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Gangliosides drive the tumor infiltration and function of myeloid-derived suppressor cells

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Abstract

While it is now widely appreciated that anti-tumor immunity is critical to impede tumor growth and progression, there remain significant gaps in knowledge about the mechanisms used by tumors to escape immune control. In tumor cells, we hypothesized that one mechanism of immune escape used by tumors involves the synthesis and extracellular shedding of gangliosides, a class of biologically active cell surface glycosphingolipids with known immunosuppressive properties. In this study, we report that tumor cells engineered to be ganglioside-deficient exhibit impaired tumorigenicity, supporting a link between ganglioside-dependent immune escape and tumor outgrowth. Notably, we documented a dramatic reduction in the numbers and function of tumor-infiltrating myeloid-derived suppressor cells (MDSC) in ganglioside-deficient tumors, in contrast to the large MDSC infiltrates seen in ganglioside-rich littermate control tumors. Transient ganglioside reconstitution of the tumor cell inoculum was sufficient to increase MDSC infiltration, supporting a direct connection between ganglioside production by tumor cells and the recruitment of immunosuppressive MDSC into the tumor microenvironment. Our results reveal a novel mechanism of immune escape that supports tumor growth, with broad implications given that many human tumors produce and shed high levels of gangliosides.

Keywords

tumor resistance to immune response; immune response to cancer; myeloid-derived suppressor cells; gangliosides

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Introduction

The formation and progression of human cancer is influenced by multiple factors. Beyond the necessary intrinsic and autonomous proliferative capability of tumor cells, interactions with the tumor microenvironment (TME) are critical to the actual growth of tumors and contribute to resistance to therapy (1, 2). These interactions in the TME can result from “signals” between tumor cells and the host in the form of soluble factors. The identification of novel tumor-derived factors and elucidation of their mechanisms of action that include, prominently, inhibition of effective antitumor immune responses, is important for developing new strategies to effectively intervene in impeding tumor progression.

Tumor cell gangliosides, biologically active, amphipathic molecules that are shed into the TME at a rapid rate (3) and by many tumors (4) are one such factor. Clinical correlations link ganglioside shedding and human tumor progression (5). Two experimental approaches—impeding tumor growth by transient pharmacological depletion of gangliosides (6) and enhancing tumor growth by enrichment of the TME by addition of exogenous gangliosides or of ganglioside-rich tumor cells (7)—further suggested that the presence of gangliosides in tumor cells and their microenvironment accelerates tumor growth. However, the mechanism(s) by which the natural, ongoing, continuous process of ganglioside synthesis and shedding by tumors enhances tumor growth have been unclear. This critical gap in understanding contrasts to the mostly in vitro identification of a number of properties of gangliosides that could impact the process of tumor formation in vivo, including upregulation of some microenvironmental processes that can promote tumor growth [e.g., fibroblast proliferation and tumor angiogenesis (8-11)] and the inhibition of others [e.g., anti-tumor immune responses (3, 12, 13)].

Addressing the issue of inhibition of the immune response, here we have identified myeloid-derived suppressor cells (MDSC) as being driven in their accumulation and function by the presence of gangliosides in the tumor cells and their microenvironment. Because tumor infiltrating MDSC are now known to have many functions, including directly inhibiting antitumor cellular immune responses (14-16), recruiting regulatory T cells (17), and inhibiting CD8⁺ effector T cell infiltration (18), the findings may explain how gangliosides allow tumors to effectively subvert antitumor immune responses in vivo.

Materials and Methods

Mice and reagents

All animal experiments were approved by the Children's National Medical Center Institutional Animal Care and Use Committee. C57BL/6 and NOD-Rag1^{-/-} mice (female, 6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed in specific pathogen-free units. MDSC isolation kits were obtained from Miltenyi Biotec (Auburn, CA). For flow cytometry, monoclonal antibodies anti-Gr-1 (RB68C5), anti-Ly-6C (AL21), anti-Ly-6G (1A8), anti-CD11b (M1/70), and anti-CD45 (F11-30) were from BD Biosciences. 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (PPPP), >98% pure by high-performance thin-layer chromatographic (HPTLC) analysis was gift from Matreya, Inc., Pleasant Gap, PA.

Tumor cells

DKO (Ras/Myc-transformed GM3S/GM2S double knockout mouse embryonic fibroblasts) and WT (transformed littermate control wild type mouse embryonic fibroblasts) (Liu et al., 2010) were cultured in DMEM medium supplemented with 10% FBS, 2-mM L-glutamine, and 1% minimum non-essential amino acids. EL4 murine lymphoma cells were maintained in DMEM supplemented with 10% FBS. All cells cultures were maintained in an atmosphere of 5% CO₂/95% air in a humidified incubator at 37 °C.

Tumor cell ganglioside radiolabelling, isolation, and HPTLC analysis of shedding

To radiolabel gangliosides, WT and DKO cells were cultured in complete medium with 1.0 µCi/ml each of D-[1-¹⁴C]-glucosamine hydrochloride and D-[1-¹⁴C]-galactose for 24 h and then washed. After an additional 24 h in culture in complete medium the total radiolabeled gangliosides of the cells and those shed into the culture supernatant were purified and analyzed by HPTLC autoradiography as described (3). Briefly, the total lipid extracts were partitioned in DIPE/1-butanol/saline and total gangliosides (lower aqueous phase) were further purified by Sephadex G-50 gel filtration. HPTLC analysis was performed using 1×20 cm precoated silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) developed in chloroform/methanol/0.25% aqueous CaCl₂.2H₂O (60:40:9, v/v/v) and visualized by autoradiography to detect shed gangliosides. Unlabeled gangliosides from WT cells were isolated and purified by the same methods, and then dissolved in PBS for use in the reconstitution experiments.

Inhibition of Glucosylceramide Synthase in EL4 cells by PPPP

PPPP was prepared as a 1 mM stock solution in ethanol, stored at 4 °C, and diluted to a final concentration of 0.5 µM in culture medium with 1% ethanol. EL4 cell cultures were incubated for five days in PPPP or in 1% ethanol alone, harvested, and washed before injection.

Tumor formation

Ganglioside depleted DKO tumor cells and WT tumor cells in 20 µl PBS were injected s.c. (10⁴ or 10⁵ cells) in C57BL/6 or NOD-Rag1^{-/-} mice. 1×10⁶ EL4 or PPPP-treated EL4 tumor cells were injected s.c. in syngeneic C57BL/6 mice. Tumor growth was monitored with a digital caliper (length, width and height) every 2-3 days. At the indicated times, mice were euthanized and tumors, and in some experiments lymph nodes, were excised and processed for analysis.

Preparation of tumor single cell suspensions

Tumors were minced into small 2×2 mm pieces and transferred into gentleMACS C Tubes containing 5 mL of RPMI-1640 medium, 300 U/mL Collagenase I, and 0.96 mg/mL Dispase II. The tubes were incubated for 20 min in a 37°C water bath with mixing to resuspend settled tissue fragments and then attached onto the sleeve of the gentleMACS Dissociator and run using the appropriate program followed by further incubation for 20 min at 37°C in a water bath. Then, 100 U/ml DNase I was added to the tube which was run on the Dissociator again. Finally, 10 mL PEB buffer was added to the tubes which were

centrifuged at 300×g for 10 minutes at 4°C. Red blood cells were removed by resuspending the pellet in 2 mL of ACK lysis buffer, followed by washing, resuspension in PBS, filtration through a 70µm cell strainer, layering on Lympholyte M and centrifugation at 400×g for 20 min at room temperature. Cells at the opaque interface were transferred into fresh tubes, washed twice with PBS, and analyzed.

Flow cytometry

Cells were washed in PBS and stained with Live/Dead Fixable Aqua stain (Invitrogen) for 30 min in the dark followed by washing with FACS wash buffer (PBS containing 05% FBS, 2 mM EDTA and 0.1% sodium azide). As a positive control for dead cell staining, 5×10^5 cells were treated with heat at 65°C for 2 min, placed on ice for 2 min and mixed with equal number of live cells. Single-cell suspensions were incubated with TruStain FcX antibody (BioLegend) to block Fc receptors and then stained with directly conjugated antibodies (BD Bioscience; BioLegend). Flow cytometric analysis was performed on a FACSCanto (BD Biosciences) and 100,000 events were recorded for each sample. Data were analyzed using FACS Diva and FlowJo software (Tree Star). Gating was on Aqua Viability Stain negative CD45 positive populations, in which further markers were analyzed.

MDSC isolation

The single cell suspensions of tumors were adjusted to 10^8 cells in 900 µl of PEB buffer containing 1% FBS. 50 µl of Gr-1(PE)/CD11b(APC)/CD45(FITC) IgG3 were then added. The cells were incubated for 30 minutes at 4°C, centrifuged at 330g for 10 minutes, and the cell pellets re-suspended at 2.5×10^7 /ml in ice-cold degassed HBSS containing 10mM HEPES and 2% FBS. The stained cells were sorted using the Influx cell sorter (BD Biosciences). MDSC (CD11b+/Gr1+) were collected after gating on the live CD45+ population, centrifuged, resuspended in HL-1 culture medium, and irradiated (2.5gy) immediately before use in the OT-1 splenocyte proliferation assay. Purity of the sorted CD11b+/Gr-1+ cells was >90%.

Inhibition of T-cell proliferation and IFN-γ secretion by MDSC

Splenocytes from OT-1 mice were prepared by dissociation using frosted-end slides, seeded in 96 well plates, and cultured for 96 hours at 37°C HL-1 medium with 1% Penicillin, 1% streptomycin, 50µM 2-ME, and 2mM Glutamine, with 0.01ug/ml OVA peptide (SIINFEKL, 257-264) or control peptide E7 (RAHYNIVTF, 49-57). The cells were pulsed with 1µCi 3H-thymidine/well during the last 18 hours, harvested, and 3H-thymidine uptake determined. The secretion of IFN-γ was quantified in supernatants OT-1 splenocyte/MDSC cultures collected on day 4. IFN-γ was assayed by ELISA.

Statistical analysis

Changes in tumor incidence with time were analyzed by the log-rank test. Changes in MDSC with tumor size and time of tumor growth were analyzed by longitudinal linear regression analysis to account for the correlation between repeated assessments over time. All bars shown in the figures represent mean±SEM values. Statistical significance was calculated using two-tailed Student's t-test.

Results and Discussion

The model

The experimental system we used to determine how immunological mechanisms are influenced in vivo by de novo ganglioside synthesis and shedding is a novel tumor cell created by oncogenic transformation (pBABE-c-Myc^{T58A}+H-Ras^{G12V}) of murine embryonic fibroblasts in which two enzymes, GM3 synthase and GM2 synthase, that together specifically control all ganglioside synthesis in these mice had been knocked out (19). Ganglioside synthesis in these DKO cells was thereby constitutively and completely blocked, in contrast to the robust ganglioside synthesis of similarly derived littermate control wild type (WT) tumor cells. This knockout renders the tumor cell completely devoid of the gangliosides (GM3, GM2, GM1, and GD1a) that are present in the WT cells (19). Only mild (less than one-fold) elevations of the ganglioside precursor molecules lactosylceramide (36%), glucosylceramide (65%), and ceramide (30%) accompanied this complete blockade of ganglioside synthesis (19). Earlier studies with the DKO cells (19) showed that the absence of gangliosides in the tumor cell was associated with markedly impeded tumor growth in syngeneic mice despite robust, unaltered proliferation kinetics of the DKO cells in vitro.

This system has enabled us to explore here a suspected relationship between active tumor cell ganglioside metabolism and subversion of antitumor immune responses. We first confirmed by autoradiography that the genetic deletion of ganglioside synthesis by DKO tumor cells is accompanied by abrogation of the release of tumor cell gangliosides into the culture supernatant (Fig 1A). Thus, alterations in immune responses observed in DKO tumor-bearing mice, compared to responses in WT tumor bearing mice, would be directly linked to the interrupted ganglioside metabolism of the tumor cell.

Ganglioside-deficient DKO tumor growth is accelerated in immunosuppressed mice

To test the hypothesis of a causal relationship between the absence of gangliosides and decreased tumor growth, and the role of anti-tumor cellular immune responses, we probed the possibility of a direct immunological effect of the presence or absence of tumor cell gangliosides on tumor growth. Here, we determined how the impeded growth of ganglioside-deficient DKO tumor cells in comparison to WT cells would be affected by the elimination of potential antitumor immune responses, using an immunodeficient host, the NOD-Rag1^{-/-} mouse (20) that is devoid of T and B lymphocytes. We compared tumor growth to that in immunologically intact normal C57BL/6 mice, assessing the impact of immunocompetence on DKO tumor growth.

In normal, immunologically intact syngeneic C57BL/6 mice, the tumor incidence of DKO cells was markedly reduced in comparison to that of WT cells; 100% of normal mice receiving 10⁴ WT cells developed tumors, in contrast to only 50% of mice receiving the same number of DKO cells (Fig. 1B, left panel, p<0.0001). Furthermore, we confirmed that consistent with our previous findings (19), the rate of DKO tumor growth was substantially impeded, taking 43 days for 10⁵ DKO tumor cells vs. 18 days for 10⁵ WT cells to reach a volume of 500 mm³ (Fig. 1C, left panel, p<0.0001). Strikingly different observations were

made when the cells were inoculated into immunodeficient NOD-Rag1^{-/-} mice. Here we could directly test whether gangliosides were acting by their immunosuppressive activities to support tumor growth. If so, ganglioside-deficient DKO tumor cells might show recovery of tumor development in the immunodeficient mouse. This was in fact the case; the tumor incidence of DKO cells in the immunodeficient NOD-Rag1^{-/-} mice (Fig 1B, right panel) was now increased from 50% to 100% ($p < 0.001$). This was the same incidence as that of the WT cells. Moreover, in NOD-Rag1^{-/-} mice, the rate of growth of these ganglioside-poor DKO tumor cells was substantially accelerated, almost that of the WT cells (Fig 1C, right panel). The rate of growth of the WT cells was not significantly different in normal vs. immunodeficient mice ($p = 0.5$). Comparing WT cell tumor growth in normal C57Bl/6 vs. immunodeficient NOD-Rag1^{-/-} mice, 10⁴ DKO cells reached a tumor volume of 500 mm³ in 22 days in the immunodeficient mice vs. 43 days in the immunocompetent mice ($p < 0.0001$). The increased incidence and faster growth of ganglioside-poor (DKO) tumors in immunodeficient mice supports the suggestion that tumor gangliosides accelerate tumor growth by interfering with cellular immune responses that normally might control tumor growth.

Tumor cell gangliosides influence immune cell and MDSC infiltration of tumors

As the next step we analyzed ganglioside-deficient DKO tumors and ganglioside-replete WT tumors for infiltration by T cells, which mediate antitumor cellular immune responses and may control tumor growth, progression, and rejection (21). Total CD45⁺ leukocyte infiltration was more than two-fold higher in DKO than in WT tumors (18.0×10^4 vs. 7.1×10^4 CD45⁺ cells/ 10^6 tumor cells, or 20% vs. 7.5% of total cells in the tumor). Both CD4 and CD8 T lymphocyte subsets were nearly four-fold higher in the DKO tumors (Table 1). We also quantified these cells in tumor draining lymph nodes (TDLN) where they are critical in effecting antitumor immune responses. Significantly higher numbers of CD4 and CD8 T cells were detected in the TDLN of ganglioside-poor DKO tumor-bearing mice than in the TDLN of ganglioside-rich WT tumor-bearing mice (Table 1), again suggesting increased effective immune responses result when tumors are ganglioside deficient.

Using a directed approach to further probe suppression of the cellular immune response, we focused on the myeloid compartment and specifically on myeloid derived suppressor cells (MDSC) for several reasons. (i) MDSC that infiltrate the TME inhibit antitumor cellular immune responses (14-16), recruit regulatory T cells (17), and inhibit CD8⁺ effector T cell infiltration (18). (ii) MDSC enhance tumor growth while MDSC depletion results in decreased tumor growth (22). (iii) Inhibition of the cellular immune responses in vitro by gangliosides has been found to involve alterations in the generation and function of various myeloid derived-cells (e.g., macrophages, dendritic cells (13, 23, 24). Together, this prior knowledge led us to determine the relationship between the presence or absence of gangliosides in the tumor cells and the TME, and the accumulation and function of MDSC in the tumors.

Gating on the CD45⁺ cell population of whole tumor single cell suspensions, by FACS we quantified tumor infiltrating MDSC (CD11b⁺Gr1⁺) in a series of 38 tumors formed from either WT or DKO cells in immunologically competent mice (Fig. 2A). The number of

infiltrating MDSC was strikingly lower in the DKO tumors than in the ganglioside rich WT tumors (Fig. 2B, 2C, $p < 0.0001$). Using longitudinal analysis, the direct relationship usually observed between increasing tumor size and increasing MDSC infiltration (14, 25), and that we observed in the ganglioside-rich WT tumors, was not seen in the DKO tumors (Fig. 2E, $p = 0.009$). With respect to tumor growth as a function of time from cell inoculation, the best longitudinal model of tumor growth over a 30-day period indicated a curvilinear growth pattern in degree of MDSC infiltration with a peak at 14 days, with consistently greater infiltration in WT tumors compared to DKO tumors; $p < 0.001$, Fig. 2F). The relative proportions of the two major currently recognized subsets of MDSC (16, 26), the predominant, granulocytic, G-MDSC ($\text{Ly6G}^+\text{Ly6C}^{\text{dim}}$) and the monocytic Mo-MDSC ($\text{Ly6G}^{\text{dim}}\text{Ly6C}^{\text{high}}$) were not significantly affected by the absence of gangliosides in the DKO tumors (Fig. 2D). Overall, the results suggest for the first time that gangliosides in tumors have a striking impact on the total number of tumor-infiltrating MDSC. They implicate tumor-associated gangliosides in facilitating MDSC accumulation and their absence to result in markedly reduced numbers of MDSC in the tumors.

We also tested whether the accumulation of MDSC in tumors under the influence of tumor gangliosides was linked, to or dependent on, lymphocyte interactions or whether MDSC accumulation is independent of adaptive immune responses, as has been suggested (22). We did this by inoculating tumor cells into the immunodeficient mice and quantifying tumor-infiltrating MDSC (Fig. 3). Tumors formed by WT tumor cells in NOD-Rag1^{-/-} mice were as highly infiltrated by MDSC ($101 \pm 17 \times 10^4$ MDSC/tumor; 16% of CD45⁺ cells) as were these same tumors in normal mice. Similarly, the number of infiltrating MDSC ($4 \pm 1 \times 10^4$ MDSC/tumor; 7.5% of CD45⁺ cells, $p < 0.0001$) in DKO tumors in the immunodeficient mice was strikingly (twenty-five-fold) lower than in the WT tumors, mirroring the reduction in MDSC in these tumors in wild type mice (Fig. 3B, 3C). Thus, the pattern of MDSC infiltration, high in WT tumors and low in DKO tumors, was essentially identical, whether the host was immunologically normal or immunodeficient. These quantitatively similar effects on MDSC infiltration by the presence versus the absence of gangliosides in the tumors, whether the tumors were propagated in wild type or in NOD-Rag1^{-/-} mice, suggest that ganglioside-dependent regulation underlying tumor accumulation of MDSC is independent of lymphoid interactions in this system.

Manipulation of tumor cell ganglioside content directly influences tumor MDSC infiltration

To further confirm the findings of the relationship between the presence or absence of gangliosides in tumors and their microenvironment, and tumor accumulation of MDSC, we used two other experimental approaches—transient membrane ganglioside enrichment and transient pharmacological ganglioside depletion of tumor cells. In the first condition, we briefly reconstituted the ganglioside complement of the ganglioside-deficient DKO tumor cells by adding purified WT cell gangliosides to DKO cells prior to injection in vivo. We recognized that in comparison to the continuous ganglioside synthesis and shedding by WT tumor cells, this attempt at reconstitution might be only temporary and the effects therefore somewhat partial or restrained. When we mixed 10^5 DKO tumor cells with 100 pmol WT gangliosides (the equivalent of the ganglioside content of 10^5 WT cells) in 20 μl PBS, DKO tumor volume on day 19, for example, was increased from 133 ± 26 to $252 \pm 33 \text{ mm}^3$ ($p = 0.01$,

Fig. 4B). And, the number of tumor infiltrating MDSC and MDSC numbers (Fig. 4C, 4D), evidenced a more than three-fold increase from $5 \pm 1 \times 10^4$ MDSC/tumor (7.5% of CD45⁺ cells) to $18 \pm 6 \times 10^4$ MDSC/tumor (11% of CD45⁺ cells) ($p=0.03$) without significant changes in MDSC subset proportions (Fig. 4E).

In the second approach, prior to injection in syngeneic mice, we incubated ganglioside-rich EL4 murine lymphoma cells with 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) to inhibit glucosylceramide synthase. This treatment blocks all ganglioside synthesis and causes complete but only transient ganglioside depletion (27). The treated cells evidenced a trend towards reduced tumor growth in vivo, which although not statistically significant, was seen as a reduction in tumor mass at 9 days (Fig. 5A), to 214 ± 32 mg from 338 ± 73 mg of the control ganglioside-rich EL4 cells ($p=0.15$). While we did not observe a change in intratumor T cell numbers, this is possibly because unlike constitutive ongoing inhibition of ganglioside synthesis in the DKO cells, inhibition by PPPP pretreatment of tumor cells is only transient (several days at most) (27), thereby possibly resulting in milder effects overall (on tumor growth, on MDSC, and by implication, on T cell numbers). Importantly, the absolute number of MDSC in the ganglioside-depleted tumors was markedly reduced (Fig. 5B), from a mean of 83×10^4 to 33×10^4 MDSC/tumor ($p=0.01$), as was their relative proportion (Fig. 5C), from $28 \pm 3.3\%$ of CD45⁺ cells in control tumors to $19.3 \pm 1.9\%$ of CD45⁺ cells in PPPP-pretreated EL4 tumors ($p=0.04$). Together, these results, which employ a second tumor system, provide further evidence for the role of tumor gangliosides in facilitating MDSC accumulation in tumors.

Tumor cell ganglioside depletion impairs function of in vivo generated tumor MDSC

Of ultimate importance to tumor progression is the function of MDSC that infiltrate tumors. MDSC in the TME have been shown to have potent inhibitory activity in many tumor systems (16), so we determined whether the reduction in the number of MDSC infiltrating the ganglioside deficient DKO tumors was accompanied by a change in the level of their immunosuppressive activity. We assessed MDSC enriched by FACS from single cell suspensions of WT and DKO tumors for their ability to inhibit antigen-specific T cell responses. Splenocytes from OT-1 T cell receptor transgenic mice were stimulated with either the cognate (OVA-257) antigen or a control peptide (E-7) and were cultured in the presence of irradiated MDSC isolated either from WT control or from DKO tumors (22). Inhibition by the MDSC of OT-1 T-cell antigen-specific proliferation (14) and release of interferon-gamma (IFN- γ) (28) were quantified. MDSC isolated from control WT tumors were twice as effective as MDSC isolated from ganglioside deficient DKO tumors in inhibiting the antigen-induced proliferative response of OT-1 splenocytes (Fig. 6A). Similar to their inhibitory effect on the proliferative response, MDSC isolated from WT tumors also markedly inhibited OT-1 cell IFN- γ production. In contrast, MDSC isolated from DKO tumors were essentially unable to inhibit OT-1 splenocyte IFN- γ release either at the optimal time point (Fig. 6B) or over a time course (Fig. 6C). Thus, in addition to markedly reduced numbers of MDSC infiltrating the ganglioside-deficient DKO tumors, those MDSC that were infiltrating the tumors were functionally impaired. Overall then, we have shown that the synthesis and shedding of tumor cell gangliosides modulates both MDSC accumulation and MDSC activity in the resulting tumors.

Tumor gangliosides are unusual molecules that, based on many in vitro and indirect in vivo findings, are assumed to be important in vivo. With relatively mild effects on their tumor cells of origin, gangliosides expressed and shed by these cells may profoundly affect “normal” stromal cells to which they can become bound in the TME although until now, whether they have a biologically significant effect on the antitumor immune response in vivo had been unclear. Benefitting from a novel model system, here we discovered that tumor gangliosides are in fact a potent immunoregulatory factor, promoting the tumor infiltration and activity of MDSC.

While the absence of tumor cell gangliosides clearly reduces tumorigenicity and tumor growth, ganglioside blockade does not completely arrest the growth of tumors once they are established. DKO tumors nevertheless eventually grow even without developing high numbers of MDSC. And, neither implantation of DKO cells in NOD-Rag1^{-/-} mice nor ganglioside addback to DKO cells implanted in immunocompetent mice yields complete recovery of growth and MDSC numbers. Based on our findings, we think that in our model the effect of tumor gangliosides on the host immune response played a key role in the initial of steps successful tumor cell implantation and growth. Without this advantage, i.e., lacking gangliosides, DKO tumors that did form grew slowly initially but then eventually enlarged, most likely through the support of other tumor-promoting factors, such as other tumor cell derived stimulants of angiogenesis, or gradual accumulation in tumors of gangliosides derived from host cells that infiltrate tumors.

Discovery of a relationship between tumor gangliosides and MDSC raises a number of important questions to be addressed in future studies, particularly regarding mechanisms by which the gangliosides are acting. This includes elucidating how MDSC accumulation is enhanced by gangliosides (possibly by chemokine/chemokine receptor expression, for example). Also, what mechanisms underlie the observed difference caused by gangliosides in the expression of inhibitory function of MDSC? These could include a specific effect on molecules that are known to cause the MDSC-induced immunosuppression, e.g., iNOS and arginase and their products, or the possibility that gangliosides directly affect MDSC activation through contact in the tumor microenvironment.

This novel immunoregulatory mechanism of tumor gangliosides—inhibition through enhancement of MDSC number and their function in tumors—is complemented by direct inhibitory effects of gangliosides on cytotoxic T cell function (29), suggesting a highly effective immunosuppressive process that tumors may use early on to implant and establish themselves, and a process that links rapid ganglioside metabolism to enhanced tumor growth. The deficit of infiltrating MDSC in ganglioside-poor DKO tumors may also in part explain the negative impact that the absence of gangliosides has on another process important to tumor growth, angiogenesis (30). In this case, it is tempting to speculate that since MDSC directly promote tumor angiogenesis (31) and the poorly vascularized, poorly growing ganglioside-poor DKO tumors have greatly reduced angiogenesis (30), tumor accumulation of MDSC (dependent on the presence of tumor gangliosides) is an “effector mechanism” that is at least in part also responsible for ganglioside enhancement of tumor angiogenesis. The ultimate outgrowth

Overall, our findings establish tumor cell gangliosides as an important immune evasion factor with potential broad significance because rapid ganglioside synthesis and shedding characterizes many tumor types. Consequently, therapeutic strategies to impede tumor cell ganglioside metabolism, such as specific inhibition of their synthesis by tumor cells, could be a promising approach to pursue to improve cancer treatment.

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References

1. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer cell*. 2012; 21:309–22. [PubMed: 22439926]
2. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nature medicine*. 2013; 19:1423–37.
3. Ladisch S, Gillard B, Wong C, Ulsh L. Shedding and immunoregulatory activity of YAC-1 lymphoma cell gangliosides. *Cancer research*. 1983; 43:3808–13. [PubMed: 6861145]
4. Lauc G, Heffer-Lauc M. Shedding and uptake of gangliosides and glycosylphosphatidylinositol-anchored proteins. *Biochimica et biophysica acta*. 2006; 1760:584–602. [PubMed: 16388904]
5. Valentino L, Moss T, Olson E, Wang HJ, Elashoff R, Ladisch S. Shed tumor gangliosides and progression of human neuroblastoma. *Blood*. 1990; 75:1564–7. [PubMed: 2317562]
6. Weiss M, Hettmer S, Smith P, Ladisch S. Inhibition of melanoma tumor growth by a novel inhibitor of glucosylceramide synthase. *Cancer research*. 2003; 63:3654–8. [PubMed: 12839955]
7. Ladisch S, Kitada S, Hays EF. Gangliosides shed by tumor cells enhance tumor formation in mice. *The Journal of clinical investigation*. 1987; 79:1879–82. [PubMed: 3584474]
8. Alessandri G, Filippeschi S, Sinibaldi P, Mornet F, Passera P, Spreafico F, et al. Influence of gangliosides on primary and metastatic neoplastic growth in human and murine cells. *Cancer research*. 1987; 47:4243–7. [PubMed: 2440560]
9. Manfredi MG, Lim S, Claffey KP, Seyfried TN. Gangliosides influence angiogenesis in an experimental mouse brain tumor. *Cancer research*. 1999; 59:5392–7. [PubMed: 10537325]
10. Li R, Manela J, Kong Y, Ladisch S. Cellular gangliosides promote growth factor-induced proliferation of fibroblasts. *The Journal of biological chemistry*. 2000; 275:34213–23. [PubMed: 10859325]
11. Liu Y, McCarthy J, Ladisch S. Membrane ganglioside enrichment lowers the threshold for vascular endothelial cell angiogenic signaling. *Cancer research*. 2006; 66:10408–14. [PubMed: 17079461]
12. McKallip R, Li R, Ladisch S. Tumor gangliosides inhibit the tumor-specific immune response. *J Immunol*. 1999; 163:3718–26. [PubMed: 10490967]
13. Caldwell S, Heitger A, Shen W, Liu Y, Taylor B, Ladisch S. Mechanisms of ganglioside inhibition of APC function. *J Immunol*. 2003; 171:1676–83. [PubMed: 12902465]
14. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol*. 2009; 182:4499–506. [PubMed: 19342621]
15. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nature reviews Immunology*. 2012; 12:253–68.
16. Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. *Nature reviews Cancer*. 2013; 13:739–52.
17. Schlecker E, Stojanovic A, Eisen C, Quack C, Falk CS, Umansky V, et al. Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J Immunol*. 2012; 189:5602–11. [PubMed: 23152559]

18. Lesokhin AM, Hohl TM, Kitano S, Cortez C, Hirschhorn-Cymerman D, Avogadri F, et al. Monocytic CCR2(+) myeloid-derived suppressor cells promote immune escape by limiting activated CD8 T-cell infiltration into the tumor microenvironment. *Cancer research*. 2012; 72:876–86. [PubMed: 22174368]
19. Liu Y, Yan S, Wondimu A, Bob D, Weiss M, Sliwinski K, et al. Ganglioside synthase knockout in oncogene-transformed fibroblasts depletes gangliosides and impairs tumor growth. *Oncogene*. 2010; 29:3297–306. [PubMed: 20305696]
20. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. *Annual review of immunology*. 2005; 23:447–85.
21. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nature immunology*. 2013; 14:1014–22. [PubMed: 24048123]
22. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nature reviews Immunology*. 2009; 9:162–74.
23. Shen W, Stone K, Jales A, Leitenberg D, Ladisch S. Inhibition of TLR activation and up-regulation of IL-1R-associated kinase-M expression by exogenous gangliosides. *J Immunol*. 2008; 180:4425–32. [PubMed: 18354163]
24. Shurin GV, Shurin MR, Bykovskaia S, Shogan J, Lotze MT, Barksdale EM Jr. Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function. *Cancer research*. 2001; 61:363–9. [PubMed: 11196188]
25. Abe F, Dafferner AJ, Donkor M, Westphal SN, Scholar EM, Solheim JC, et al. Myeloid-derived suppressor cells in mammary tumor progression in FVB Neu transgenic mice. *Cancer immunology, immunotherapy : CII*. 2010; 59:47–62.
26. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol*. 2008; 181:5791–802. [PubMed: 18832739]
27. Olshefski R, Ladisch S. Synthesis, shedding, and intercellular transfer of human medulloblastoma gangliosides: abrogation by a new inhibitor of glucosylceramide synthase. *Journal of neurochemistry*. 1998; 70:467–72. [PubMed: 9453539]
28. Mundy-Bosse BL, Lesinski GB, Jaime-Ramirez AC, Benninger K, Khan M, Kuppusamy P, et al. Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. *Cancer research*. 2011; 71:5101–10. [PubMed: 21680779]
29. Lee HC, Wondimu A, Liu Y, Ma JS, Radoja S, Ladisch S. Ganglioside inhibition of CD8+ T cell cytotoxicity: interference with lytic granule trafficking and exocytosis. *J Immunol*. 2012; 189:3521–7. [PubMed: 22956583]
30. Liu Y, Wondimu A, Yan S, Bobb D, Ladisch S. Tumor gangliosides accelerate murine tumor angiogenesis. *Angiogenesis*. 2014; 17:563–71. [PubMed: 24165965]
31. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, et al. Expansion of myeloid immune suppressor Gr⁺CD11b⁺ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer cell*. 2004; 6:409–21. [PubMed: 15488763]

Fig. 1. Diminished tumor incidence and tumor growth of ganglioside-poor DKO cells is reversed in immunodeficient mice

(A) HPTLC autoradiogram of radiolabeled shed tumor gangliosides recovered from the 24-hour culture supernatant of WT or DKO cells. S= radiolabeled (rat brain) GM1 and GD1a ganglioside standards. (B) Life table analysis of tumor incidence of ganglioside-rich WT and ganglioside-deficient DKO tumor cells in immunocompetent C57BL/6 or immunodeficient NOD-Rag1^{-/-} mice. Significance of the increase in tumor incidence by ganglioside-deficient DKO tumor cells in immunodeficient NOD-Rag1^{-/-} mice versus in immunocompetent C57BL/6 mice was $p < 0.0001$. (C) Tumor growth. Significance of the acceleration in tumor growth by ganglioside-deficient DKO tumor cells in immunodeficient NOD-Rag1^{-/-} mice versus in immunocompetent C57BL/6 mice was $p < 0.0001$. Bars are the mean $\pm$ SEM of the combined results of 2-3 experiments, using 18 C57BL6/J and 8 NOD-Rag1^{-/-} mice.

Fig. 2. Tumor gangliosides cause accumulation of myeloid derived suppressor cells

Immunocompetent female C57BL/6 mice were injected s.c. with 10⁵ WT or DKO tumor cells. Single cell suspensions of the tumors were stained with antibodies to CD45, CD11b, and Gr-1. (A) Flow cytometric analysis, gating on CD45⁺ cells. (B) Total MDSC (CD11b⁺+Gr-1⁺) in WT (n=42) or DKO (n=50) tumor-bearing mice (C) MDSC, % of CD45⁺ cells; (D) granulocytic G-MDSC (Ly6G⁺Ly6C^{dim}) and monocytic Mo-MDSC (Ly6G^{dim}Ly6C^{high}) subset distribution from tumors on day 14, quantified by established methods (16, 26); (E): MDSC increased in WT but not in DKO tumors as a function of tumor size (n=38, p<0.009);

(F) MDSC infiltration was consistently higher in WT than in DKO tumors as a function of time of tumor growth ($p < 0.001$); Each time point (mean $\pm$ SEM) includes 4-12 mice except for day 14 (38 mice), from 1-4 experiments.

Fig. 3. Tumor gangliosides enhance tumor infiltration by MDSC in immunodeficient NOD-Rag1^{-/-} mice

Immunodeficient female NOD-Rag1^{-/-} mice were injected s.c. with 10⁵ WT or DKO tumor cells. Single cell suspensions of the tumors were stained with antibodies to CD45, CD11b, and Gr-1. (A) Representative flow cytometric analysis, gated on CD45⁺ cells. (B) Absolute number of MDSC in WT or DKO tumors (C) MDSC, % of CD45⁺ cells. Each bar indicates the mean ± SEM of 8 tumors in 2 separate experiments.

Fig. 4. Transient ganglioside reconstitution increases MDSC accumulation in DKO tumors

Female C57BL/6 mice were inoculated s.c. with 10^5 WT or DKO tumor cells together with or without 100 pmol WT tumor-derived gangliosides (gsl) and analyzed as in Fig. 2. (A) Representative flow cytometric analysis. (B) Tumor growth curves. Each bar represents 15 tumors (5/group in 3 separate experiments); effect of ganglioside addback to DKO cells, day 19, $p=0.01$. (C) MDSC number and (D) MDSC, % of total CD45⁺ cells from tumors harvested on day 15 ($n=9$ mice in 3 experiments). (E) Tumor granulocytic (Ly6G⁺Ly6C^{dim}) and monocytic (Ly6G^{dim}Ly6C^{high}) MDSC subset distribution on day 15. The vertical bars indicate the mean $\pm$ SEM.

Fig. 5. Transient ganglioside depletion reduces MDSC accumulation in EL4 tumors
 10^6 EL4 tumor cells, cultured for 5 days with or without 0.5 μ M PPPP to deplete cellular gangliosides and washed, were injected s.c. into C57BL/6 mice. (A) Tumor mass, (B) total MDSC, and (C) MDSC as % of CD45⁺ cells on day 9. Bars: mean $\pm$ SEM of total of 8 mice from 2 separate experiments.

Fig. 6. MDSC infiltrating DKO tumors are poor inhibitors of OVA-induced OT-1 splenocyte proliferation and IFN- γ release

MDSC inhibitory activity was assessed by co-culture of 10^4 - 10^5 MDSC from WT or DKO tumors with 10^5 OT-1 splenocytes stimulated with OVA or control (E7) peptide as in Materials and Methods. **(A)** Inhibition of OT-1 splenocyte proliferation by MDSC; MDSC isolated from WT and ganglioside-deficient DKO tumors ($n=12$, 5 individual experiments). Proliferation stimulated by E7 (control peptide) was consistently 5% of OVA-induced proliferation. **(B)** IFN- γ secretion by OT-1 splenocyte cultures, measured at 96 hours as IFN- γ accumulation in the medium of triplicate cultures of 10^5 OT-1 cells and 5×10^4 MDSC in two separate experiments. Bars indicate the mean $\pm$ SEM. **(C)** Time course of IFN- γ release, determined at 48, 72, and 96 hours as in Fig. 6B.

Table 1
Tumor gangliosides decrease T cell infiltration in tumors and tumor-draining lymph nodes

	Tumor* (n=27)		Tumor-draining lymph nodes** (n=46)	
	CD4	CD8	CD4	CD8
WT	3.7±1.3	1.6±0.4	1.7±1.1	1.6±0.7
DKO	17.2±4.9	5.7±1.1	2.7±1.7	2.1±1.1
<i>p</i>	0.012	0.002	0.002	0.006

Single cell suspensions from WT and DKO tumors harvested on day 14 were analyzed by FACS, gated on CD45+ cells. Results are from the indicated number of mice in 9 (tumor) or 12 (lymph node) separate experiments. Control lymph nodes contained a mean of 1.2×10^6 CD4+ cells and 0.9×10^6 CD8+ cells.

* lymphocytes/ 10^3 tumor cells

** lymphocytes $\times 10^6$ /lymph node