Expert Opinion

Direct validation of NGcGM3 ganglioside as a new target for cancer immunotherapy

Mayrel Labrada†, Marilyn Clavell, Yanín Bebelagua, Joel de León, Daniel F Alonso, Mariano R Gabri, Roberto C Veloso, Vicente Vérez & Luis E Fernández
Center of Molecular Immunology, Vaccine Department, 216 St and 15th Ave., Atabey, Playa, P.O. Box 16040, Havana, 11600, Cuba

Objective: The target concept means not only an aberrant expression of a particular molecule in tumour tissues but also evidence of a clear therapeutic advantage, as a consequence of immune-intervention, in an antigen-positive relevant tumour model. Since we reported the presence of NGcGM3 ganglioside in human breast tumours years ago and though Phase I clinical trials of a ganglioside containing vaccine have been conducted, a definitive direct validation of this peculiar molecule as target for cancer immunotherapy has remained unperformed. *Methods*: Two animal models were used: leghorn chickens and C57BL/6 mice. The murine 3LL-D122 cell line, the derived subcutaneous tumours and metastatic lung lesions were processed for gangliosides identification. Active immunotherapy experiments in the 3LL-D122 spontaneous lung metastasis model were performed with NGcGM3/VSSP vaccine prepared by conjugation of NGcGM3 with the outer membrane proteins of *Neisseria meningitides*. *Results*: The 3LL-D122 Lewis lung carcinoma results were consistent with an increased expression of NGcGM3 from primary tumours to metastatic lesions, as observed in human breast cancer samples. Both vaccines, prepared with synthetic or natural-source-derived ganglioside, showed similar anti-tumour and immunogenicity profiles. Finally, a clear involvement of $NK1.1^+$ cells and $CD8^+$ T cells in the anti-metastatic effect elicited by the vaccine was manifested. *Conclusions*: While 'proof of concept' Phase II and III clinical trials with the NGcGM3/VSSP vaccine in cancer patients are currently ongoing these results reasonably sustain the validation of this peculiar ganglioside as a novel target for cancer immunotherapy.

Keywords: cancer immunotherapy, ganglioside, NGcGM3, VSSP

Expert Opin. Biol. Ther. (2010) ***10****(2):153-162*

1. Introduction

Since the discovery that the immune system could be manipulated to destroy tumours in cancer patients [1] and the identification of tumour antigens, it has been possible to direct a specific attack to tumours, involving antibodies or cytotoxic effector cells [2]. Moreover, the validation of cancer immune-surveillance hypothesis confirmed that Antigen Presenting Cells (APC) recognize transformed cells and induce the activation of other components of the immune system [3,4]. The cancer vaccines concept, which arose because of this knowledge, implies the achievement of robust antigen specific immune responses in tumour-bearing hosts, causing a significant antitumor response.

Recently a prioritized list of cancer vaccine target antigens, using well-vetted criteria generated by expert panels was described [5]. From the selected nine basic properties, four of them (i.e., therapeutic function, immunogenicity, specificity and oncogenicity) accounted for 80% of the relative weight. While selectivity of immune

informa
healthcare

10.1517/14712590903443084 © 2010 Informa UK Ltd ISSN 1471-2598

effectors' action is one of the major attractions of active specific immunotherapy (ASI), the chosen target might be present almost exclusively in tumour tissues. Probably the best example of such an specific target is a B cell lymphoma idiotype [6,7].

As mentioned before another important feature in target antigen validation is the therapeutic function. For this purpose, the availability of a proper animal model, though not always available for each type of antigen [6,8] is especially helpful, bearing in mind the time-consuming process associated with advanced stage clinical trials.

Gangliosides are another interesting type of molecules, different from proteins, that have been identified as tumour antigens [9]. These molecules are the most variable group of glycosphingolipids present on the plasma membrane of mammalian cells [10-13]. Gangliosides have been considered attractive targets for cancer immunotherapy based on their higher abundance in tumours when compared with the corresponding normal tissues [14-17]. They have an important role in tumour progression and metastization events [18,19]. Gangliosides are also powerful stimulators of *in vivo* tumour growth [20] and they impair multiple events of the immune response, operating as soluble factors in the immunosuppression induced by tumors [21-23]. They are composed by a ceramide portion, followed by at least two monosaccharide units, linked to one or more sialic acid (SA) residues. The most commonly found SA in most animal species is *N*-acetylneuraminic acid (NAcNeu), but also found, less commonly is *N*-glycolylneuraminic acid (NGcNeu) [24,25]. The synthesis of the NGcNeu acid is carried out by the CMP-*N*-acetylhydroxylase enzyme, which use the NAcNeu acid as a precursor [26]. Humans have a partial depletion in the gene that encodes this enzyme [27,28], which causes the loss of the NGcNeu SA in normal human tissues. However expression of *N*-glycolylated gangliosides has been observed in human cancers [14,29]. Of particular relevance is the report of increased amounts of NGcGM3 ganglioside in human breast tumours [30,31] and melanoma [32]. NGcGM3 has also been implicated in various immunological events. In particular, it was recently reported that NGcGM3 ganglioside impairs dendritic cell differentiation and maturation [33]. Additionally de León and colleagues also reported the impairment of T helper cells by NGcGM3 ganglioside [34].

Phase I clinical trials in cancer patients with a NGcGM3-containing vaccine have been conducted [35] and Phase II and III clinical trials are currently ongoing, but a definitive direct validation of NGcGM3 as target for cancer immunotherapy has remained unperformed.

Herein we describe the immunogenicity and the anti-metastatic effect of an experimental NGcGM3-containing vaccine in a relevant spontaneous lung metastasis murine model. This vaccine consisted in very small sized proteoliposomes (VSSP) obtained by the incorporation of NGcGM3 into the outer membrane protein (OMP) complex of *Neisseria meningitides* [36]. After surgery, mouse lungs significantly increased their weight due to the development of detectable spontaneous metastasis [37]. A particular attraction of this murine metastases model is related to the fact that it resembles what is happening in most human cancers, in which recurrence of metastatic disease occurs after removal of primary tumours by surgery. Finally, a clear involvement of effectors cells of the innate and acquired immune system in the anti-metastatic effect elicited by the vaccine was also manifested.

2. Materials and methods

2.1 Animals and tumour cell lines

Two animal models were used: white leghorn chickens, 10 – 12 weeks-old, maintained at the National Centre for Agricultural Health (CENSA; Havana, Cuba); and C57BL/6 female mice, 8 – 12 weeks-old, purchased from the Centre for Laboratory Animal Production (CENPALAB; Havana, Cuba). Chickens were maintained at the animal house facility of CENSA and mice at the Centre of Molecular Immunology (CIM; Havana, Cuba). All the animals were treated according to the Cuban National Laboratory Animal Use Guidelines.

The murine 3LL-D122 cell line [37] of the Lewis lung carcinoma, of C57BL/6 origin, and the P3X63 myeloma cell line used as control for NGcGM3-positive cells [38], were grown in DMEM-F12 (Gibco) supplemented with 10% fetal calf serum (FCS) (Hyclone), 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 µg/ml; Life Technologies).

2.2 NGcGM3 ganglioside and NGcGM3/VSSP vaccine

NGcGM3 ganglioside was obtained from a natural source (NAg) [36] or by synthesis (SAg) by the methods described by Duclos and Sherman *et al.* [39,40]. Purity was monitored by High Performance Thin Layer Chromatography (HPTLC) [36].

NGcGM3/VSSP vaccine was prepared by hydrophobic conjugation of the NGcGM3 ganglioside with the OMP complex of *N. meningitides*, as described by Estevez *et al.* [36], either with NAg (NAg/VSSP) or SAg (SAg/VSSP). Briefly, NGcGM3 and OMP were dissolved in 0.1 M Tris-HCl buffer (pH 8.5), containing sodium deoxycholate and SDS, and then dialysed to remove detergents. This procedure allowed ganglioside and proteins to hydrophobically incorporate into VSSP and conferred high solubility to the conjugate. All animals received 200 µg of NGcGM3/VSSP per dose.

Chickens received SAg/VSSP or NAg/VSSP, intramuscularly in Montanide ISA 51, on days 0 and 14. Sera were collected on day 21 and then analysed for anti-NGcGM3 IgY antibodies content, by an ELISA technique [36], but using alkaline-phosphatase-conjugated goat antibodies anti-IgY immunoglobulin (Sigma). Mice received two doses of NGcGM3/VSSP subcutaneously (s.c.) at 14-day interval.

2.3 Spontaneous murine tumour model

Mice inoculated with 3LL-D122 clone (2×10^5/mouse) into the right hind footpad, were treated twice with

NGcGM3/VSSP s.c. 7 and 21 days after tumour implantation. Tumours were measured with a caliper. Primary tumours were surgically removed when tumour diameter reached 8 – 9 mm. Mice were killed 21 days after tumour amputation and the spontaneous lung metastases quantified by weighing the lungs. The control group received PBS.

2.4 Analysis of tumour gangliosides

Cultured 3LL-D122 cells were growth to obtain 10^8 cells, detached from the culture surface by 5-min incubation in trypsin/EDTA (0.1% and 1mM) solution, pelleted and processed for ganglioside extraction.

To develop subcutaneous tumours, 3LL-D122 cells (5×10^5) were grown on flanks of mice. When tumours reached 10 – 15 mm they were removed, weighed and processed for ganglioside extraction.

To obtain lung metastatic lesion, 3LL-D122 cells (2×10^5) were injected into the right hind footpad of mice, when tumour diameter reached 8 – 9 mm, tumours were surgically removed. At 21 days after tumour amputation metastatic lesions were carefully dissected from metastatic lungs, weighed and processed for ganglioside extraction.

Gangliosides from described tumour samples were homogenized and lipids were extracted in chloroform:methanol: water (4:8:3). These extracts were centrifuged (1000*g*, 15 min) and the supernatant recovered was evaporated and dissolved in chloroform/methanol (1:1) for overnight incubation at 4°C. After centrifugation the supernatant recovered was evaporated and the dry samples dissolved in chloroform/methanol/water (30:60:8) to be applied to DEAE-Sephadex A-25 column. The acidic lipid fraction was eluted with 0.02 M sodium acetate in methanol. Monosialo gangliosides contained in these fractions were visualized by spraying orcinol reagent and heating at 100°C for 10 min, after separation on HPTLC plates. The HPTLC images were captured with a calibrated Powerlook III prepress colour scanner (Amersham Biosciences). The percentage of NGcGM3 ganglioside in the whole mono-sialio ganglioside obtained was calculated by densitometric analysis, with the TotalLab120 program (Nonlinear Dynamics).

2.5 Detection of NGcGM3 by mass spectrometry

Matrix-associated laser desorption ionization mass spectrometry (MALDI/MS) was carried out on a Shimadzu Biotech Axima Performance time-of-flight mass spectrometer. Gentisic acid (2,5-dihydroxybenzoic acid; 10 mM in water) was purchased from Sigma and used as matrix. Samples (5 μg/μl) were deposited on the target, covered with 1 μl of the matrix in aqueous solution and dried. Analyte ions were desorbed from the matrix with pulses from a 337 nm nitrogen laser. Spectra were obtained in the reflectron positive ion mode with an average of 128 pulses, the ion gate was fixed in 1000 – 1500 Da. The masses are average masses.

2.6 Detection of NGcGM3 by immunochemistry

Immunostaining of HPTLC plates was performed according to the protocol described by Saito *et al.* with minor modifications [41]. Briefly, ganglioside samples were separated on HPTLC, plate soaked in a 0.1% solution of polyisobutylmethacrylate (Aldrich Chemical) in hexane for 75s and then allowed to air dry. Plates blocked in PBS buffer containing 1% BSA (Sigma) were incubated at 4°C (overnight) with 10 μg/ml of 14F7mAb, that specifically recognize NGcGM3 ganglioside [32]. After washing, alkaline-phosphatase-conjugated goat anti-mouse IgG antibodies (Jackson) were added. Spots were visualized with a 1 mg/ml solution of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M glycine buffer, (pH 10.4). The reaction was stopped by washing with water.

For immunohistochemical detection of NGcGM3 ganglioside in 3LL-D122 subcutaneous tumour and lung metastasis, the Vectastain ABC kit (Vector Laboratories) was employed according to the instructions of the manufacturer. Formalin-fixed 3LL-D122 subcutaneous tumour or lung specimens were processed using the usual paraffin technique, as previously reported [42]. Tumour sections (5 μm) were incubated for 1 h with the 14F7 mAb (20 μg/ml in PBS). Slides were then incubated with biotinylated horse anti-mouse IgG for 30 min, followed by peroxidase-conjugated avidin–biotin complex for 30 min. Bound antibodies were detected by incubation with diaminobenzidine substrate and tumour sections were then counterstained with haematoxylin. Pertinent specificity tests were performed, including blocking of endogenous peroxidase, omission of the first antibody and utilization of unrelated mAb.

2.7 Flow cytometry analysis

Cultured P3X63 cells were diluted at 1×10^6 cells per sample and labeled with sera of chickens (diluted 1/10) immunized with SAg/VSSP or NAg/VSSP in Montanide ISA 51 in FACS buffer (0.1% BSA and 0.1% NaN3 in PBS). Afterwards, biotin-conjugated goat antibodies anti-IgY immunoglobulin (Sigma) and FITC-Streptavidin (Jackson) were added.

Cultured 3LL-D122 cells were diluted at 2×10^5 cells per sample. Cells were labeled with 1 μg of 14F7 mAb or a control monoclonal antibody of the same isotype (IgG) in FACS buffer for 15 min at 4°C. Afterwards FITC-conjugated goat antibodies (anti-IgG immunoglobulin; BD Pharmingen) was added. Cells were analysed with a FACS can flow cytometer (Becton Dickinson & Company).

2.8 *In vivo* cell depletion study

mAbs to CD8 and NK1.1 were purified from the culture supernatants of the YTS 169 (anti-CD8, ECACC) and PK136 (anti-NK1.1, ATCC) hybridomas by ammonium sulphate precipitation (anti-CD8) and affinity chromatography (anti-NK1.1). *In vivo* depletion was done by intraperitoneal injection with 1 mg of anti-CD8 mAb or 0.2 mg of anti-NK1.1 mAb, one day after each vaccine immunization. These doses have been previously shown to deplete more than 95% of corresponding cell subset.

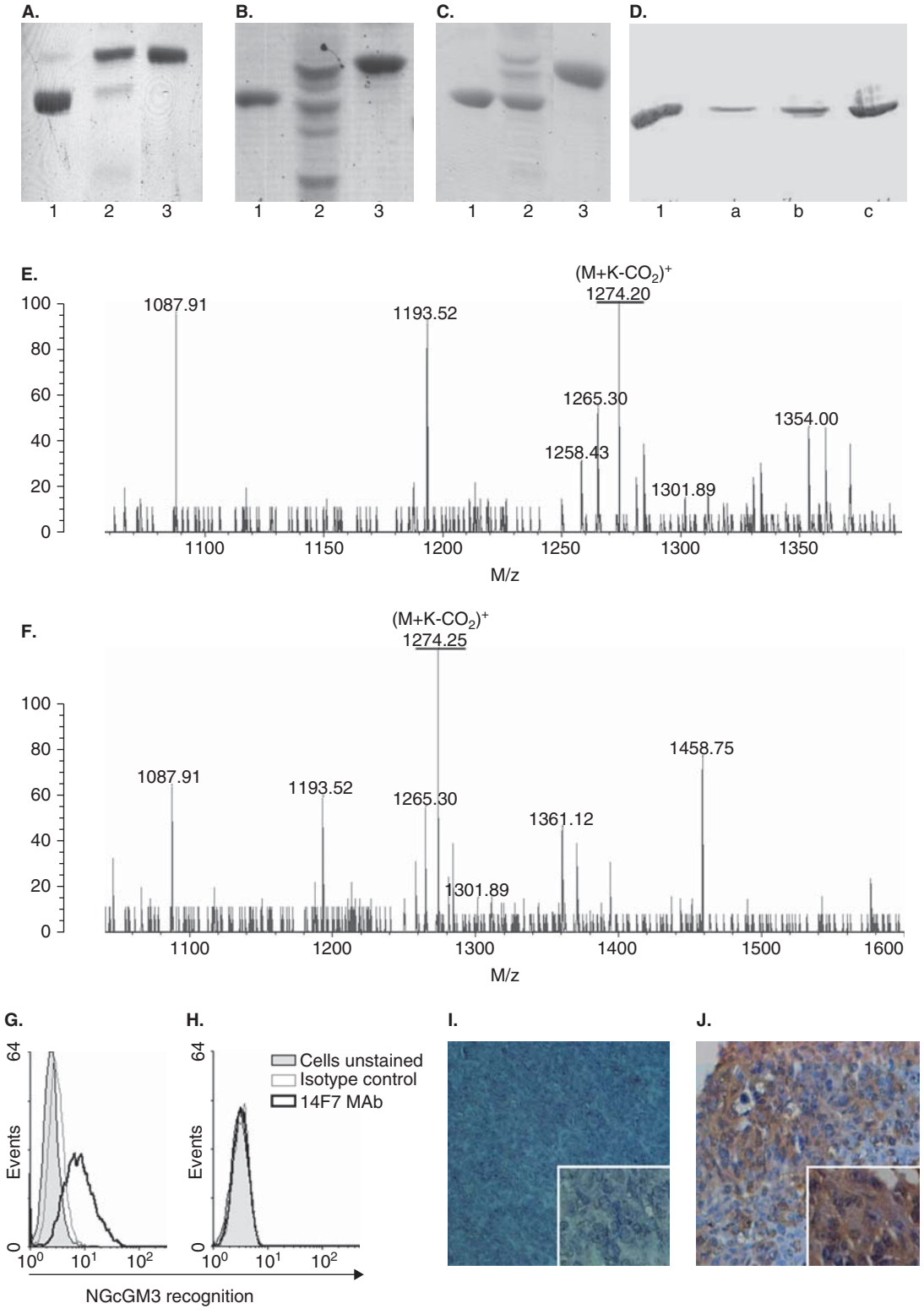

Figure 1. NGcGM3 ganglioside on the spontaneous metastasis model of 3LL-D122 lung carcinoma. (Continued).

2.9 Statistical analyses

Mann Whitney U test for paired comparison of values or One-way ANOVA, combined with Tukey's Test, for multiple comparisons were employed. Differences were considered significant if $p < 0.05$ (* in the figures).

3. Results

3.1 An increased expression of NGcGM3 ganglioside in metastasis compared with primary tumours of the 3LL-D122 Lewis lung carcinoma

Considering the relevance of the model of spontaneous lung metastasis 3LL-D122 Lewis lung carcinoma and the fact that the expression of NGcGM3 in this tumour type has not been previously reported, we decided to evaluate the presence of this molecule on tumours and metastatic samples.

3LL-D122 cells, either cultured *in vitro,* derived from subcutaneous tumours or from metastatic lung lesions were processed according to certain optimized chemical extraction and purification protocols for monosialogangliosides.

The corresponding isolated mixtures of this type of gangliosides were analysed first by HPTLC, (**Figure 1A – C**). The presence of NGcGM3 in the monosialogangliosides fraction of the three different tumour derived materials was apparent considering the Rf values relative to the ganglioside standard. The major component of the ganglioside fraction derived from the cell line in culture was NAcGM3, representing 87.4% of the total lipid-bound sialic acid while its glycolylated variant accounted for the 12.6%. On the other hand in primary tumours the difference in expression between NAcGM3 (56.1%) and NGcGM3 (32.2%) was less significant. Strikingly, in metastatic lesions the situation was totally inverted with NGcGM3 accounting for 73.4% of total lipid bound sialic acid while NAcGM3 accounts for just 11.2%. These data might support a malignancy marker character for NGcGM3 expression in this experimental model.

Further validation of the presence of NGcGM3 in these tumour cell samples was achieved by TLC-ELISA and MALDI-TOF mass spectrometry. Immunostaining experiments with 14F7 mAb confirmed the presence of this sialyl-lactosyl ceramide in the monosialo ganglioside fraction isolated from 3LL-D122 cells, either cultured *in vitro,* derived from subcutaneous tumours or from metastatic lung lesions (see **Figure 1D**).

Similarly, mass spectrometric analysis evidenced the typical decarboxylation process (m/z 1274) often seen as part of this molecules ionization process, before suffering the complete loss of the sialic acid moiety, in the corresponding spectra belonging to the mixtures from subcutaneous tumours (**Figure 1E**) and metastasis (**Figure 1F**).

Once the presence the chemical entity in the lipid extracts of the different cell samples was unequivocally established we wondered if in the intact biological material the ganglioside could be detected as well. First, the 3LL-D122 tumour cell line was analysed by FACS with the 14F7 mAb. While a marked staining of the NGcGM3-positive P3X63 myeloma cells was observed in the experiment (**Figure 1G**) an almost absent recognition of the carcinoma cell line by the specific antibody was apparent (**Figure 1H**), confirming the previous chemical results.

Secondly, a comparison of the expression pattern of NGcGM3 between primary tumours and metastatic lesions was conducted by immunohistochemistry. Again 14F7 mAb revealed its notable specificity, reducing the background even when a sensitive immunoperoxidase assay was employed. Consistently with the HPTLC results, primary tumours displayed a distinct and less intensive staining for NGcGM3 (**Figure 1I**) than the corresponding lung metastatic lesions (**Figure 1J**). In this case a heterogeneous pattern of immunoreactivity was observed with patches of lung carcinoma cells showing a diffuse cytoplasmatic staining.

3.2 Similar immunological profiles of vaccines formulated either with the ganglioside isolated from horse erythrocytes or fully synthetic

As part of the validation process of NGcGM3 as target we reasoned that the gangliosides coming from two different sources should be similar in antigenicity and also that a vaccine, prepared with the two molecules, should be equally immunogenic. NGcGM3, either purified from a natural source (NAg) or that one chemically synthesized (SAg) reacted identically with 14F7 mAb in a TLC-ELISA experiment (**Figure 2A**,**B**), confirming the similar antigenicity of both N-glycolylated variants of GM3 ganglioside.

The immunogenicity profiles of SAg and NAg were assessed through the incorporation of these antigens into the outer membrane protein complex of *N. meningitides,* obtaining the nanoparticulated vehicle named VSSP (very small size

Figure 1. NGcGM3 ganglioside on the spontaneous metastasis model of 3LL-D122 lung carcinoma. (Continued). Chemical ganglioside detection by orcinol reagent on **A.** 3LL-D122 tumour cell line; **B.** 3LL-D122 primary tumours and **C.** Metastatic lesions, lane 1: standard NGcGM3; lane 2: gangliosides from each tumour stage; lane 3: standard NAcGM3. **D.** HPTLC immunostaining using 14F7 Mab, a: tumour cell line, b: primary tumours, c: metastatic lesions. **E.** MALDI-TOF mass spectra of ganglioside samples from primary tumors and **F.** metastatic lesions obtained in the reflectron positive ion mode with an average of 128 pulses, the ion gate was fixed in 1000 – 1500 Da. The masses are average masses. **G.** NGcGM3 detection on P3X63 myeloma cell line and **H.** on 3LL-D122 tumour cell line, by FACS using 14F7 mAb. Detection of NGcGM3 in sections of **I.** 3LL-D122 primary tumours and **J.** metastatic lesions, by means of immunohistochemistry with 14F7 mAb, original magnification × 400, insets × 1000.

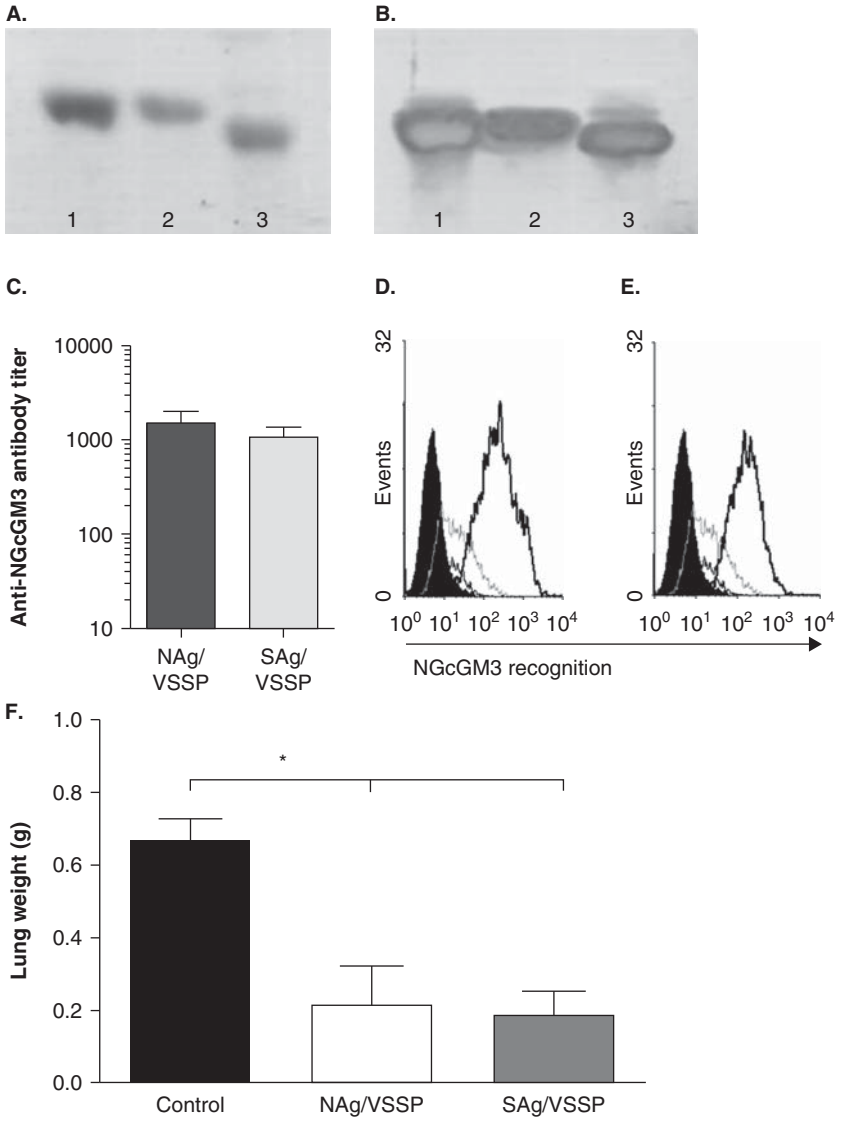

Figure 2. Purity and identity of NGcGM3 ganglioside. A. HPTLC stained with orcinol reagent, **B.** HPTLC immune staining with 14F7 mAb, lane 1: standard NGcGM3, lane 2: natural NGcGM3, lane 3: synthetic NGcGM3. **C.** IgY response against NGcGM3 in chickens immunized with NAg Vac or SAg Vac in Montanide ISA 51. Each bar represents the mean of 10 animals and the error bars represent the standard error of the mean. **D.** P3X63 cells recognized by sera of chicken immunized with NAg Vac or **E.** SAg Vac in Montanide ISA 51. Solid black line: P3X63 cells unstained, open grey line: preimmune sera, open black bold line: immune sera, open black line: unrelated antibody control. Each arrangement of histograms is representative of ten animals per group. **F.** Anti-metastatic effect of NGcGM3-containing vaccine on spontaneous lung metastases murine model, C57BL/6 mice were inoculated subcutaneously (s.c.) with 3LL-D122 cells (2×10^5/mouse) into the right hind footpad, and treated twice with NAg/VSSP or SAg/VSSP or PBS (control), s.c. 7 and 21 days after tumour inoculation. Both NGcGM3/VSSP-treated groups had significantly fewer pulmonary metastases measured as lung weight, relative to control animals ($p < 0.001$). No significant differences were observed between NAg/VSSP and SAg/VSSP treatments ($p > 0.05$).
*$p < 0.05$.

proteoliposomes). Both vaccine preparations were inoculated in chickens separately and significant levels of IgY antibodies against SAg or NAg ganglioside were detected (**Figure 2C**) in sera of injected animals. Mean anti-NGcGM3 antibody titers detected in immunized chickens were 1280 irrespective of which antigen was used in the vaccine ($p > 0.05$, unpaired t test), indicating that both ganglioside vaccines similarly stimulated the immune system. In addition an almost identical fine specificity of both specific anti-sera in recognizing NGcGM3, normally expressed in the external membranes of P3X63 myeloma cells, was manifested in a FACS experiment (**Figure 2D,E**).

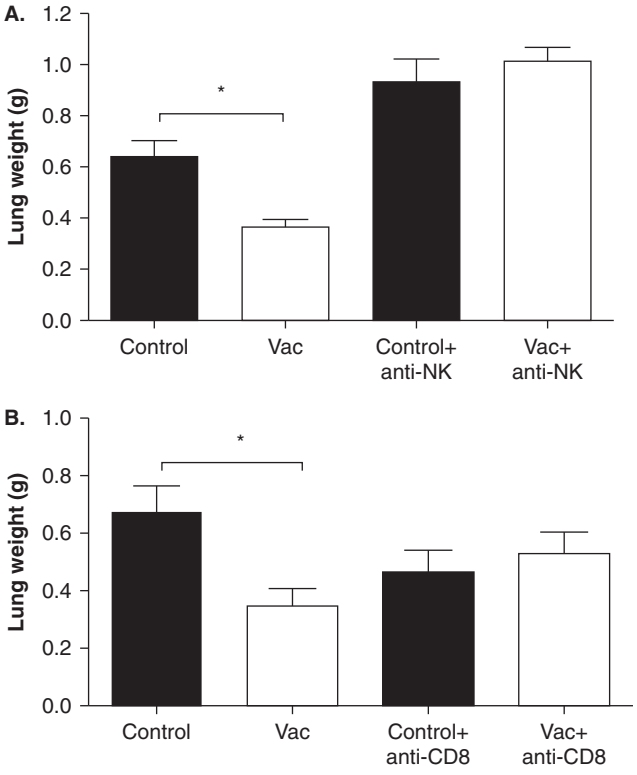

Figure 3. C57BL/6 mice inoculated with 3LL-D122 cells (2 × 10^5/mouse) into the right hind footpad, and treated twice with NGcGM3/VSSP, s.c. 7 and 21 days after tumour inoculation were treated with antibodies that depleted immune effectors populations. A. Effect of depleting NK1.1$^+$ cells by injecting anti-NK1.1 mAb, one day after each vaccine immunization, control groups received PBS or PBS+anti-NK1.1 mAb. The injection of anti-NK1.1 mAb eliminated the effect of the vaccine. **B.** Exhaustion of CD8$^+$ T cells was done by injecting anti-CD8 mAb, one day after each vaccine immunization, control groups received PBS or PBS+anti-CD8 mAb. The administration of anti-CD8 mAb abolished the effect of the vaccine. Column bars represent media values and error bars correspond to standard deviation. The *p* value was generated with ANOVA and Tuckey's multiple comparison tests. Data are representative of two independent experiments.
*$p < 0.05$.

3.3 The NGcGM3/VSSP vaccine inhibits spontaneous lung metastasis of the 3LL-D122 Lewis lung carcinoma

Finally, the definitive value of NGcGM3 as target for cancer immunotherapy was tested in experiments on active immunotherapy in the spontaneous lung metastasis model described before. Both NGcGM3/VSSP vaccines (formulated with NAg or SAg) were administered after tumour cells' implantation. In mice injected with the two different vaccine preparations a reduced amount of metastasis was detected in the lungs, compared with the corresponding control animals just receiving PBS ($p < 0.001$, One-way ANOVA, **Figure 2F**). As expected the weights of the lungs removed from animals vaccinated with the NAg preparation were similar to those coming from mice vaccinated with SAg/VSSP ($p > 0.05$, One-way ANOVA). Additionally, in a similar experiment injecting OMP into mice inoculated with 3LL-D122 Lewis Lung Carcinoma, the OMP did not avoid metastatic colonization in the lungs, while NGcGM3/VSSP did (data not shown).

3.4 Involvement of cellular immune effectors in the anti-metastatic response generated with NGcGM3/VSSP

NK1.1$^+$ cells have been shown to mediate strong anti-metastatic activities in several models of lung metastasis. The role of NK1.1$^+$ cells in the capacity of the NGcGM3 vaccine to avoid metastization of 3LL-D122 tumours implanted in C57BL/6 mice was investigated in experiments in which depletion of these cells was achieved by injecting an anti-NK1.1 mAb, 24h after each vaccine inoculation. The vaccine preparation was administered twice in the spontaneous metastasis model, as described before. Control groups received PBS or PBS plus the anti-NK1.1 mAb. As shown in **Figure 3A** the anti-metastatic effect of the NGcGM3/VSSP vaccine was abolished in mice deficient in NK1.1$^+$ cells, suggesting that these types of innate immunity cells are involved in the pharmacological effect of the vaccine.

On the other hand determining if CD8$^+$ T cells also contributed to the anti-tumour effects evidenced by vaccines produced with NGcGM3 was interesting as well. In the same fashion *in vivo* cell-type specific depletion procedures were conducted in the spontaneous metastasis model described in Materials and methods. C57BL/6 mice bearing intra-foot pad tumours were treated twice with NGcGM3/VSSP as previously described. Depletion of CD8$^+$ T cells was achieved by injection of an anti-CD8 mAb 24h after each vaccine injection. Corresponding control animals received PBS or PBS plus anti-CD8 mAb. After euthanasia the common reduction in metastatic dissemination observed in mice treated with the NGcGM3 vaccine was lost in the group of animals that also received the depleting dose of the anti-CD8 antibody (**Figure 3B**). These results suggested the involvement of CD8$^+$ T cells in the anti-tumour immune response generated by vaccination with the NGcGM3/VSSP preparation.

4. Discussion

In this paper we considered the murine model of spontaneous lung metastasis with the 3LL-D122 Lewis lung carcinoma to study the presence of NGcGM3 ganglioside on tumour samples. This murine spontaneous metastasis model is much related to the disease occurring in cancer patients, who in many cases develop metastatic lesions after surgical treatment. Then we studied the ganglioside content at three stages of tumour development: first the cell line stage, second the primary tumour stage and finally the metastatic stage. The 3LL-D122 Lewis lung carcinoma spontaneous metastasis murine model was consistent with an increased expression of NGcGM3 from primary tumours to metastatic lesions, as observed in human breast cancer samples. Our results are in

accordance with other published studies that associate the metastatic capacity of tumour cells with the gangliosides pattern and/or with changes in sialylation [20,43,44]. Earlier studies in the natural brain tumour of the VM mouse strain, showing analogous results regarding the total ganglioside sialic acid content of the subcutaneously grown tumour (70% of SA was in the form of NGcNeu acid) in relation to cultured VM tumour cells (almost exclusively NAcNeu acid) [45].

The insertion of the synthetic NGcGM3 ganglioside on VSSP vaccine confirmed the value of this target for cancer immunotherapy. Our results proved equivalent immunogenicity profiles against NGcGM3 despite the source of the antigen. Previous reports of immunization of mice with other ganglioside-conjugated vaccines have been successful in the generation of humoral response against these molecules [46-51]. Antibody response against gangliosides is mostly characterized by the IgM isotype, and these have shown to mediate protection in animal models challenged with ganglioside-expressing tumours [52]. We also confirmed the identification of the gangliosides on tumour cells by the sera of vaccinated chickens on positive NGcGM3 myeloma cells.

The therapeutic benefit due to the use of NGcGM3-containing vaccine in tumour-bearing mice was demonstrated in the spontaneous lung metastasis murine model with 3LL-D122. Two therapeutic subcutaneous administration of the vaccine, in the neoadjuvant setting, inhibited spontaneous metastasis on the lungs of treated mice. Additional preclinical experiments into the adjuvant setting will be performed to evaluate the effect of the vaccine after tumour surgical procedure.

Several adjuvants have been used for vaccines based on gangliosides for the treatment of patients with cancer, but in all cases only generation of humoral response have been reported [50,51,53-57]. A Phase I clinical trial in advanced breast cancer patients was performed with NGcGM3/VSSP vaccine. All treated patients developed anti-NGcGM3 antibody titers (IgM and IgG). The hyper-immune sera increased complement-mediated cytotoxicity versus P3X63 myeloma cells, and a marked IgG differential reactivity against human mammary ductal carcinoma samples [35].

The involvement of cytotoxic lymphocyte has not been related to gangliosides response due to its T-independent antigen nature [58]. Our results, in addition to the validation of NGcGM3 as target for cancer immunotherapy, suggest the involvement of cellular immune response in the antitumor effect generated with NGcGM3/VSSP. Further experiments will be decisive to elucidate the nature and the specificity of cell response generated.

Attending to recently studies revealing NGcGM3 ganglioside influences on immune cells functions [33,34], and considering that anti-ganglioside antibodies are capable of eliminating these immunosuppressive molecules from the tumour microenvironment and circulation [59], we could speculate that NGcGM3/VSSP vaccine will reverse the immune suppressor effect of NGcGM3 on dendritic and T helper cells. Moreover, *N. meningitides*-derived OMP vesicles have demonstrated a capacity to stimulate immune system response [60]. Future experiments will be focused on deeply understanding the mechanism of action of the NGcGM3/VSSP vaccine.

Acknowledgments

The authors thank A López and Y González for technical assistance with animal handling, and Y Prieto for helping us with densitometric analysis of HPTLC plates.

Declaration of interest

M Labrada has received the Center of Molecular Immunology (CIM) budget for Research & Development and has no financial conflict of interest. M Clavell, Y Bebelagua, J de León and LE Fernández have received CIM's budget for Research & Development and have no financial conflict of interest.

DF Alonso and MR Gabri have received a grant from the Quilmes National University and have no financial conflict of interest. RC Veloso and V Vérez have received the center of Biomolecular Chemistry budget for R&D and have no financial conflict of interest.

Bibliography

1. Wiemann B, Starnes CO. Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmacol Ther 1994;64(3):529-64
2. Rosenberg SA. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 1999;10(3):281-7
3. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci USA 1998;95(13):7556-61
4. Sahin U, Tureci O, Schmitt H, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 1995;92(25):11810-3
5. Cheever MA, Allison JP, Ferris AS, et al. The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res 2009;15(17):5323-37
6. Bendandi M. Idiotype vaccines for lymphoma: proof-of-principles and clinical trial failures. Nat Rev Cancer 2009;9(9):675-81
7. Inoges S, Rodriguez-Calvillo M, Zabalegui N, et al. Clinical benefit associated with idiotypic vaccination in patients with follicular lymphoma. J Natl Cancer Inst 2006;98(18):1292-301
8. Lollini PL, Cavallo F, Nanni P, Forni G. Vaccines for tumour prevention. Nat Rev Cancer 2006;6(3):204-16
9. Lage A, Perez R, Fernandez LE. Therapeutic cancer vaccines: at midway between immunology and pharmacology. Curr Cancer Drug Targets 2005;5(8):611-27
10. Saito M, Kitamura H, Sugiyama K. Liver gangliosides of various animals ranging from fish to mammalian species. Comp Biochem Physiol B Biochem Mol Biol 2001;129(4):747-58
11. Saito M, Sugiyama K. Major and c-series gangliosides in lenticular tissues: mammals to molluscs. Comp Biochem Physiol B Biochem Mol Biol 2001;130(3):313-21
12. Suzaki K. The pattern of mammalian brain gangliosides. 3. Regional and developmental differences. J Neurochem 1965;12(12):969-79
13. Wang B, Miller JB, McNeil Y, McVeagh P. Sialic acid concentration of brain gangliosides: variation among eight mammalian species. Comp Biochem Physiol A Mol Integr Physiol 1998;119(1):435-9
14. Hakomori S. Tumor malignancy defined by aberrant glycosylation and sphingo (glyco)lipid metabolism. Cancer Res 1996;56(23):5309-18
15. Livingston PO, Hood C, Krug LM, et al. Selection of GM2, fucosyl GM1, globo H and polysialic acid as targets on small cell lung cancers for antibody mediated immunotherapy. Cancer Immunol Immunother 2005;54(10):1018-25
16. Livingston PO, Ragupathi G. Cancer vaccines targeting carbohydrate antigens. Hum Vaccin 2006;2(3):137-43
17. Portoukalian J, Zwingelstein G, Dore JF. Lipid composition of human malignant melanoma tumors at various levels of malignant growth. Eur J Biochem 1979;94(1):19-23
18. Birkle S, Zeng G, Gao L, et al. Role of tumor-associated gangliosides in cancer progression. Biochimie 2003;85(3-4):455-63
19. Deng W, Li R, Ladisch S. Influence of cellular ganglioside depletion on tumor formation. J Natl Cancer Inst 2000;92(11):912-7
20. Alessandri G, Filippeschi S, Sinibaldi P, et al. Influence of gangliosides on primary and metastatic neoplastic growth in human and murine cells. Cancer Res 1987;47(16):4243-7
21. Ladisch S. Tumor cell gangliosides. Adv Pediatr 1987;34:45-58
22. Ladisch S, Kitada S, Hays EF. Gangliosides shed by tumor cells enhance tumor formation in mice. J Clin Invest 1987;79(6):1879-82
23. Ladisch S, Wu ZL, Feig S, et al. Shedding of GD2 ganglioside by human neuroblastoma. Int J Cancer 1987;39(1):73-6
24. Varki A. Selectins and other mammalian sialic acid-binding lectins. Curr Opin Cell Biol 1992;4(2):257-66
25. Varki A. Diversity in the sialic acids. Glycobiology 1992;2(1):25-40
26. Muchmore EA, Milewski M, Varki A, Diaz S. Biosynthesis of N-glycolyneuraminic acid. The primary site of hydroxylation of N-acetylneuraminic acid is the cytosolic sugar nucleotide pool. J Biol Chem 1989;264(34):20216-23
27. Varki A. Loss of N-glycolylneuraminic acid in humans: mechanisms, consequences, and implications for hominid evolution. Am J Phys Anthropol 2001;(Suppl 33):54-69
28. Varki A. N-glycolylneuraminic acid deficiency in humans. Biochimie 2001;83(7):615-22
29. Malykh YN, Schauer R, Shaw L. N-Glycolylneuraminic acid in human tumours. Biochimie 2001;83(7):623-34
30. Marquina G, Waki H, Fernandez LE, et al. Gangliosides expressed in human breast cancer. Cancer Res 1996;56(22):5165-71
31. Oliva JP, Valdes Z, Casaco A, et al. Clinical evidences of GM3 (NeuGc) ganglioside expression in human breast cancer using the 14F7 monoclonal antibody labelled with 99mTc. Breast Cancer Res Treat 2006;96(2):115-21
32. Carr A, Mullet A, Mazorra Z, et al. A mouse IgG1 monoclonal antibody specific for N-glycolyl GM3 ganglioside recognized breast and melanoma tumors. Hybridoma 2000;19(3):241-7
33. de Leon J, Fernandez A, Mesa C, et al. Role of tumour-associated N-glycolylated variant of GM3 ganglioside in cancer progression: effect over CD4 expression on T cells. Cancer Immunol Immunother 2006;55(4):443-50
34. de Leon J, Fernandez A, Clavell M, et al. Differential influence of the tumour-specific non-human sialic acid containing GM3 ganglioside on CD4 +CD25– effector and naturally occurring CD4+CD25+ regulatory T cells function. Int Immunol 2008;20(4):591-600
35. Carr A, Rodriguez E, Arango Mdel C, et al. Immunotherapy of advanced breast cancer with a heterophilic ganglioside

(NeuGcGM3) cancer vaccine. J Clin Oncol 2003;21(6):1015-21

36. Estevez F, Carr A, Solorzano L, et al. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). Vaccine 1999;18(1-2):190-7
37. Eisenbach L, Hollander N, Greenfeld L, et al. The differential expression of H-2K versus H-2D antigens, distinguishing high-metastatic from low-metastatic clones, is correlated with the immunogenic properties of the tumor cells. Int J Cancer 1984;34(4):567-73
38. Muthing J, Steuer H, Peter-Katalinic J, et al. Expression of gangliosides GM3 (NeuAc) and GM3 (NeuGc) in myelomas and hybridomas of mouse, rat, and human origin. J Biochem 1994;116(1):64-73
39. Duclos RI Jr. The total synthesis of ganglioside GM3. Carbohydr Res 2000;328(4):489-507
40. Sherman AA, Yudina ON, Shashkov AS, et al. Preparative route to N-glycolylneuraminic acid phenyl 2-thioglycoside donor and synthesis of Neu5Gc-alpha-(2→3′)-lactosamine 3-aminopropyl glycoside. Carbohydr Res 2002;337(5):451-7
41. Saito M, Kasai N, Yu RK. In situ immunological determination of basic carbohydrate structures of gangliosides on thin-layer plates. Anal Biochem 1985;148(1):54-8
42. Alonso DF, Gabri MR, Guthmann MD, et al. A novel hydrophobized GM3 ganglioside/Neisseria meningitidis outer-membrane-protein complex vaccine induces tumor protection in B16 murine melanoma. Int J Oncol 1999;15(1):59-66
43. Coulombe J, Pelletier G. Gangliosides and organ-specific metastatic colonization. Int J Cancer 1993;53(1):104-9
44. Makatsori E, Fermani K, Aletras A, et al. Screening of N-acylneuraminic acids in serum and tissue specimens of mouse C57BI with Lewis' lung cancer by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 1998;712(1-2):23-9
45. El-Abbadi M, Seyfried TN, Yates AJ, et al. Ganglioside composition and histology of a spontaneous metastatic brain tumour in the VM mouse. Br J Cancer 2001;85(2):285-92
46. Dickler MN, Ragupathi G, Liu NX, et al. Immunogenicity of a fucosyl–GM1–keyhole limpet hemocyanin conjugate vaccine in patients with small cell lung cancer. Clin Cancer Res 1999;5(10):2773-9
47. Dumontet C, Rebbaa A, Portoukalian J. Very low density lipoproteins and interleukin 2 enhance the immunogenicity of 9-O-acetyl-GD3 ganglioside in BALB/c mice. J Immunol Methods 1997;206(1-2):115-23
48. Livingston PO, Calves MJ, Helling F, et al. GD3/proteosome vaccines induce consistent IgM antibodies against the ganglioside GD3. Vaccine 1993;11(12):1199-204
49. Livingston PO, Calves MJ, Natoli EJ Jr. Approaches to augmenting the immunogenicity of the ganglioside GM2 in mice: purified GM2 is superior to whole cells. J Immunol 1987;138(5):1524-9
50. Livingston PO, Ritter G, Calves MJ. Antibody response after immunization with the gangliosides GM1, GM2, GM3, GD2 and GD3 in the mouse. Cancer Immunol Immunother 1989;29(3):179-84
51. Livingston PO, Ritter G, Srivastava P, et al. Characterization of IgG and IgM antibodies induced in melanoma patients by immunization with purified GM2 ganglioside. Cancer Res 1989;49(24 Pt 1):7045-50
52. Zhang H, Zhang S, Cheung NK, et al. Antibodies against GD2 ganglioside can eradicate syngeneic cancer micrometastases. Cancer Res 1998;58(13):2844-9
53. Helling F, Zhang S, Shang A, et al. GM2-KLH conjugate vaccine: increased immunogenicity in melanoma patients after administration with immunological adjuvant QS-21. Cancer Res 1995;55(13):2783-8
54. Kim SK, Ragupathi G, Cappello S, et al. Effect of immunological adjuvant combinations on the antibody and T-cell response to vaccination with MUC1–KLH and GD3–KLH conjugates. Vaccine 2000;19(4-5):530-7
55. Livingston PO. Experimental and clinical studies with active specific immunotherapy. Prog Clin Biol Res 1989;288:309-21
56. Livingston PO. Approaches to augmenting the immunogenicity of melanoma gangliosides: from whole melanoma cells to ganglioside-KLH conjugate vaccines. Immunol Rev 1995;145:147-66
57. Livingston PO, Ragupathi G. Carbohydrate vaccines that induce antibodies against cancer. 2. Previous experience and future plans. Cancer Immunol Immunother 1997;45(1):10-9
58. Livingston PO. Augmenting the immunogenicity of carbohydrate tumor antigens. Semin Cancer Biol 1995;6(6):357-66
59. Ravindranath MH, Kelley MC, Jones RC, et al. Ratio of IgG:IgM antibodies to sialyl LewisX and GM3 correlates with tumor growth after immunization with melanoma-cell vaccine with different adjuvants in mice. Int J Cancer 1998;75(1):117-24
60. Ambrosino DM, Bolon D, Collard H, et al. Effect of Haemophilus influenzae polysaccharide outer membrane protein complex conjugate vaccine on macrophages. J Immunol 1992;149(12):3978-83

Affiliation

Mayrel Labrada[†1], Marilyn Clavell[1], Yanín Bebelagua[1], Joel de León[1], Daniel F Alonso[2], Mariano R Gabri[2], Roberto C Veloso[3], Vicente Vérez[3] & Luis E Fernández[1]
[†]Author for correspondence
[1]Center of Molecular Immunology, Vaccine Department, 216 St and 15th Ave., Atabey, Playa, P. O. Box 16040, Havana, 11600, Cuba
Tel: +53 7 271 6810; Fax: +53 7 272 0644; E-mail: mayrel@cim.sld.cu
[2]Quilmes National University, Department of Science and Technology, Laboratory of Molecular Oncology, R. Saenz Peña 180, Bernal B1876BXD, Buenos Aires, Argentina
[3]Center of Biomolecular Chemistry, Laboratory of Synthetic Antigens, 200 St, Atabey, Playa, Havana, 11600, Cuba