REPORT

# *The* N*-glycolylated variant of ganglioside GM3 in tumor biology: An attractive target for cancer immunotherapy*

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***ABSTRACT***

**Gangliosides are one of the immunosuppressive molecules released by tumors to their microenvironment. These glycosphingolipids are differentially distributed in tumoral *vs.* normal tissues, and gangliosides containing an *N*-glycolylated variant of sialic acid have been found to be preferentially tumor-associated in humans. One such ganglioside is *N*-glycolyl GM3 (NGcGM3), which has become an attractive target for antigen-specific antitumor therapy. Since the differential expression of this ganglioside in advanced human tumors contrasts with the trend towards reduced immunogenicity typical of cancer cells, it has been presumed that this molecule plays an important role in tumor biology. Therefore, we have studied the relevance of NGcGM3 for tumoral development and, specially, its influence on the immune response. Our results show that NGcGM3 contributes to tumoral progression by modifying helper T lymphocyte function. This ganglioside reduces the expression of CD4 in T lymphocytes, inserting into the plasma membrane of these cells. Furthermore, it affects cell proliferation and promotes a differentiation towards an anti-inflammatory cytokine secretion profile in $CD4^+CD25^-$ T lymphocytes. The latter effect is not caused by increased suppression mediated by naturally occurring regulatory T lymphocytes. Additionally, NGcGM3 also affects the differentiation and maturation of dendritic cells.**

## *I*ntroduction

The identification of molecules with altered expression patterns in tumoral cells when compared to normal tissues is the main strategy for the development of active or passive antigen-specific antitumoral immunotherapies. The targets selected for this strategy should ideally fulfill an important role in tumor biology, allowing the induction of a specific antitumoral immune response that interferes with key biological events associated to tumoral progression.

In spite of the implementation of multiple alternatives for the generation of an effective immune response against tumors, cancer remains a serious public health problem worldwide with a high mortality rate. The immunotherapies used so far have not been sufficiently effective, due among other factors to the nature of the interactions between the tumor and the immune system of the host. Proof argue for the existence of an immunological surveillance system against neoplasias, which involves different effector mechanisms of the immune system that prevent the appearance of tumors or limit their progression once they are established. However, the selective pressure exerted by the immune system favors the selection of resistant tumor variants. This phenomenon, known as tumor editing, is facilitated by the high mutation rates that characterize cancer cells [1], and also leads to the activation of immunosuppressive mechanisms in tumors. In this context, it has been shown that tumor cells can release molecules to their microenvironment that modulate antigen presentation and have a negative impact on lymphocyte proliferation, affecting the activity of immune effector cells [2].

It has also been shown that tumor progression is correlated with an increased frequency of cells with suppressive properties [3]. Gangliosides are a well-known example of immunosupressive molecules [4]. They constitute the most variable glycosphingolipid group, and are characterized by the presence of at least one sialic acid moiety in their structure. Gangliosides are expressed on the plasma membrane of vertebrate cells, where they constitute an essential component of the so-called lipid micro-domains, which are cholesterol-rich structures that function as anchoring points for a large variety of proteins involved in cellular signal transduction.

The distribution of gangliosides among different tissues is highly heterogeneous and depends on the ex-tent of cellular differentiation. There are changes in ganglioside composition in the plasma membrane of cancer cells, leading to the emergence of tumor-associated antigens [5]. That is the case of the *N*-glycolilated variant of sialic acid which, although widely distributed on different mammalian species, are almost absent in normal human tissues [6]. This scarcity is caused by the absence in humans of a functional gene for cytidine monophosphate-*N*-acetyl sialic acid hydroxylase, the enzyme that catalyzes the *N*-glycolylation of *N*-acetyl sialic acid [7].

It has been shown that dietary intake and latter incorporation of this modified variant into the cellular sialic acid pool account for the very low levels of *N*-glycolyl gangliosides detectable in normal human cells [8]. In cancer cells, however, the incorporation of this precursor is favored due to the increased expression of sialic acid transporters in the hypoxic environment of the tumor, leading therefore to increased levels of *N*-glycolyl gangliosides [9]. This phenomenon has turned these molecules into an attractive target for antigen-specific antitumoral therapy; and specially so for the *N*-glycolyl GM3 ganglioside (NGcGM3), which has been detected in human tumor cells such as those from infiltrating ductal carcinoma of the breast [10] and melanoma [11].

The expression of this ganglioside in advanced tumors contrasts with the fact that cancer cells down regulate those molecules that can become a target for

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immune surveillance systems. In addition, little is known about the role of NGcGM3 in tumor biology and its interaction with cells from the immune system. These gaps in the existing knowledge about this system limit the design of more effective antitumoral therapies targeting NGcGM3, and restrict our understanding of the mechanisms of action for vaccine preparations currently under study.

## Results

### Influence of NGcGM3 on tumor growth *in vivo*

The murine myeloma X63, a cell line in which more than 85% of the total ganglioside content is formed by NGcGM3, was selected as a model for studying the influence of this molecule on tumor growth *in vivo*. X63 cells were cultured in the presence or absence of D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), a chemical compound that inhibits the activity of glucosylceramide synthetase. This procedure decreased by more than 60% the NGcGM3 contents of treated myeloma cells. Cell variants with varying ganglioside contents were then inoculated into Balb/c mice, followed by an evaluation of tumoral growth. The percentage of tumor-free animals was significantly higher in the group inoculated with cells expressing lower amounts of membrane-bound NGcGM3, and the average tumor diameter for this group was also lower [12]. The mutual influence between two X63 tumors with normal or reduced ganglioside expression levels, inoculated into the opposite sides of the same individual, was also evaluated. In this case the co inoculation of both variants resulted in decreased growth for the variant expressing higher amounts of ganglioside, suggesting the successful induction of an effector immune response against the D-PDMP-treated line that was in turn effective against the normal myeloma. This result led to the study of the link between the effect of NGcGM3 in tumoral progression and the possibility of modifying the functional activity of the immune system.

Considering the importance of $CD4^+$ T helper lymphocytes in the induction of a specific immune response, it was decided to evaluate whether the influence of NGcGM3 on tumoral progression was associated to its interaction with this cell population. Balb/c mice inoculated with the cell variants of X63 (pretreated or not with D-PDMP) were treated with a murine CD4-specific monoclonal antibody (mAb) with the aim of depleting the cell population expressing this protein on their membrane. The elimination of $CD4^+$ Th lymphocytes restored the *in vitro* proliferation rates of X63 cells with reduced ganglioside contents to normal levels [12], indicating that NGcGM3 influences tumor growth by modulating the functional activity of these lymphocytes (Figure 1a). In order to confirm this result, the gangliosides from both X63 variants (treated or not with D-PDMP) were extracted and incubated with lymphocytes obtained from lymph nodes of Balb/c mice. The incubation of these cells with different dilutions of the ganglioside extract induced a significant, dose-dependent reduction in their CD4 expression levels [12]. Based on this result, it was decided to study in deeper detail the influence of the NGcGM3 ganglioside on the functional properties of $CD4^+$ Th lymphocytes.

### Effect of NGcGM3 on $CD4^+$ Th lymphocytes

Highly purified NGcGM3 was used to determine the degree of negative regulation this molecule could exert over the expression of CD4 in murine and human lymphocytes. The results indicated a significant, dose-dependent decrease in CD4 expression caused by the ganglioside. The expression of other molecules relevant for lymphocyte function, such as CD3 and CD8, was not affected by this treatment [12].

The effect of the ganglioside on CD4 expression was reversible, since human lymphocytes pre-incubated with NGcGM3 and then cultured in its absence recovered CD4 expression levels up to 80% of their original values. The recovery was mediated by *de novo* synthesis, as evidenced when lymphocytes cultured in the presence of cycloheximide (an inhibitor of protein synthesis) failed to recover pre-treatment CD4 levels [12]. The sensitivity of the lymphocytes to NGcGM3-mediated CD4 down-regulation was unaffected after full recovery of CD4 expression.

In order to evaluate whether NGcGM3 was inserted into the plasma membrane of T lymphocytes, cells obtained from Balb/c lymph nodes and human peripheral blood mononuclear cells were incubated with different ganglioside concentrations. This treatment increased the levels of plasma membrane-associated NGcGM3 for both cell populations, in a dose-dependent manner [12]. The assay showed a statistically significant negative correlation between the phenomena of NGcGM3 membrane insertion and CD4 down-regulation in both murine and human Th lymphocytes (Figure 1b).

Murine non-activated and activated, as well as naturally occurring regulatory T lymphocytes were also used for studying the effect of the NGcGM3 ganglioside on CD4 expression. According to the results, the most pronounced down-regulation of CD4 mediated by NGcGM3 takes place in non-activated lymphocytes, with a less pronounced and similar effect for both activated and naturally occurring regulatory lymphocytes [13]. This indicates that the influence of the ganglioside on CD4 is independent from the expression of CD25, the receptor for interleukin 2 (IL2); in fact, the membrane levels of this molecule are not affected by NGcGM3.

During the last years it has been recognized that regulatory T lymphocytes play an important role not only in the regulation of the immune response, but also on tumoral escape to immune surveillance [14]. Taking this into account, the effect of the ganglioside on lymphocyte function was compared between $CD4^+$ $CD25^-$ Th lymphocytes and naturally occurring regulatory $CD4^+CD25^+FoxP3^+$ cells, purified by separation on magnetic beads.

The first experiments examined the influence of NGcGM3 on the proliferation of these cells, stimulated with an anti-CD3 mAb and IL2. Increasing ganglioside concentrations led to a reduction of the proliferative capacity for both cellular populations. However, this effect was statistically significant and showed a dose-dependent behavior only for $CD4^+CD25^-$ Th lymphocytes. Additionally, there were differences bet-

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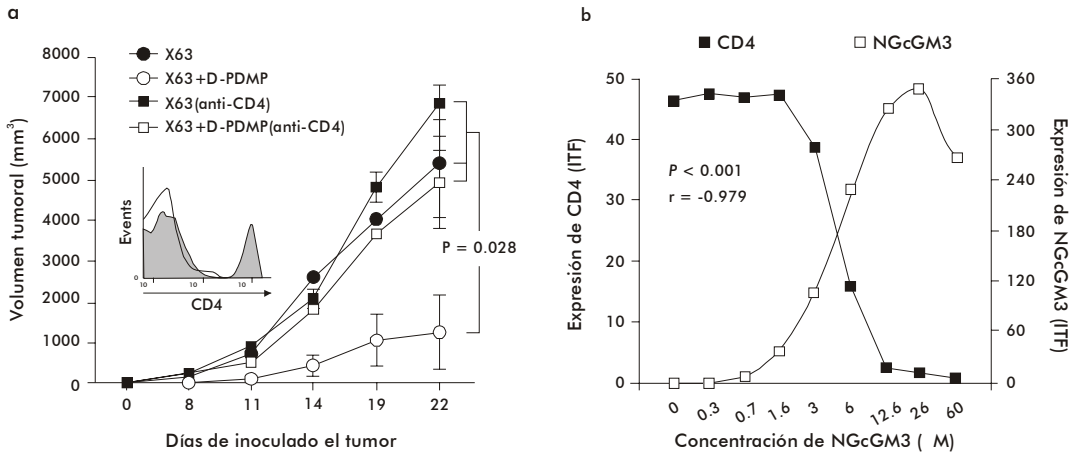


Figure 1. Ganglioside NGcGM3 contributes to tumoral progression *in vivo* by modulating the function of CD4+ T lymphocytes. (a) Balb/c mice were inoculated with $10^6$ X63 cells, treated or not with D-PDMP. Additionally, phosphate buffer solution (SSTF) or an anti-CD4 mAb were inoculated intraperitoneally. The average tumoral volume was significantly reduced in the animals inoculated with the cell variant expressing lower amounts of gangliosides. The elimination of CD4+ T-lymphocytes restored tumoral growth (Dunn's test); (b) Lymphocytes isolated from Balb/c lymph nodes were incubated for 1 h with different concentrations of NGcGM3, measuring the insertion of the ganglioside on the plasma membrane and the expression of CD4 by flow cytometry. The ganglioside-mediated down-regulation of CD4 correlated with the incorporation of NGcGM3 on the cell membrane (Pearson's test). The data are presented as total fluorescence intensity (TFI) values.

ween the influence of NGcGM3 on the proliferation of the activated Th lymphocytes, depending on their previous activation status (*i.e.* whether they were resting lymphocytes or were already activated before treatment with anti-CD3 and IL2). The proliferation of previously resting lymphocytes was significantly affected by the presence NGcGM3, whereas the proliferation of already stimulated Th cells did not decrease [13]. This result confirmed that the effects of NGcGM3 treatment are most relevant during the stage of lymphocyte activation, rather than on activated cells.

Since the main biological activity characterizing the phenotype of naturally occurring regulatory T cells is the inhibition of Th lymphocyte proliferation, we next decided to evaluate whether an increase in the inhibitory capacity of these cells was part of the immunosuppressive mechanism of NGcGM3. For this objective, the suppressive activity of naturally occurring $CD4^+$ $CD25^+$ T lymphocytes previously treated or not with NGcGM3 on the proliferation of $CD4^+CD25^-$ Th lymphocytes stimulated with an anti-CD3 mAb and in the presence of dendritic cells (DC) was evaluated. The result indicated that a pre-incubation with the ganglioside did not modulate the suppressive activity of these cells (Figure 2c). A similar assay was also implemented in which the ganglioside was added to the co-culture of effector and regulatory cells, proving that the presence of NGcGM3 did not enhance regulatory T cell-mediated suppression; additionally, NGcGM3 inhibited Th lymphocyte proliferation by 75% even in the absence of regulatory lymphocytes [13]. These results suggest that the modulation of the activity of naturally occurring regulatory T lymphocytes is not involved in the immunosuppressive mechanism of NGcGM3.

## Influence of NGcGM3 on dendritic cell function

Based on the results outlined above and taking into account the role played by DC in the development of antigen-specific immune responses, it was decided to evaluate if NGcGM3 had any influence on DC function in the context of their interaction with Th lymphocytes, as well as any direct effects of the ganglioside on the differentiation and maturation of DC.

First, the impact of NGcGM3 on the proliferation of Th lymphocytes co-cultured with DC and stimulated with an anti-CD3 mAb was evaluated. The ganglioside decreased lymphocyte proliferation in a dose-dependent fashion for as much as 70% (Figure 2a). The remaining proliferative activity was accompanied by a switch of the cytokine secretion profile of these lymphocytes [13]. The presence of NGcGM3 during cell culture increased three-fold and two-fold the frequency of IL4- and IL10-secreting cells respectively, without increasing the percentage of interferon (IFN) gamma-producing lymphocytes (Figure 2b). This finding suggested that NGcGM3 not only changes the proliferation of Th cells, but also promotes the appearance of an anti-inflammatory cytokine secretion profile.

The evaluation of the effect of NGcGM3 on the differentiation of DC from their bone marrow precursors was performed with an *in vitro* differentiation assay in the presence or absence of the ganglioside. Dendropoiesis was decreased by NGcGM3, as evidenced by a significant reduction in the number of DC recovered from each culture well [13]. There was detectable IL10 mRNA (as evaluated from cDNA) in the DC differentiated in the presence of NGcGM3, but not in the cells differentiated without the ganglioside. Additionally, the maturation upon an inflammatory stimulus with lipopolysaccharide (LPS) of DC differentiated in the presence of NGcGM3 was impaired, as shown by a 50% reduction of the expression levels of the co-stimulatory molecule CD40 [13].

In order to study the direct influence of NGcGM3 on DC maturation, DC cells differentiated from their bone marrow precursors were stimulated with LPS in

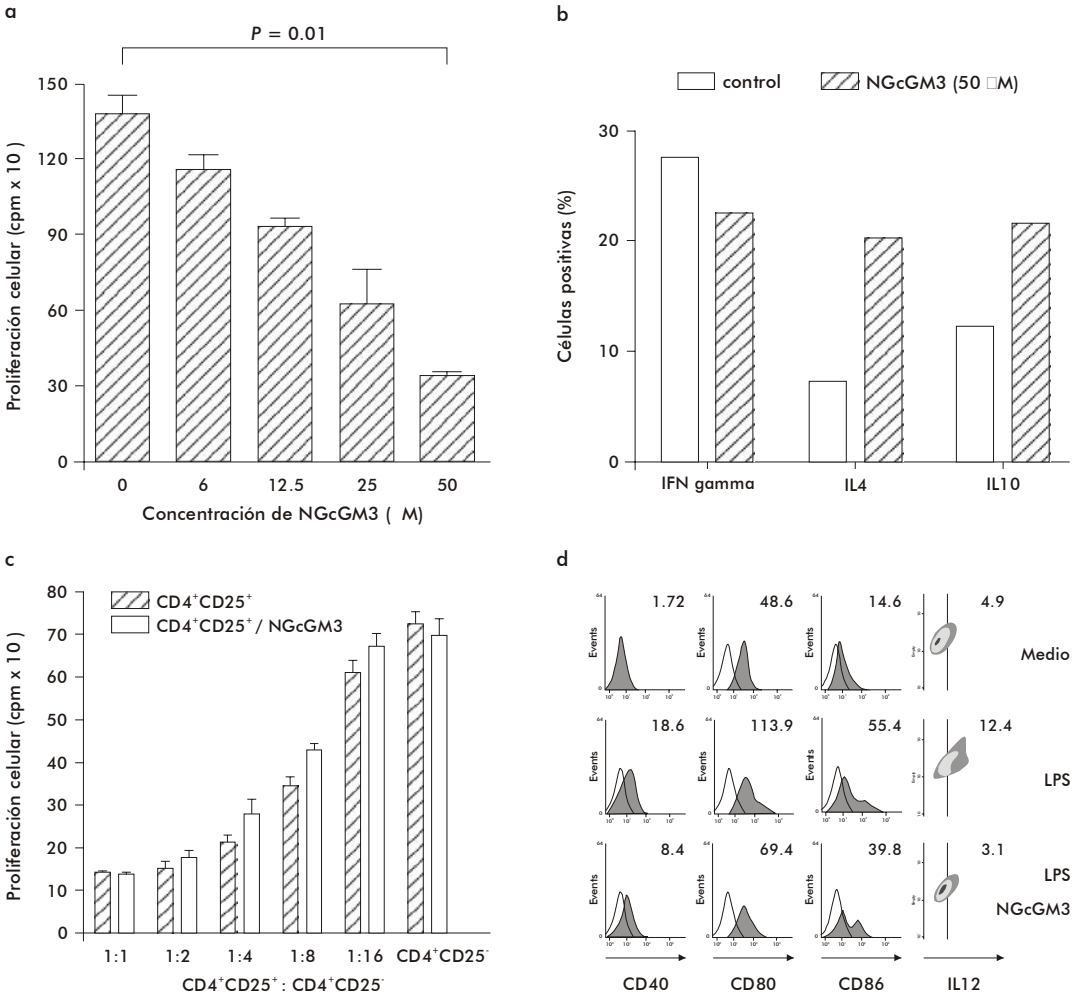


Figure 2. NGcGM3 affects Th lymphocyte and DC function without increasing the suppressive properties of naturally occurring regulatory T-lymphocytes. (a) $CD4^+CD25^-$ T lymphocytes were incubated with DC, an anti-CD3 mAb and different concentrations of NGcGM3. The ganglioside decreased significantly the proliferation of the lymphocytes (Tukey's test); (b) $CD4^+CD25^-$ lymphocytes were incubated in the presence or absence of NGcGM3, an anti-CD3 mAb, and DC, using flow cytometry to measure the frequency of lymphocytes producing IFN gamma, IL4 and IL10. The ganglioside increased the percentage of cells producing anti-inflammatory cytokines; (c) $CD4^+CD25^+$ T lymphocytes were incubated for 48 h in the presence (50 μM) or absence of NGcGM3, after which the cells were collected and co-incubated at different ratios with $CD4^+CD25^-$ lymphocytes in the presence of DC and an anti-CD3 mAb. The suppressive activity of regulatory T cells was not changed upon incubation with the ganglioside (Mann-Whitney's U test); (d) DC were differentiated from their bone marrow precursors. Their maturation was stimulated by incubation with lipopolysaccharide (LPS) in the presence or absence of the ganglioside. Flow cytometry was then used to compare the expression of the co-stimulatory molecules CD40, CD80 and CD86, as well as the frequency of IL12-producing cells. NGcGM3 affected the maturation of DC upon the potent inflammatory stimuli afforded by LPS. The data are presented as total fluorescence intensity (TFI) values. The open histograms correspond to the unlabelled cell control.

the presence of the ganglioside. NGcGM3 was also able to directly affect DC maturation, evidenced by a reduction in the levels of the co-stimulatory molecules CD40, CD80 and CD86. Furthermore, the production of IL12 by these cells decreased four-fold when stimulated with LPS in the presence of the ganglioside (Figure 2d). IL12 is a key player during the induction of a proinflammatory profile in Th lymphocytes by DC.

The effect of NGcGM3 on DC differentiation or ma-turation as reflected on their capacity for modulating the production of cytokines in Th lymphocytes was also studied. $CD4^+CD25^-$ Th cells were co-cultivated with DC differentiated or matured in the presence of the ganglioside. Afterwards, the lymphocytes were sti-mulated with a murine anti-CD3 mAb in the presence or absence of NGcGM3. The presence of NGcGM3 during T lymphocyte stimulation decreased at least two-fold the levels of IFN gamma and increased signi-ficantly the concentration of IL4 in culture supernatants. This effect was detected only in the presence of the ganglioside, independently of the presence or absence of NGcGM3 during the previous differentiation or maturation of DC. However, the secretion of IL4 was further enhanced if, in addition to the stimulation of the lymphocytes in the presence of NGcGM3, the DC employed in the experiment had differentiated or matured in the presence of the ganglioside. A similar phenomenon was detected when the frequency of IL10-producing $CD4^+$ T-lymphocytes was measured instead. The presence of NGcGM3

during stimulation lead, at a minimum, to a three-fold increase in the percentage of cells expressing this cytokine, and again, previous differentiation of the DC in the presence of the ganglioside further augmented the frequency of IL10-producing T lymphocytes by 20% [13]. The results suggest that although the presence of NGcGM3 is absolutely necessary for a detectable change in the response of Th lymphocytes upon a stimulus such as TCR-mediated signaling, its influence on DC also extends to the promotion of an anti-inflammatory cytokine secretion profile. This finding underscores the potential significance of metastasis to secondary lymphoid organs within the context of tumor interaction with the immune system, where the buildup of ganglioside shed from tumor cells would have a direct influence on the activation of lymphocyte populations.

## Conclusions

Considering the importance of DC and Th lymphocytes as coordinators of the specific immune response, it can be asserted that NGcGM3 has a negative influence on the cellular populations that guarantee the efficacy of immune surveillance on cancer. Our results as a whole validate the *N*-glycolylated variant of GM3 as a target for cancer immunotherapy; based not only on its preferential expression in human tumoral cells, but also on its relevance for tumor biology. There are antitumoral diagnostic and therapeutic strategies already under development based on this target.