Antitumor Protection by NGcGM3/VSSP Vaccine Against Transfected B16 Mouse Melanoma Cells Overexpressing *N*-Glycolylated Gangliosides

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Abstract. *Background: Cancer vaccines are designed to modulate immunological responses against tumor cells through the presentation of tumor antigens. Materials and Methods: The mouse mRNA of the cytidine monophospho-N-acetylneuraminic acid hydroxylase (Cmah) gene, the enzyme that catalyzes the synthesis of N-glycolylneuraminic acid (NGc), was cloned and transfected into the B16 melanoma cell line. Transfected cells (B16-H) were characterized and used as an NGcGM3-positive primary tumor model for the evaluation of the therapeutic activity of the NGcGM3/VSSP vaccine. Results: The presence of NGcGM3 in B16-H cells promoted proliferation and adhesion in vitro, but resulted in reduced tumorigenicity in vivo. However, B16-H cells developed growing tumors in mice where NGcGM3/VSSP vaccination induced a therapeutic antitumor activity. NGcGM3/VSSP was ineffective in mice inoculated with parental B16 or B16-H cells that had lost NGcGM3 expression. Conclusion: The presence of NGcGM3 in tumor cells is critical for the antitumor activity of NGcGM3/VSSP vaccine.*

Target-specific cancer immunotherapy is currently linked to passive approaches using monoclonal antibodies such us rituximab (1) and trastuzumab (2). As expected for a target-directed approach, the inclusion criteria of patients for the above treatments involve the evaluation of the level of expression of the transmembrane protein CD20 and the one of cancer receptor HER2/neu, in their corresponding tumor

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samples, as appropriate. It is to be assumed that target antigen expression in tumor cells plays a crucial role in the efficacy of these therapeutic agents.

Active specific immunotherapy is a promising field in cancer research. It is accepted that relevance of the target antigen in developing these types of cancer vaccines contemplates some properties of the antigen, such as oncogenicity, specificity, expression level, cellular location and the percentage of positive cells in the tumor (3). Nevertheless, only in two of the currently running late-stage clinical trials of active specific immunotherapies (MAGEA3 vaccine and CDX110, ClinicalTrials.gov identifier: NCT00145145 and NCT01498328, respectively), is the presence of the selected antigen in tumor samples considered as one of the inclusion criteria.

Cancer vaccines are not just composed as a result of the antigen but also of the delivery system, which is crucial to achieve maximal response, and by the immunological adjuvant, which in turn can play a significant role in the modulation of the immune response and the tumor microenvironment. Thus, effective therapeutic responses are a consequence of the proper choice of antigen and immunological modulation of the vaccine adjuvant. Actually, the question as to what extent the presence of the vaccine target antigen in the tumor decides the effect of the treatment remains open for active immunotherapy.

Gangliosides are a broad family of glycosphingolipids, which contain at least one molecule of neuraminic acid. Proposed as potential targets for cancer immunotherapy, based on their higher abundance in tumors when compared with the matched normal tissues, ganglioside-based vaccines have been clinically tested in different types of advanced cancer, mainly melanomas, based on the observation that altered expression of these glycolipids in the cells correlates with their metastatic potential (4, 5). GM3 is the precursor molecule of the ganglioside family members which may contain either *N*-glycolylneuraminic acid or *N*-acetylneuraminic acid.

Very small size proteoliposomes (VSSP) is an immunological adjuvant based on the outer membrane vesicles derived from *Neisseria meningitidis,* used so far in ganglioside and peptide vaccines (6-8). Mesa *et al.* established that VSSP has the ability to activate, both *in vitro* and *in vivo*, mouse and human dendritic cells, and the subsequent secretion of IL-12p40/p70, TNF-α (*Tumor necrosis factor*) and IL-6. They also reported that VSSP adjuvant is able to condition a Th1 phenotype on stimulated naïve T-cells (9) and to activate strong specific cytotoxic T-lymphocyte (CTL) responses for soluble peptides (10). Additionally, Fernández *et al.* demonstrated that VSSP is able to circumvent the function of tumor-induced myeloid-derived suppressor cells, a lymphoid population reported to be suppressors of antitumor immunity, through inhibition of CD4^{+} and CD8^{+} T-cell function (11).

We have already published findings for the activity of two GM3-based vaccines combined with VSSP, called NAcGM3/VSSP and NGcGM3/VSSP. Strong antitumor activity of NAcGM3/VSSP vaccine was found on the B16 mouse melanoma model (naturally NAcGM3-expressing cells) (6). Considering that VSSP is a potent immunomodu-latory adjuvant, the participation of the target antigen in the antitumor activity of NGcGM3/VSSP vaccine is the main focus of this article. NGcGM3 has been described as a tumor antigen in several types of human cancers including melanoma, lung and breast cancer (12, 13). However, most mouse cancer cell lines are negative for the expression of this antigen, although murine somatic cells frequently express it. We have already published data describing the null expression of NGcGM3 in the B16 melanoma tumor cell line (14). The presence of *N*-glycolylneuraminic acid in the cells is a consequence of the expression of the enzyme cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (*Cmah*) (15).

In order to address the participation of the target antigen in the NGcGM3/VSSP vaccine we developed a mouse model based on a genetically modified variant of B16 melanoma cell line. By stable transfection of the murine *Cmah* gene mRNA sequence into the B16 melanoma cell line, we generated a novel NGcGM3-positive mouse melanoma model where the antitumor activity of the NGcGM3/VSSP vaccine was tested. Characterization of the transfected cells and the mouse melanoma model is also presented in this work.

Materials and Methods

Monoclonal antibodies. 14F7 monoclonal antibody (mAb) is a murine IgG mAb directed against NGcGM3 (kindly provided by the Centro de Inmunología Molecular, La Havana, Cuba) (12, 16). M2590 mAb is a murine IgM directed against NAcGM3 (Cosmo Bio Co. Ltd., Tokyo, Japan).

Tumor cells and culture conditions. B16 and B16-H cells were maintained in Dulbecco's Modified Eagle Media – Nutrient Mixture

F12 (DMEM-F12) culture medium (Gibco BRL, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (PAA, Pasching, Austria) and were subcultured approximately three times a week using a Trypsin-EDTA solution (Gibco BRL). Cell viability was assessed using the trypan blue exclusion technique. B16 Cmah-transfected cells (B16-H) were cultured with DMEM plus G418.

Ethics statement. Pathogen-free C57BL/6 mice (approximately 10 weeks old, with an average weight of 25 g), were obtained from the Animal Care Division of La Plata National University (La Plata, Argentina). Five mice per cage were kept with water and food *ad libitum* in the animal house facility at Quilmes National University. All animal experimental protocols were approved by the Quilmes National University Animal Review Board. Maintenance of animals was conducted under accepted international standards (17).

Cell transfection. *Cmah* mRNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) from total mouse liver RNA obtained using the RNAqueous Midi RNA kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. RT-PCR assay was carried out with specific primers (forward primer: 5'-CGCCTTCCTGGTGTGA-3'; reverse primer: 5'-GTTGGGTGG TGTTAGAGG-3'). The RT step consisted of 5 μg total RNA, 10 mM dNTPs, 50 ng random hexamers pd(N)6 (GE Heathcare, Chalfont St. Giles, Buckinghamshire, UK) as first strand primer, 0.1 M Dithiothreitol (DTT), 40 U RNAseOUT (Invitrogen, Carlsbad, CA, USA) and 200 U Superscript III retrotranscriptase (Invitrogen) in a final volume of 20 μl. The RT step was performed at 50°C for 1 h. The Cmah sequence was then amplified by means of a PCR reaction comprised of 45 μl Supermix High Fidelity PCR mix (Invitrogen), 10 pmol forward primer, 10 pmol reverse primer and 1 μg cDNA obtained in the RT step. The amplification profile consisted of a single initial denaturation step (95°C, 5 min), followed by 35 cycles of 95°C, 30 s; 53.7°C, 1 min and 72°C, 1.5 min; ending with a final extension step (72°C, 5 min). The amplification products were then cloned into the pcDNA3.1/V5-His TOPOTA vector (Invitrogen). After determining the identity of the inserts, B16 cell line (ATCC CRL-6322) was transfected using Lipofectamine 2000 (Invitrogen).

RT-PCR. Expression of the *Cmah* mRNA was evidenced by an RT-PCR assay, using total RNA from normal mouse liver, B16 or B16-H cells as template. Total RNA was obtained using the RNAqueous Midi RNA kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. The RT-PCR amplification protocol was conducted as described above in *Cell transfection*.

Flow cytometric assay. Cells were harvested with a trypsin-EDTA solution, resuspended in serum-free medium and incubated at 5×10^5 to 1×10^6 cells per sample with 10 μg or 5μg of the mAbs M2590 or 14F7, respectively, for 30 min at 4°C. Control antibodies of the same isotype (IgM or IgG respectively) were included. Tumor cells were washed with Phosphate buffered saline (PBS) containing 2 mM EDTA, 1% Fetal calf serum (FCS) and further incubated with an 1:100 dilution of R-Phycoerythrin (RPE)-conjugated goat anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark) for 30 min at 4°C. A total of 5×10^4 cells were analysed with a FACSCan Flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). WinMDI 2.9 (en.bio-soft.net) was used for data analysis.

Proliferation assay. A total of 1×10^4 B16 or B16-H cells/well were seeded in 96-well plates in DMEM-F12, supplemented with 5%

FCS. Forty-eight hours after plating, cells were washed with PBS and fixed with 10% formalin in PBS buffer. Afterwards, cells were stained with a 0.5% toluidine blue solution for 15 min at room temperature and then washed with PBS. Finally, cells were resuspended in a 0.1% dodecyl sulfate (SDS) solution and the absorbance was measured at 595 nm.

Adhesion assay. A total of 4×10^4 B16 or B16-H cells/well were seeded in 96-well plates in DMEM-F12 supplemented with 2% FCS. At different times after plating, cells were carefully washed with PBS buffer and fixed with methanol. After 10 min incubation at room temperature, methanol was discarded and cells were stained with a 0.1% crystal violet solution. Stain dissolution was achieved by incubation of cells with 60 µl/well of a 10% methanol/5% glacial acetic acid solution. Finally, the absorbance was measured at 570 nm.

Cell migration assay. B16 or B16-H cells (1×10^6/well) were seeded in 6-well plates in DMEM-F12 supplemented with 10% FCS. When cells reached 95% confluence, three wounds were inflicted in the monolayer with a sterile p200 pipette tip through the cells and the plate was incubated at 37˚C, for 13 h approximately. A control wound was done and after 1 h of incubation at 37˚C cells were fixed with 10% formalin in PBS buffer and stained with a 10% toluidine blue solution. The invaded area of 10 random sections per well were quantified using the ImageJ Software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Tumor cell challenge. B16 or B16-H cells were trypsinized, washed with PBS buffer, resuspended in serum-free medium and injected into the subcutis of the flank of C57BL/6 mice. Animals were injected with 5×10^3 to 5×10^5 B16-H cells. The time of appearance of local tumors was monitored by palpation. Tumors were periodically measured with a caliper, and tumor diameter was calculated as $\sqrt{(width \times length)}$. Animals were sacrificed by cervical dislocation 60 days after tumor inoculation or when they became moribund.

Vaccine antitumor activity in B16-H. The NGcGM3/VSSP vaccine was produced and generously provided by the Center of Molecular Immunology (La Habana, Cuba). Briefly, NGcGM3 was purified from horse red blood cells and hydrophobically conjugated with the outer membrane protein complex from *N. meningitides*, as previously reported (18). The method allowed the gangliosides to be hydrophobically incorporated into VSSP and conferred high solubility on the conjugate. For antitumor experiments, mice were vaccinated with 200 µg of the ganglioside mixed with an equal volume of the immunological adjuvant Montanide ISA 51 (Seppic, Paris, France). Vaccines were administered *s.c.* four times at 14-day intervals. Control animals received saline solution only. One week after the third dose, animals were challenged with 5×10^3 or 2×10^4 B16 or B16-H cells in 200 µl of DMEM-F12 (day 0) and tumor growth was monitored as described above (Figure 1).

Indirect Immunofluorescence assay. A total of 1×10^5 cells/well were cultured for 48 h on coverslides (Fisher Scientific, Pittsburgh, PA, USA) in DMEM-F12, supplemented with 10% FCS. After washing with PBS buffer, cells were fixed with a 3% paraformaldehyde in PBS solution for 15 min at room temperature. Subsequently, cells were incubated with 500 µl of a 0.1% BSA in PBS solution and stained with the 14F7 mAb at a concentration of 400 µg/ml for 1 h at 37˚C. Afterwards, bound antibody was detected by incubation with a 1:400 dilution fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Caltag, Burlingame, CA, USA). Mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc, Burlingame, CA, USA) was used to contrast nuclei.

Statistical analysis. Differences in adhesion capacity of B16 and B16-H cells were measure by a two-way ANOVA test contrasted with a Bonferroni test. Comparison of the proliferative capacity was carried out by means of the Student's *t*-test. Differences in vaccine antitumor activities in B16 or B16-H cells were analysed using Fisher's exact test. Statistical analyses were carried out using the GraphPad Prism version 3.0 (GraphPad Software, La Jolla, CA, USA).

Results

Transfection of Cmah sequence increased the presence of membrane NGcGM3 in B16 melanoma cells. The isolation of the *Cmah* mRNA sequence was conducted by RT-PCR using *Cmah*-specific self-designed primers from healthy mouse liver. Three different molecular weight bands ranging between 1600 and 2000 bp were obtained. The sequencing of these products confirmed that the middle band of around 1800 bp had an identical nucleotide sequence as the reported *Cmah* mRNA gene (GenBank, Gene ID: 12763). The other two amplicons were probably normal alternative splicings of the gene. The 1800-bp band was cloned and transfected into B16 melanoma cells in order to obtain mouse tumor cells with positive Cmah activity. *Cmah*-transfected cells (designated B16-H) were cultured with DMEM plus G418 and the expression of *Cmah* was tested by RT-PCR using specific primers as shown in Figure 2A. Afterwards, to assess the activity of the Cmah enzyme we evaluated NGcGM3 presence in transfected and non-transfected cells. Using immunofluorescence with an anti-NGcGM3 mAb, we observed that this ganglioside was overexpressed in B16-H cells as can be seen in Figure 2B. Interestingly, results obtained by flow cytometric analysis showed the presence of NAcGM3, a well-known ganglioside highly expressed in B16 cells, was down-regulated in transfected B16-H cells decreasing from more than 90% to 59%. On the other hand, NGcGM3 expression was dramatically increased in B16-H cells, from less than 3% to 30% (Figure 2C). These results show that the presence of Cmah in B16-H cells alters the neuraminic acid synthesis pathway, leading to an increase in the *N*-glycolylneuraminic acid form.

Expression of NGcGM3 in B16-H cells modified the in vitro and in vivo tumor cell behavior. We investigated whether the presence of NGcGM3 has an effect on cell growth, attachment and motility. Interestingly, a significant increase of nearly 50% in *in vitro* proliferation of B16-H cells was observed when compared to that of parental B16 cells (Figure 3A). Moreover, a similar increase in B16-H cells was observed when *in vitro* cell adhesion was evaluated (Figure 3B). In the cell migration assay, we did not observe any difference between B16 and

Table I. *Effect of NGcGM3/VSSP vaccine on tumor formation using B16-H cells. B16-H cells were injected into the subcutis of the right flank of C57BL6 mice 7 days after the third immunization with NGcGM3/VSSP. Tumor incidence is expressed as positive/total animals at 60 days after tumor cell challenge. *p=0.005 vs. control mice challenged with 2×10^4 cells, Fisher's exact test.*

	Tumor incidence	
Challenge (no. of cells)	Control	NGcGM3/VSSP
5×10^3	2/5 (40%)	1/11 (9%)
2×10^4	4/5 (80%)	0/9 (0%)*

Figure 1. *NGcGM3/VSSP vaccine immunization scheme, as described in the Materials and Methods.*

B16-H cells in the quantification of the invaded area (Figure 3C). However, a clear difference in cell contact and spreading was observed. Parental B16 cells grew in monolayer showing very poor cell-to-cell contact, while B16-H cells grew as crowded cells with scant space between them (Figure 3D).

Stable transfection of *Cmah* into B16 cells also altered their *in vivo* behavior. Growing in syngeneic C57BL/6 mice, parental B16 cells developed palpable melanoma tumors when challenged with 5×10^3 cells, with a tumor incidence of 80%, as reported previously (6). This high tumor incidence was achieved by B16-H cells when mice were challenged with 2×10^4 cells (Table I). As shown in Figure 4, B16-H cells were able to develop melanoma tumors in the syngeneic host. However, these transfected cells were less tumorigenic since a higher tumor cell burden was needed to obtain a similar tumor incidence in comparison with parental B16 cells.

NGcGM3/VSSP vaccine had antitumor activity towards B16-H tumor model in vivo. As far as we know, there is no reported mouse melanoma model with positive expression of NGcGM3. In order to evaluate the potential use of the B16-H tumor model in the assessment of the antitumor activity of NGcGM3/VSSP vaccine, we challenged syngeneic mice treated with an immunization protocol of four bi-weekly doses with 5×10^3 or 2×10^4 B16-H cells. We previously reported that the NGcGM3/VSSP vaccine had no antitumor effects when mice were challenged with B16 cells (19). As shown in Table I, the present results showed that this vaccine exerted significant antitumor activity in mice challenged with B16-H cells, demonstrating the pivotal role of NGcGM3 as the vaccine target in tumor cells.

NGcGM3/VSSP antitumor activity was dependent on NGcGM3 expression on tumor cells. We observed that NGcGM3 expression was reduced in B16-H cells during long-term *in vitro* culture (Figure 5). As a result, after approximately 30 *in vitro* passages, NGcGM3 expression had diminished to nearly half the level of newly transfected cells.
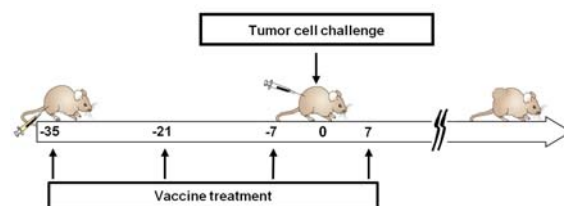
We tested the antitumor activity of the NGcGM3/VSSP vaccine in mice challenged with B16-H cells with more than 30 *in vitro* passages and we observed that the vaccine was indeed ineffective against B16-H cells having a poor expression of NGcGM3, showing no differences compared with the control group (Figure 6). These results support the idea that antitumor protection by NGcGM3/VSSP vaccine strongly depends on NGcGM3 expression in tumor cells.

Discussion

Gangliosides have been identified as tumor antigens in several types of human cancer, including melanoma and lung cancer (20-23). Malignant transformation is often associated with changes in several glycan structures, including alterations in ganglioside expression. Actually, a number of gangliosides, such as GM2, GD3 and NGcGM3 are considered attractive targets for cancer immunotherapy (24-26). It is reported that they have a relevant role in tumor progression and metastasis (27), they are also powerful stimulators of *in vivo* tumor growth (28), and modulate multiple events of the immune response (29-31).

NGcGM3 is an attractive target for cancer vaccines since it is expressed in human tumor cells but not in normal tissue (32, 33). However, a different expression profile is observed in other mammal species, including mice. We have already reported the null expression of the Cmah enzyme in the B16 melanoma cell line and the observation that the presence of *N*-glycolylated gangliosides in these cells were almost absent (14). In attempts to develop NGcGM3-positive mouse cancer cells, we showed that the presence of this glycolipid could be promoted by *in vitro* culture with rich *N*-glycolylneuraminic acid bovine mucin or purified *N*-glycolylneuraminic acid. As a result, the presence of NGcGM3 in the cell membrane promoted *in vitro* cell proliferation and cell adhesion, as well as tumor growth *in vivo* (14). Using this NGcGM3 transient-expression model we evaluated the antitumor activity of NGcGM3/VSSP vaccine but we were unable to find a positive antitumor activity of the NGcGM3/VSSP vaccine, perhaps due to the temporary presence of NGcGM3 in the cells, during the initial few days of tumor growth.
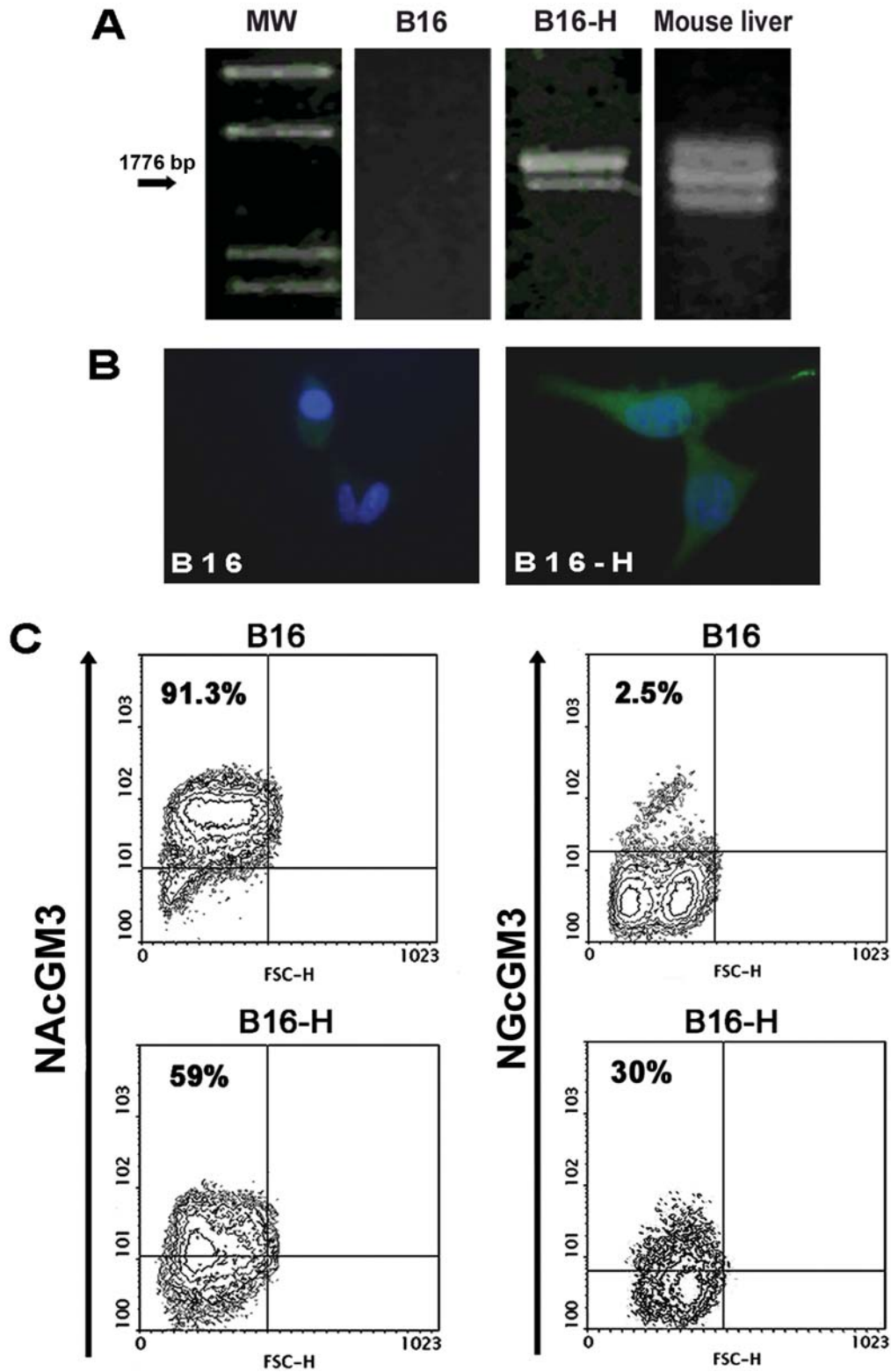
Figure 2. *A: RT-PCR amplification of Cmah mRNA sequence from B16, B16-H and mouse liver. Cmah-negative B16 mice melanoma cells were transfected with Cmah mRNA sequence obtained from mouse liver. Transfection rendered positive Cmah expression in B16-H cells. MW: Molecular weight marker. B: Immunofluorescence with anti-NGcGM3 antibody on B16 and B16-H cells. As a consequence of Cmah transfection, amount of NGcGM3 in cell membrane was increased in B16-H cells. C: Flow cytometric analysis of B16 and B16-H cells labeled with anti-NGcGM3 and anti-NAcGM3. B16-H cells exhibited an increase in NGcGM3 expression at the expense of NAcGM3.*
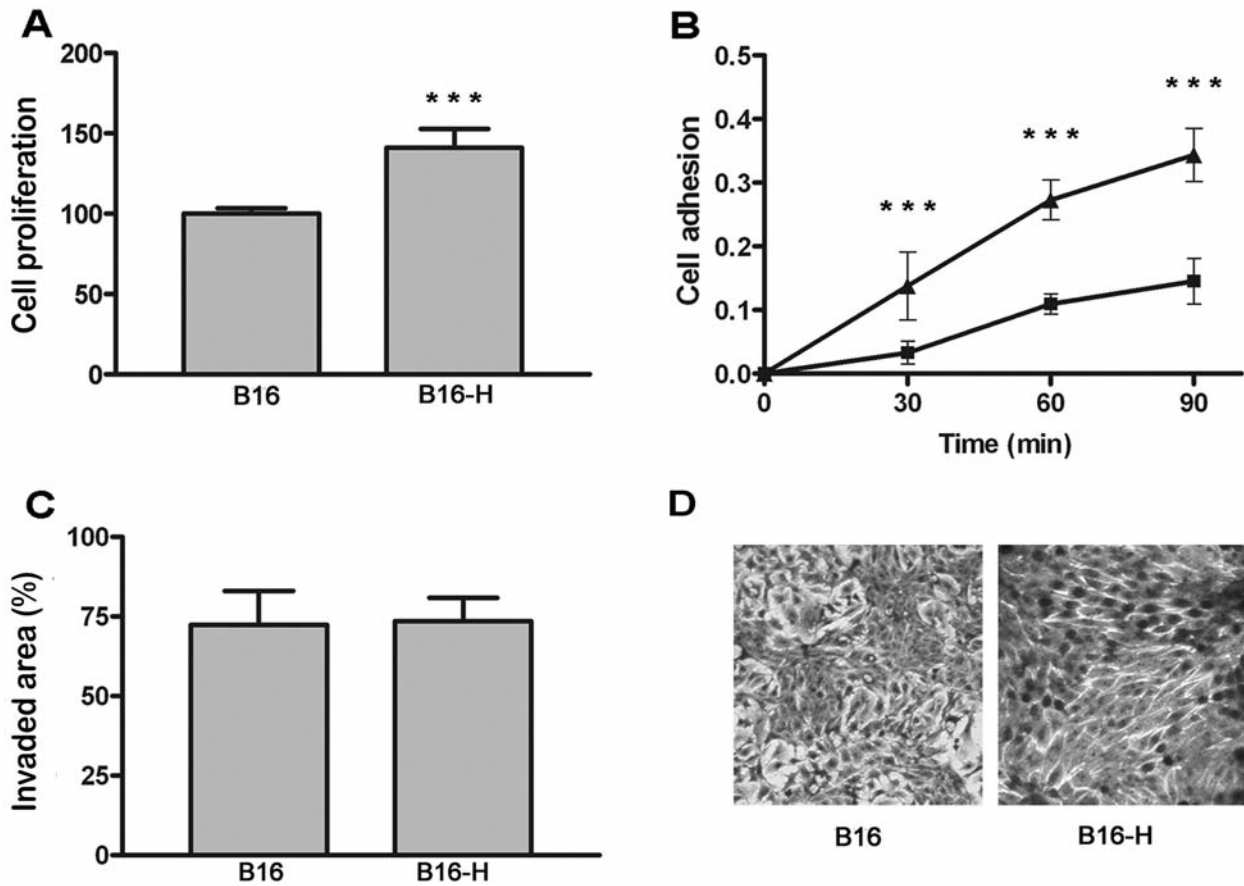
Figure 3. *A: In vitro cell proliferation assay. B16 and B16-H cells were grown for 48 h and stained with toluidine blue solution. The presence of NGcGM3 promoted the proliferation of B16-H cells compared to non-transfected cells ***p<0.0001 (t-test). B: In vitro cell adhesion assay. B16 (squares) and B16-H (triangles) cell adhesion was measured at different times. B16-H cells exhibited an increase in adhesion rate compared to the parental cells ***p<0.0001 (t-test). Results are shown as the mean±SD. C: In vitro cell migration assay. B16 and B16-H cell migration was evaluated by monolayer wound assay, as described in the Materials and Methods. Quantification of cell migration did not show significant differences between cell lines. D: Representative images of B16 and B16-H cells growing in monolayer. The photographs show the different spatial distribution displayed by B16 and B16-H cells. Original magnification, ×1000.*

The participation of NGcGM3 as a relevant target for immunotherapy was initially suggested by experiments conducted with the 3LL-D122 metastatic Lewis lung carcinoma mouse model. When injected subcutaneously in syngeneic mice these cells develop spontaneous metastases in the lung. We observed that NGcGM3 is expressed in metastatic cells but not in primary tumors, perhaps due to the incorporation of the ganglioside during its transit throughout the host vessels (34). Interestingly, NGcGM3/VSSP vaccine treatments in this model inhibited spontaneous lung metastases, but not the growth of subcutaneous primary tumors, suggesting the active participation of NGcGM3 in achieving the adequate antitumor response.

The transfection of the *Cmah* sequence into B16 cells allowed us to answer the question regarding the role of NGcGM3 in the antitumor activity of the NGcGM3/VSSP

vaccine and also to explore the consequences of the expression of this ganglioside in cell biology.

As mentioned, we amplified the sequence of the mRNA of *Cmah* by RT-PCR and as a consequence, three bands of different molecular weight were obtained. The genetic sequencing of these bands indicated that the middle one is equal to the complete sequence of the gene mRNA. The remaining two were mRNA variants, without a 46 amino acid fragment, as was reported by Koyama *et al.* (35).

As it is shown in the Results, *Cmah* sequence transfection into B16 cells resulted in a significant increase in NGcGM3 at the expense of the naturally expressed NAcGM3. *N*-Acetylneuraminic acid can be metabolically produced or exogenously incorporated by the cells. All the *N*-acetylneuraminic acid is catalyzed to cytidine monophospho-*N*-acetylneuraminic acid (CMP-NeuAC) by the cytidine
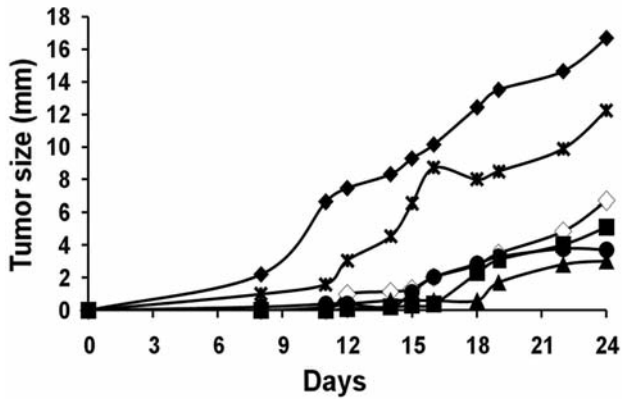
Figure 4. *Subcutaneous tumor growth of B16 and B16-H cells in syngeneic mice. Different groups of C57BL6 mice were inoculated s.c. with 5×10^3 (black circles), 1.5×10^4 (black triangles), 2×10^4 cells (black squares), 5×10^4 (crosses) or 5×10^5 (black rhombus) of B16-H cells. As a comparison 5×10^3 B16 cells (white rhombus) are also shown. No significant differences were observed between tumor growth in mice injected with 5×10^3 B16 and 2×10^4 B16-H cells. (ANOVA contrasted with Bonferroni's multiple comparison test). Results are shown as the mean of five animals per group.*
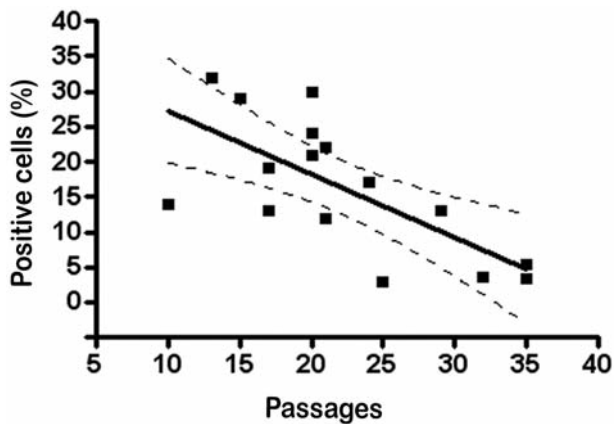

Figure 5. *Expression of NGcGM3 in B16-H cells is related to in vitro culture passage by evaluation of the percentage of NGcGM3-positive cells in B16-H cultures by flow cytometry using anti-NGcGM3 mAb. Cells were maintained in vitro in the presence of a selective antibiotic and subcultured three times a week. p<0.005 (Pearson test).*

Figure 6. *Tumor response after immunization with NGcGM3/VSSP in mice inoculated with NGcGM3-positive or NGcGM3-negative B16-H cells. Different groups of mice inoculated s.c. with 2×10^4 B16-H cells, with more than 30 in vitro culture passages (passage 35) or with newly transfected cells (passage 12), were treated (white squares) or not (black rhombus) with NGcGM3/VSSP vaccine as described in the Materials and Methods. The vaccine had antitumor activity in treated mice challenged with NGcGM3-positive B16-H cells (newly transfected cells). *p<0.005 (Fisher's exact test).*

monophosphate *N*-acetylneuraminic acid synthetase (CMAS) inside the nucleus, and finally transferred to newly synthesized glycoconjugates by one of the sialyl transferases passing through Golgi compartments. The forced presence of Cmah in B16-H cells, promotes the catalysis of CMP-NeuAc into CMP-NeuGc which in turn is transferred to new glycans as NGcGM3 ganglioside, among which it is included (36).

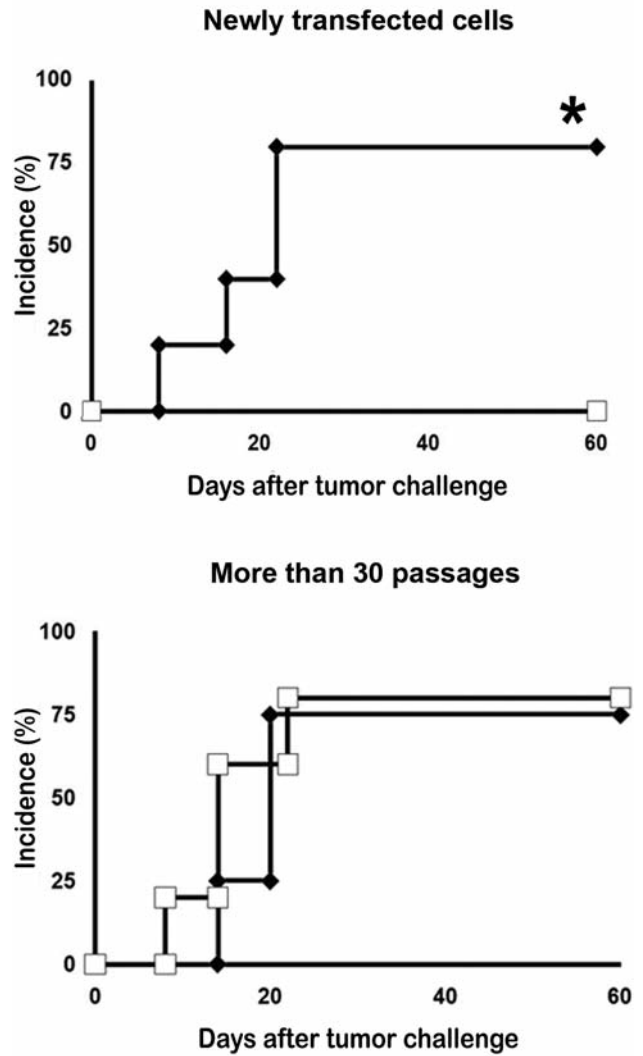Overexpression of NGcGM3 (and/or the underexpression of NAcGM3) in B16 cells results in a modification of the native parental *in vitro* cell behavior. In transfected cells, we observed promotion of *in vitro* cell proliferation and cell adhesion. It is reported that gangliosides participate in cell signalling and act as modulators of cell behavior. Regarding GM3, Prinetti *et al*. recently reported the inhibition of cell motility by the interaction of NAcGM3 with caveolin-1 (37). B16 cells are positive for caveolin-1 expression, thus the modification of the relation between NAcGM3 and NGcGM3 could be part of the explanation of the observed results.

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Although the *in vitro* proliferation rate of B16-H was higher than that of parental cells, they were less tumorigenic when injected into syngeneic mice. A four-fold higher B16-H tumor cell burden was needed to obtain the same tumor incidence, as the one in mice challenged with B16 cells. This observation suggests that rising *N*-glycolylneuraminic acid concentration in the cells could impede tumor organization, or develop a host response against tumor cells. However, it is possible to develop a melanoma tumor by increasing the tumor cell challenge.

We then used this model to evaluate the antitumor activity of NGcGM3/VSSP vaccine, showing a decrease in the tumor incidence compared to the one of B16 cell-challenged animals by the vaccine treatment. No animals developed tumors when vaccinated mice were challenged with 2×10^4 B16-H cells, a burden which induced a tumor incidence of 80% in non-treated animals.

Measuring ganglioside expression in B16-H cells, we observed a reduction in the presence of NGcGM3 in cell membrane during long-term culture, even when cells were grown with G418 in the culture medium. These observations allowed us to evaluate the antitumor activity of NGcGM3/VSSP vaccine in aged B16-H cells (from monolayers with more than 30 *in vitro* passages). Our results confirm the critical role that the expression of NGcGM3 has in the antitumor activity of the NGcGM3/VSSP vaccine, since antitumor activity was found under these conditions, confirming that NGcGM3 is the main target.

The ganglioside NGcGM3 has been described in human neoplasms, including breast carcinoma and melanoma. van Cruijsen *et al*. examined 176 samples of NSCLC by immunohistochemistry in tissue microarrays, and found NGcGM3 to be widely expressed in more than 90% of cases (38). More recently, Scursoni *et al*. reported, for the first time, the expression of NGcGM3 in a pediatric solid tumor. They detected the ganglioside in 88% of cases of Wilms tumor (nephroblastoma) (39), in 81% of cases of neuroblastoma and in 100% of the Ewing sarcoma family of tumors (40) using the specific anti-NGcGM3 mAb 14F7.

In this work, we demonstrated that the presence of NGcGM3 in the cell membrane is a critical factor to ensure the antitumor activity of the NGcGM3/VSSP vaccine, establishing a clear relationship between the vaccine and its target molecule. These results highlight the importance of target expression assessment in tumor samples as biomarker, for prospective selection of patients able to respond to therapeutic cancer vaccines.

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