RESEARCH ARTICLE

Exosomes from Murine-derived GL26 Cells Promote Glioblastoma Tumor Growth by Reducing Number and Function of CD8+T Cells

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Abstract

Aim: Brain tumors almost universally have fatal outcomes; new therapeutics are desperately needed and will only come from improved understandins of glioma biology. Methods: Exosomes are endosomally derived 30~100 nm membranous vesicles released from many cell types. Examples from GL26 cells were here purified using density gradient ultracentrifugation and monitored for effects on GL26 tumor growth in C57BL/6j mice (H-2b). Lactate dehydrogenase release assays were used to detect the cytotoxic activity of CD8+T and NK cells. Percentages of immune cells producing intracellular cytokines were analyzed by FACS. Results: In this study, exosomes from murine-derived GL26 cells significantly promoted *in vivo* tumor growth in GL26-bearing B6 mice. Then we further analyzed the effects of the GL26 cells-derived exosomes on immune cells including CD8+T, CD4+T and NK cells. Inhibition of CD8+T cell cytotoxic activity was demonstrated by CD8+T cell depletion assays *in vivo* and LDH release assays *in vitro*. The treatment of mice with exosomes also led to a reduction in the percentages of CD8+T cells in splenocytes as determined by FACS analysis. Key features of CD8+T cell activity were inhibited, including release of IFN-gamma and granzyme B. There were no effects of exosomes on CD4+T cells and NK cells. Conclusion: Based on our data, for the first time we demonstrated that exosomes from murine derived GL26 cells promote the tumor growth by inhibition of CD8+T cells *in vivo* and thus may be a potential therapeutic target.

Keywords: Glioblastoma tumor - exosomes - tumor growth - cytotoxic activity

Asian Pacific J Cancer Prev, **14 (1)**, 309-314

Introduction

Immunosuppression of tumor cells against organisms is considered to be a pivotal factor in tumor growth and progression, and this factor also play a significant role in tumor response to immunotherapeutics (Rabinovich et al., 2007; Kudo-Saito et al., 2009).

Several mechanisms have reported may contribute to the ability of tumor cells to survive in the context of an active immune response, including inhibition of cytotoxic activity by secretion of unique factors (Kim et al., 2007), systematically inhibition of immune response by up-regulation of inhibitory cytokines such as interleukin 10 (IL-10) (Yang et al., 2003), or escaping immune destruction by down-regulation of the expression of MHC molecules on the surface of tumor cells (Igney et al., 2002; Rivoltini et al., 2005).

Exosomes are small membrane vesicles found in cell culture supernatants and in different biological fluids. They are 30 to 100 nm vesicles secreted by range of mammalian cells including tumor cells, reticulocytes, intestinal epithelial cells, as well as hematopoietic cells (Mallegol et al., 2005; Hendrix et al., 2011; Martin-Jaular et al., 2011). Types of tumor cells such as ovarian cancer, chronic myelogenous leukemia were demonstrated to secret functional exosomes (Cho et al., 2011; Clayton et al., 2011; Hood et al., 2011;Taverna et al., 2012). Studies have reported that the exosomes implicated in the cell-to-cell signaling (Lotvall et al., 2007), involve in presentation of Ags to T cells in the immune system (Prado et al., 2008) and the exosomes from tumor cells promote the immune response to tumors by presentation of tumor Ags (Rountree et al., 2011). However, it has also been reported that exosomes from murine mammary tumor cells including TS/A and 4T1 cells can inhibit NK cells cytolytic activity by down-regulation of the expression of perforin, exosomes from breast tumors inhibit the activation of T cells (Liu et al., 2006).

In this study, we first surprisedly found that GL26 cells-derived exosomes effectively promote GL26 tumor growth in vivo. So based on the previous studies, we further investigated the promotion mechanisms using immune cells depletion assaies in vivo and lactate dehydrogenase release assay ex vivo. Results in our study demonstrated

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that GL26 growth in vivo promoted by the exosomes from GL26 cells due to the functional suppression and reduction of CD8+T cells.

Materials and Methods

Cell culture

Luciferase expression mice glioblastoma cells GL26-Luc cells was perchased from and cultured in DMEM medium supplemented with 10% fetal bovine serum and and antibiotics.

Isolation and purification of exosomes

GL26 cells were cultured in DMEM culture medium with 10% fetal bovine serum previously deprived of bovine microvesicles by ultracentrifugation (16 h at 100,000 × g) and antibiotics in a humidified 5% CO_2 incubator. Exosomes from culture supernatants were isolated by gradient centrifugations as reported and then purified on a sucrose gradient. In brief, exosomes were isolated by successive centrifugation (300 g for 5 min, 1,200 g for 20 min, 10,000 g for 30 min) and a final ultra-centrifugation step at 100,000 g for 1 hour, followed by resuspension in PBS. For further purification, exosomes were resuspended in 2.5 M sucrose in 20 mM Hepes buffer (pH 7.4) and were subsequently loaded on the bottom of a SW41 tube. Hepes buffer (20 mM) with 2 M sucrose followed by Hepes buffer (20 mM) with 0.25 M sucrose was carefully loaded on top of the exosomes to produce a discontinuous 2-0.25 M sucrose gradient. After centrifugation overnight at 100,000 μg in a SW41 swing rotor, 1ml of each fraction was collected from the top of the tube.

Mice and glioblastoma model

C57BL/6j mice (H-2b) (6-8weeks, female) were purchased and keeped in the Wuhan University Center for Animal Experiment/A3-lab. To establish a syngeneic tumor model, we used GL26 cells that were derived from a chemically induced glioma in a female C57BL/6 mouse. Mice were anesthetized with an IP injection of Ketamine and Medetomidine, 1× 10^5 GL26-Luc cells in 2 μl PBS were injected through an entry site at the bregma of mice. To study the function of the GL26-derived exosomes, mice were treated with exosomes (20 μg) at 3, 6, 9, 12, 15, 18 days after tumor implantation. Luciferase activity was also detected at different time points (5th day, 10th day, 15th day and 20th day).

For in vivo depletion assays, mice CD4+T cells, CD8+T cells and NK cells were first respectively depleted before tumor implantation. 5 days after tumor implantation, CD4+T cells, CD8+T cells, NK cells or exosomes pretreated CD4+T, CD8+T cells and NK cells were intravenously administrated in corresponding depletion mice for 2 times a week. Then the tumor growth was monitored as described above.

In vivo depletion of CD8+ T cells, CD4+ T cells, and NK cells

Briefly, mice were intravenously injected with anti-CD8 mAb, anti-CD4 mAb or anti-NK mAb 3 days prior to tumor implantation. As an isotype control, mice were intraperitoneally injected with rat IgG2b mAb (Invitrogen). To confirm the depletion of CD8+ T cells, CD4+ T cells, and NK cells in vivo, splenocytes from each treated mouse were stained using FITC-conjugated anti-mouse CD8 mAb, FITC conjugated anti-mouse CD4 mAb, or anti-DX5 mAb, respectively, and then analyzed by flow cytometry. FITC-conjugated anti-rat IgG (eBioscience) was used as an isotype-matched negative control.

Cytotoxic activity of CD8+T cells, CD4+T cells and NK cells

Mice were pretreated with GL26 cells-derived exosomes for 3 weeks (20 mg each time, 2 times every week) or PBS. CD4+T cells, CD8+ T cells and NK cells were purified from the immunized mouse splenocytes using the BD™ IMag Mouse CD4+, CD8+ T lymphocytes and NK cells enrichment set-DM and the BD™ IMagnet (BD Biosciences Pharmingen, USA). Then the CD4+T cells, CD8+T cells and NK cells from immunized mice as effecter cells in vitro were resuspended in RPMI-1640 with 10% FCS and analyzed for cytotoxic activity. GL26 cells as target cells. The cytotoxic activity were respectively tested at E:T ratios of 10:1, 5:1, and 2.5:1. GL26 cells were incubated with CD4+T cells, CD8+T cells or NK cells at the indicated lymphocyte to target cell ratio (E/T) in 96-well plates in a total volume of 200μl of RPMI-1640 medium. Released lactate dehydrogenase (LDH) was measured according to the manufacturer's protocol after 4 h of incubation at 37 °C in 5% CO_2. The percentage of specific killing was calculated as: % specific killing = (experimental release-spontaneous release)/(total release-spontaneous release). The data are represented as the mean percentages of the specific lysis values from six mice.

Intracellular cytokine and granzyme B (GrB) analysis

Splenocytes from the exosomes pretreated mice were harvested and cultured in 6-well plates (5×10^6 cells/well). Then cells were stimulated with or without PMA (10 ng/ml) at 37 °C in 5% CO_2 for 24 h. Monensin (eBioscience, 1 μg/ml), an inhibitor of intracellular protein transport, was added for 4 h to block cytokines releases before cells collection. After 4 h of incubation, CD4+, CD8+ T cells and NK cells were purified from the splenocytes using the BDTM IMag Mouse CD4+, CD8+ T lymphocytes and NK cells enrichment set-DM and the BDTM IMagnet (BD Biosciences Pharmingen, USA). The purified CD4+, CD8+T cells and NK cells were then fixed in 2% paraformaldehyde in PBS at room temperature for 15 min, permeabilized and stained with PE-labeled anti-mouse IFN-gamma or granzyme B antibodies at 4 °C for 45 min according to the manufacturer's instructions. A production of IFN-γ was analyzed using FACS.

Flow Cytometry for splenocytes analysis

Single-cell suspensions were prepared from spleens of GL26 cells-derived exosomes or PBS pretreated mice. Lysis of RBCs was performed using ACK Lysis Buffer (Lonza). Samples were then stained with the CD4, CD8α and DX5 antibodies. After incubation for 45 min at 4 °C,

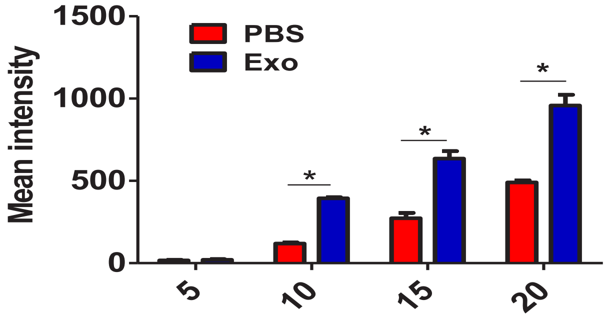

Figure 1. GL26 Cells-derived Exosomes Promote Tumor Growth in Vivo. Mice were implanted with GL26-Luc tumors and then treated with Exo from GL26 tumor cells or PBS. Then the luciferase signals which represented the tumor size was analyzed using D-luciferin substrate through the Kodak image station. As shown in Figure 1 GL26-bearing mice treated with Exo grow faster than the PBS group. *p<0.05, vs. PBS group

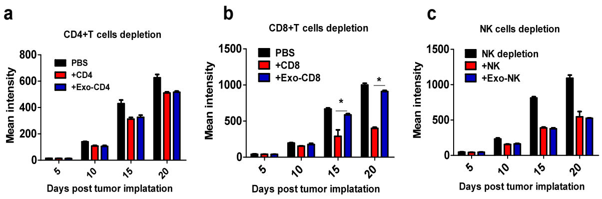

Figure 2. Exo from GL26 Tumor Cells Inhibit the Cytolytic Activity of CD8+T Cells in vivo. To investigate the correlation of the Exo and immune factors, CD4+T cells, CD8+T cells and NK cells in mice were respectively depleted, mice were then implanted with GL26 tumors. 5 days after tumor implantation, mice were then adoptively injected with CD4+T cells, CD8+T cells, NK cells from normal mice or Exo pretreated CD4+T cells, CD8+T cells and NK cells. Results demonstrated that adoptively transfer CD4+T cells, exosomes pretreated CD4+T cells, NK cells or exosomes pretreated NK cells into CD4+T cells or NK cells depletion mice significantly inhibit the GL26 tumor growth (Figures 2a and 2c). But the CD8+T cells from Exo pretreated mice could not effectively inhibit the tumor growth (Figure 2b, *p<0.05)

cells were washed with pre-iced PBS. Finally, cells were resuspended with PBS and analyzed using FACS.

Statistical analysis

One-way, two-way analysis of variance (ANOVA) and t-test were used to determine statistical significance, Tukey's Multiple Comparison Test was used to compare all pair of columns (*p<0.05 and **p<0.01).

Results

GL26 cells-derived exosomes promote GL26 tumor growth

To investigate the effects of GL26 cells-derived exosomes on GL26 tumor growth, we pretreated syngeneic C57BL/6j mice with gradient-sucrose purified exosomes from cultured mice glioblastoma GL26 cells, and evaluated the effect of the exosomes on the growth of implanted GL26 tumor cells. As shown in Figure 1, the growth rate (luciferase signals) of the implanted GL26 tumor cells was significantly greater in the mice treated with GL26 cells-derived exosomes than in the mice pretreated with PBS.

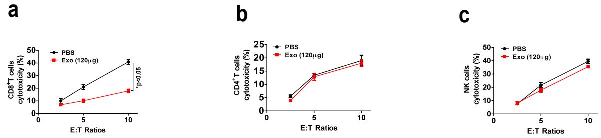

Figure 3. Exo from GL26 Tumor Cells Inhibit the Cytolytic Activity of CD8+T Cells in Vitro. LDH release assay was used to study the cytolytic activity of CD4+T cells, CD8+T cells and NK cells from Exo immunized mice. As shown in Figure 3, the cytolytic activity of CD8+T cells from Exo treated mice was obviously inhibited (Figure 3a, *p<0.05, vs PBS group). There is no inhibition effects of CD4+T cells and NK cells from Exo pre-immnunized mice (Figures 3b and 3c)

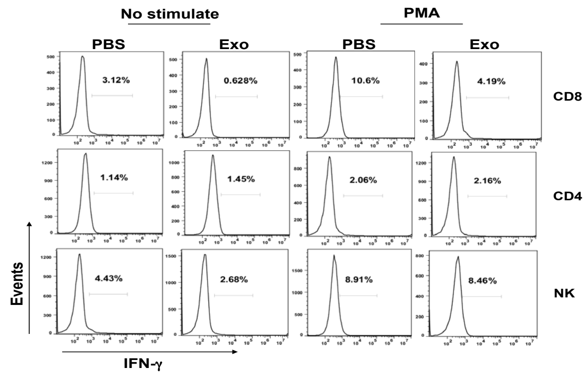

Figure 4. GL26 Cells-derived Exo Down-regulated the Expression of IFN-g. As shown in Figure 4, expression of IFN-g was dramatically down-regulated in CD8+ T cells of exsomes pretreated group compared with the PBS group

Exosomes from GL26 inhibit the activity of CD8+T cells in vivo

In vivo tumor growth model have demonstrated that exosomes from cultured GL26 cells effectively promote the GL26-luc tumor growth in vivo. Based on the previously reports that promotion of tumor growth may due to the immunomodulation, so we subsequently study the effects of GL26 cells-derived exosomes on the immune responses via CD4+T, CD8+T and NK cells depletion in vivo. As shown in Figure 2a and 2c, adoptively transfer CD4+T cells, exosomes pretreated CD4+T cells, NK cells or exosomes pretreated NK cells into corresponding CD4+T cells or NK cells depletion mice effectively inhibit the GL26 tumor growth. And there is no difference between CD4+T cells, NK cells and exosomes pretreated CD4+T cells, NK cells transfer groups. But, we surprisely found that in CD8+T cells depletion mice, tumor growth in CD8+T cells transferred group was significantly inhibited, but the exosomes pretreated CD8+T cells almost have no inhibitory effects (Figure 2b).

GL26 cells-derived exosomes inhibit the cytolytic activity of CD8+T cells ex vivo

To determine the effect of the GL26 cells-derived exosomes on the cytolytic activity of immune cells in spleens, CD4+T cells, CD8+T cells and NK cells were isolated from the spleens of mice that had been treated with GL26 exosomes or PBS. Cytotoxic activity was determined in vitro using lactate dehydrogenase (LDH) release assay with GL26 target cells. We found that the CD8+T cells isolated from the mice treated with the GL26 cells-derived exosomes exhibited significantly

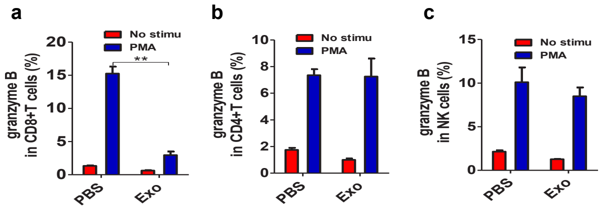

Figure 5. Granzyme B was Decreased in Exo Exposed CD8+T Cells. As shown in Figure 5, granzyme B was significantly inhibited in CD8+T cells exposed to GL26 cells-derived exosomes (Figure 5a). And the exosomes could not inhibit the expression of granzyme B in CD4+T cells and NK cells (Figures 5b and 5c)

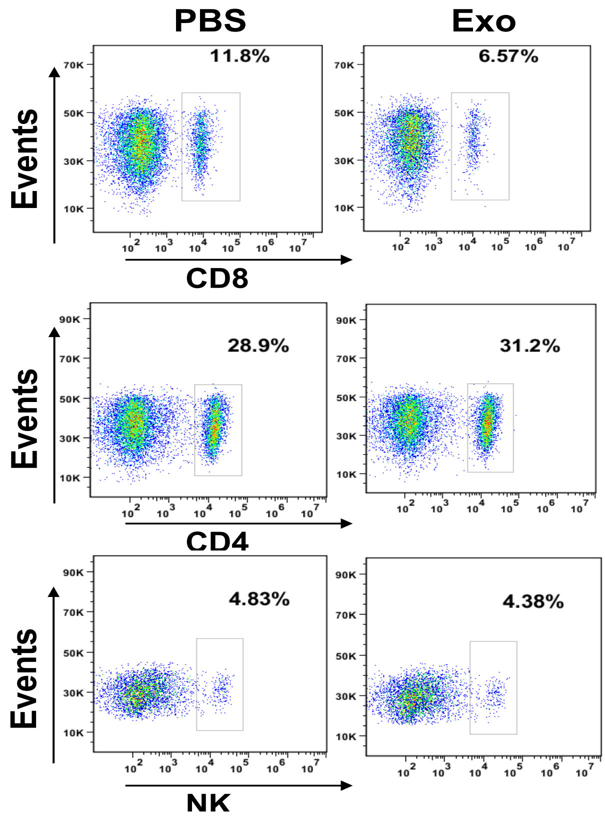

Figure 6. GL26-derived Exosomes Reduce Percentages of CD8+T Cells. The percentage of CD8+T cells but not CD4+T cells and NK cells in GL26 cells-derived exosomes pretreated mice was significantly decreased

lower cytotoxicity activity than did the CD8+T cells isolated from the PBS treated mice (Figure 3a). But the cytolytic function have no difference between the PBS and exosomes pretreated groups (Figure 3b, 3c). These data indicated that GL26 cells-derived exosomes promote tumor growth due to cytolytic activity inhibition of CD8+T cells.

GL26 derived exosomes effectively downregulated the expression of IFN-γ and granzyme B in CD8+T cells

The effect of GL26 cells-derived exosomes on the expression of the major cytotoxicity effectors including IFN-γ and granzyme B in CD4+T cells, CD8+T cells and NK cells using FACS analysis. As shown in Figure 4 and Figure 5, after stimulated with PMA, expression of IFN-γ and granzyme B were dramatically down-regulated in CD8+T cells of exsomes pretreated group compared with the PBS group.

Pretreatment with GL26-derived exosomes results in a reduction of the percentages of CD8+T cells

The immunomodulation of GL26 cells-derived on the CD8+T cells may through different mechanisms, including inhibition of cytolytic activity of CD8+T cells and reduction of the numbers of the CD8+T cells in spleen. So the percentage of CD8+T cells in GL26 cells-derived exosomes pretreated mice was analyzed using FACS. As shown in Figure 6, there was a significant reduction in the percentages of CD8+T cells but not CD4+T cells and NK cells in spleens of exosomes pretreated mice.

Discussion

The immune system plays irreplaceable roles in tumors elimination. It also serves as a watchdog against transformed cells that may lead to cancer. The key cells of the immune system for tumor surveillance are T cells, which are part of the adaptive immune response and NK cells which are part of the innate immune component (Smyth et al., 2000; Töpfer et al., 2011). After recognition of an antigen on the tumor cells via the T cell receptor (TCR), activated CD8+T cells and CD4+T cells in some certain conditions can effectively kill the tumor cells (Friedman et al., 2012; Wilde et al., 2012). NK cells also play a important role in immune surveillance by killing the MHC I-deficient tumor cells (Smyth et al., 2001).

Despite existence of two immune barriers, in many cases the immune system does not get activated but "ignores" the tumor. Many mechanisms of tumor escape from immune systems have been reported previously: absent or low expression of molecules on tumor cells involved in tumor target cell recognition (Diermayr et al., 2008); absence of co-stimulation leading to tolerization of T cells (den et al., 2004); soluble factors secreted by tumor cells inhibiting T cell response; and regulatory T cells, myeloid suppressor cells, and stromal cells may impair immune-cell responses to tumors (Johann et al., 2010; Bacić et al., 2011; Zamarron et al., 2011). Furthermore, tumors can release soluble molecules such as HLA-I (sHLA-I). This, in turn, reduces T cell-mediated immune response and induces apoptosis of cytolytic effector cells such as natural killer and CD8+T lymphocytes through the engagement of HLA-I receptors such as CD8 and/or activating isoforms of the inhibitory receptor superfamily. Furthermore, the elimination of anti-tumor effector cells may be achieved by induction of apoptosis consequent to triggering elicited via activating molecules, such as receptors responsible for natural cytotoxicity, upon their binding with ligands expressed on tumor cells.

In the 1980's Dr. Douglas Taylor first described microvesicles secreted by tumor cells (Poutsiaka et al., 1985). They were estimated to be between 50–200 nanometers in diameter and associated with a variety of immune inhibitory effects. Previously studies mainly focused on the development of tumor vaccines. Tumor-derived exosomes usually contain tumor antigens and have been used as a source of tumor antigens to stimulate anti-tumor immune response (Zhong et al., 2011; Lv et al., 2012). But, recent studies have demonstrated that such microvesicles also act as immunosuppressive roles in process of tumor formation and invasion (Yang et al., 2012). It secreted from tumor or cultured tumor cells mediated cell-cell communication has grown increasingly important in cancer immune escape associated research. Recent findings on vesicle-based information transfer by exosomes have changed the view of the tumor microenvironment. Exosomes represent the main extracellular processes implicated in the regulation of multiple physiological processes. Importantly, in cancer, exosomes contribute to the formation of the tumor microenvironment, promoting invasion, angiogenesis, immune regulation and metastasis. Therefore, exosomes could be considered one of the major factors acting locally or systemically to promote the continuous crosstalk between the tumor and its microenvironment, influencing the behavior of different cell types such as stromal, endothelial and bone marrow-derived cells and finally result in the immune escape of tumor cells. It not only induces T cell apoptosis, but also blocks various aspects of T cell signaling, proliferation, cytokine production, and cytotoxicity.

van Oijen M et al. have demonstrated that immune responses against cancer cells detectable in the peripheral blood of melanoma patients lose their efficacy and may even turn, in some cases, into indicators of tumor progression (van Oijen et al., 2004). This evidence proves the concept of immunosuppressive mechanisms negatively modulating tumor immunity and nullifying its ability to control tumor growth. Earlier studies have reported that tumor exosomes might contribute in blunting cancer-specific T cells, at least in defined phases of their activation state, derives from studies focused on the expression by these organelles of a bioactive membrane-bound form of FasL. Apoptosis via Fas/FasL interaction represents indeed one of the major pathways controlling T cell homeostasis through the selective elimination of over-reactive Fas-expressing T cells. Several years ago, tumor cells, particularly from melanoma and colorectal carcinoma, were found to express FasL and to exploit this expression as a novel pathway of immune escape. Cunren Liu et al. have demonstrated that breast cancer cells can communicate with NK cells through the production of exosomes by the tumor cells that are able to inhibit NK cell activation and promote tumor growth (Liu et al., 2006). Aled Clayton et al. have demonstrated that proliferation of CD8+T cells in response to IL-2 was inhibited by mesothelioma cell line-derived exosomes (Clayton et al., 2007).

In this study, we have shown for the first time the role of GL26 cells-derived exosomes in the promotion of growth of implanted GL26 tumors in vivo. Compelling evidence of GL26 cells-derived exosome-mediated inhibition of the CD8+T cell immune response was provided in three different assays: more rapid growth of the implanted GL26 tumors in mice that were pretreated with GL26 cells-derived exosomes, inhibition of the cytotoxic activity of CD8+T cells both in vivo and in vitro, reduction of CD8+T cells in spleen and inhibition of cytolytic associated IFN-γ and granzyme B. As discussed above, GL26 cells-derived exosomes plays an immune escape role by blunting the cytotoxic killing function of CD8+T cells including down-regulation of the IFN-γ and granzyme B secreted from it and proliferation of CD8+T cells. Studies in Huangge Zhang's group have demonstrated that mice breast cancer cells derived exosomes are able to inhibit NK cell but not CD8+T cells activation and promote tumor growth. This difference may be due to the source of exosomes, different components in exosomes and different types of tumors.

We noticed that GL26 tumors grow faster about 5 days after implantation. Many factors could result in this phenomenon, and based on the previously studies, we proposed that this phenomenon may be associated with the exosomes which plays an immunesuppressive effect on tumor growth and invasion. And we do in this study confirmed our speculation that GL26 cells-derived exosomes plays a key role in GL26 tumors growth. These data in this study may provide us a new way to understand the growth or tumorigenicity of brain tumors.

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