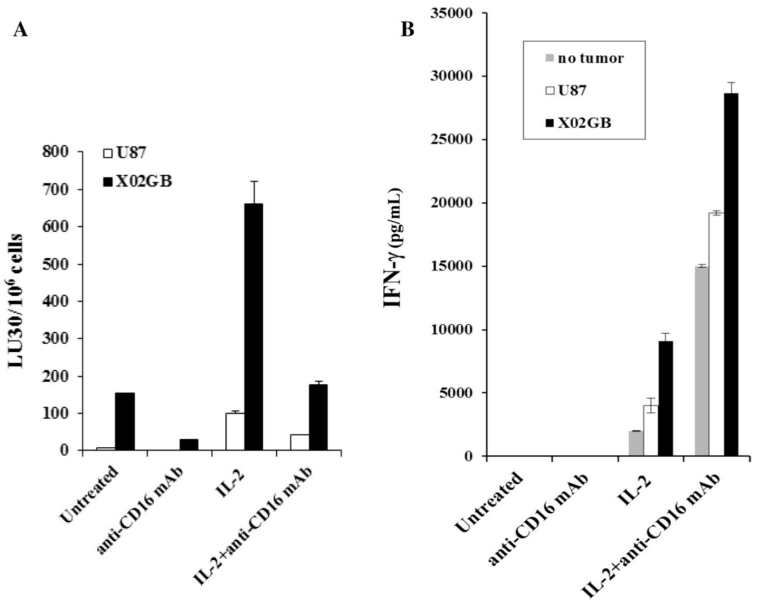

Fig. 1.

X02GB cells were more susceptible to NK cells and triggered higher levels of IFN-γ than U87. Highly purified NK cells (1×10^6 cells/mL) were either left untreated or treated with IL-2 (1000 units/mL) or anti-CD16mAb (3 μg/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μg/mL) (condition to induce split anergy) for 12–24 h and used in cytotoxicity assay against U87 GBM and X02GB CSCs. NK cell cytotoxicity was determined using a standard 4-h ^{51}Cr release assay. One of the three representative experiments is shown in this figure (**a**). NK cells were treated as described in (**a**), and each NK cells sample (1×10^5/mL) was either cultured in the absence or presence of U87 and X02GB cells at an NK to GBM ratio of 0.5:1. After an overnight culture, supernatants were removed from the cultures and the levels of IFN-γ secretion were determined using specific ELISA (**b**). One of the two representative experiments is shown in this figure

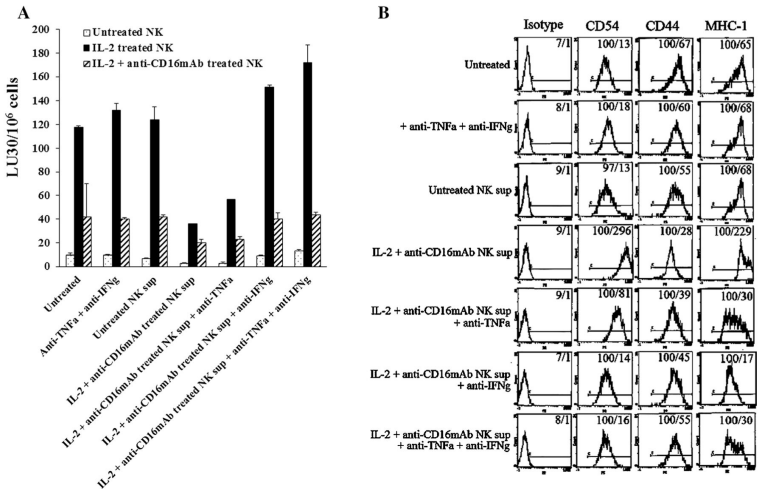

Fig. 2.

Increased resistance to NK cell-mediated cytotoxicity and increased expression of CD54 and MHC-I on X02GB differentiated with supernatants from split anergized NK cells. Highly purified NK cells were left untreated or treated with IL-2 and anti-CD16mAb for 24 h, after which the supernatants were used for differentiation of X02GB cells as described in "Materials and methods" section. Untreated X02GB and those treated with anti-TNF-α (1:100) and anti-IFN-γ (1:100) in the absence of NK cell supernatants were also used as controls. Similar amounts of supernatants from untreated NK cells and those cultured with split anergized NK cells in the presence and absence of anti-TNF-α (1:100) and/or anti-IFN-γ (1:100) were used to treat X02GB for a period of 7 days to induce differentiation. Afterward, the cells were used in a standard ^{51}Cr release assay against freshly isolated untreated IL-2 (1000 units/mL) or the combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μg/mL)-treated NK cells (**a**). X02GB was differentiated with NK supernatants in the presence and absence of anti-TNF-α and anti-IFN-γ antibodies as described in (**a**), and then, CD54, CD44 and MHC-I surface expression on untreated and split anergized NK cell supernatant-treated cells was assessed after PE-conjugated antibody staining followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers in the right-hand corner are the percentages and the mean channel fluorescence intensities in each histogram (**b**)

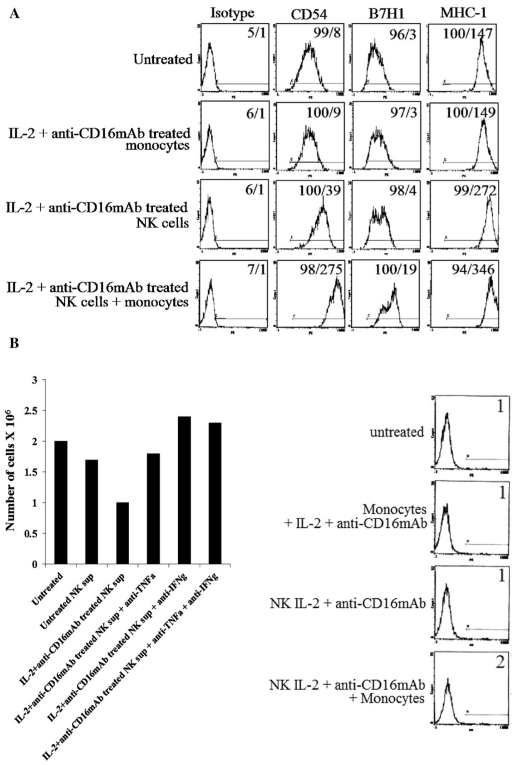

Fig. 3.

Monocytes induced split anergy in NK cells. NK cells were treated with a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μg/mL) to induce split anergy and subsequently cultured with autologous monocytes at 1:1 (NK:monocytes) ratio for 18–24 h. Monocytes were treated with IL-2 (1000 units/mL) and anti-CD16mAb (3 μg/mL) for 18–24 h and used as control. Afterward, supernatants from split anergized NK cells and monocytes were harvested and added to X02GB for 7 days. Surface expression of CD54, B7H1 and MHC-I was assessed after PE-conjugated antibody staining followed by flow cytometric analysis. Isotype control antibodies were used as controls. The numbers in the right-hand corner are the percentages and the mean channel fluorescence intensities in each histogram (**a**). At the end of the incubation of X02GB with split anergized NK cell supernatants, X02GB cells, which remained attached to the plate during the incubation period, were collected and the number of cells was assessed using microscopy (**b**, *left*). After X02GB was differentiated with split anergized NK cell supernatants, as described in (**a**), the percentage of cell death was assessed with propidium iodide staining followed by cytometric analysis. The number in the right-hand corner is the percentage for each histogram (**b**, *right*)

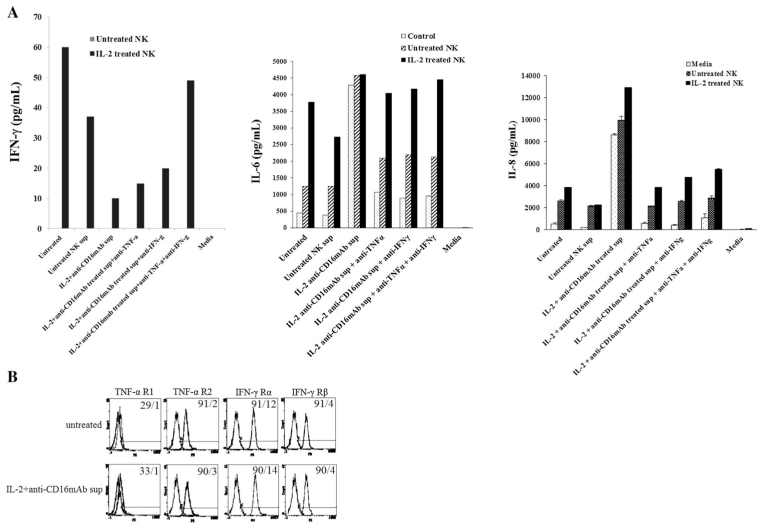

Fig. 4.

X02GB differentiated by split anergized NK cell supernatants inhibited IFN-γ secretion but triggered increased IL-8 and IL-6 secretion by NK cells. NK cells were left untreated or treated with a combination or IL-2 (1000 units/mL) and anti-CD16mAb (3 μg/mL) in the presence of anti-TNF-α (1:100), anti-IFN-γ (1:100) or a combination of anti-TNF-α (1:100) and anti-IFN-γ (1:100) for 18 h. Afterward, supernatants were used to differentiate X02GB as described in “Materials and methods” section. Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/mL) for 18 h. Afterward, NK cells were added to X02GB treated with split anergized NK cell supernatants at an effector to target ratio of 0.5–1. After an overnight incubation, the supernatants were removed from the co-cultures and the levels of IFN-γ, IL-8 and IL-6 secretions were determined using specific ELISAs (**a**). Untreated and split anergized NK supernatant-differentiated X02GB cells were prepared as described in “Materials and methods” section, and the levels of TNFRI, TNFRII, IFN-γRα and IFNγRβ on X02GB cells were determined using PE-conjugated antibodies followed by flow cytometric analysis. The histogram on the *left* is the isotype control staining and on the *right* the receptor staining. The numbers in the right-hand corner are the percentage of positive cells and the mean channel fluorescence intensity for each receptor expression (**b**)

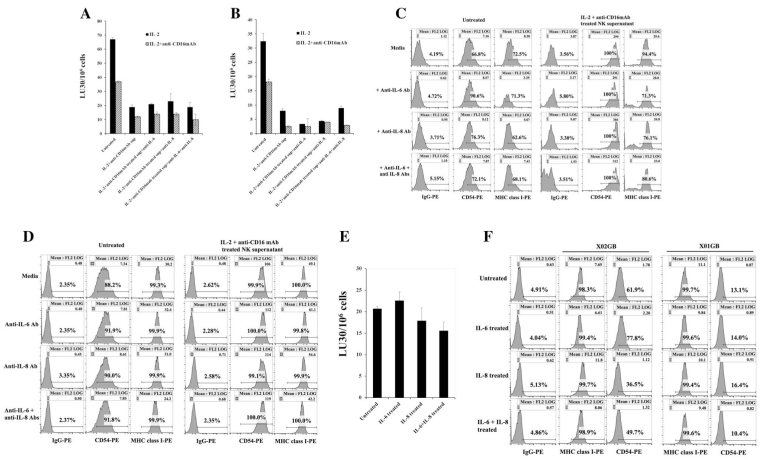

Fig. 5.

Differentiation of GBM by split anergized NK cell supernatants was independent of the effects of IL-6 and IL-8. X01GB (**b**) and X02GB (**a**) cells were differentiated with split anergized NK cell supernatants as described in “Materials and methods” section in the presence or absence of anti-IL-6 (1:100), anti-IL-8 (1:100) or a combination of anti-IL-6 (1:100) and anti-IL-8 (1:100). Afterward, the cells were used in a standard ^{51}Cr release assay against freshly isolated NK cells treated overnight with IL-2 (1000 units/mL) or the combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μg/mL) (**a**, **b**). Untreated and split anergized NK supernatant-differentiated X01 and X02GB cells were prepared as described in (**a**, **b**), and the levels of MHC-I and CD54 on X01GB (**c**) and X02GB (**d**) were determined using PE-conjugated antibodies followed by flow cytometric analysis. The number in the upper right-hand corner of each histogram is the mean channel fluorescence intensity for each receptor expression (**c**, **d**). X02GB cells were plated and then treated with hrIL-6 (8 ng/mL), hrIL-8 (8 ng/mL) or a combination of hrIL-6 (8 ng/mL) and hrIL-8 (8 ng/mL) for 24 h. Afterward, the cells were used in a standard ^{51}Cr release assay against freshly isolated NK cells treated overnight with IL-2 (1000 units/mL) (**e**). Untreated and split anergized NK supernatant-differentiated X01 and X02GB cells were prepared as described in (**e**), and the levels of MHC-I and CD54 on X01 and X02GB were determined using PE-conjugated antibodies followed by flow cytometric analysis. The number in the upper right-hand corner of each histogram is the mean channel fluorescence intensity for each receptor expression (**f**)

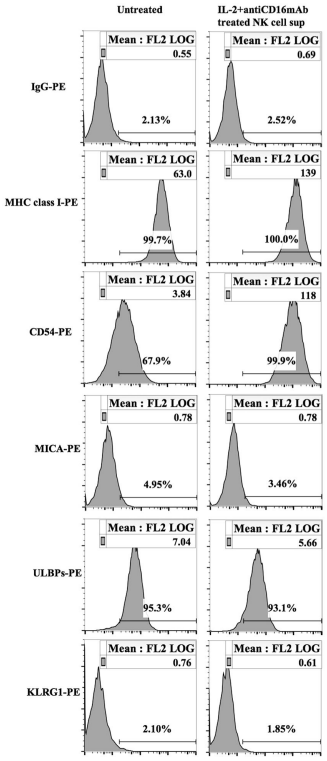

Fig. 6.

NK cell ligand expression on untreated and split anergized NK cell supernatant-differentiated X02GB. X02GB cells were differentiated with split anergized NK cell supernatants as described in “Materials and methods” section. Untreated X02GB was used as control. Afterward, the cells were washed with 1× PBS, detached from the tissue culture plate and surface expression of CD54, MHCI, MICA, KLRG1 and ULBP 1–6 was assessed after PE-conjugated antibody staining followed by flow cytometric analysis. The number in the upper right-hand corner of each histogram is the mean channel fluorescence intensity for each receptor expression