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ORIGINAL ARTICLE

The tumor antigen *N*-glycolyl-GM3 is a human CD1d ligand capable of mediating B cell and natural killer T cell interaction

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Abstract The expression of *N*-glycolyl-monosialodihexosyl-ganglioside (NGcGM3) in humans is restricted to cancer cells; therefore, it is a tumor antigen. There are measurable quantities of circulating anti-NGcGM3 antibodies (aNGcGM3 Abs) in human serum. Interestingly, some people have circulating Ag-specific immunoglobulins G (IgGs) that are capable of complement mediated cytotoxicity against NGcGM3 positive cells, which is relevant for tumor surveillance. In light of the chemical nature of Ag, we postulated it as a candidate ligand for CD1d. Furthermore, we hypothesize that the immune mechanism involved in the generation of these Abs entails cross talk between B lymphocytes (Bc) and invariant natural killer T cells (iNKT). Combining cellular techniques, such as flow cytometry and biochemical assays, we demonstrated that CD1d binds to NGcGM3 and that human Bc present NGcGM3 in a CD1d context according to two alternative strategies. We also showed that paraformaldehyde treatment of cells expressing CD1d affects the presentation. Finally, by co-culturing primary human Bc with iNKT and measuring Ki-67 expression, we detected a reproducible increment in the proliferation of the iNKT population when Ag was on the medium. Our findings identify a novel, endogenous, human CD1d ligand, which is sufficiently competent to stimulate iNKT. We postulate that CD1d-restricted Bc presentation of NGcGM3 drives effective iNKT activation, an immunological mechanism that has not been previously described for humans, which may contribute to understanding aNGcGM3 occurrence.

Keywords CD1d · B cells · Invariant natural killer T cells · Tumor antigen · Gangliosides

Abbreviations

18:1 Biotinyl PE	1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-*N*-(Biotinyl)
Abs	antibodies
aIgG1	anti-IgG1
aKi-67	anti-Ki-67
aNGcGM3	anti-NGcGM3
Bc	B lymphocytes or B cells
CIM	Centro de Inmunología Molecular
GM3	monosialodihexosyl-ganglioside
hs	hours
IgGs	immunoglobulins G
iNKT	invariant natural killer T cells
MeOH	methanol
min	minutes
NAc	*N*-Acetylneuraminic acid
NAcGM3	*N*-Acetyl-GM3
NGc	*N*-Glycolylneuraminic acid
NGcGM3	*N*-Glycolyl-GM3
NKT	natural killer T cells
ON	overnight
PFA	paraformaldehyde

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SEM	standard error of the mean
TCR	T cell receptor
TMC	tonsillar mononuclear cells
β-GluCer	β-Glucosylceramide
α-GalCer	α-Galactosylceramide

Introduction

Gangliosides with sialic acid are normal components of the cell membrane for almost all eukaryotic cells [1]. The most common sialic acids in gangliosides from mammalian tissues are *N*-acetylneuraminic acid (NAc) and *N*-glycolylneuraminic acid (NGc). When conjugated with GM3, they comprise the oligosaccharide head structure of *N*-Acetyl-GM3 (NAcGM3) and *N*-Glycolyl-GM3 (NGcGM3), respectively. Of note, NGcGM3 is not expressed in normal human tissues because of a species-specific genetic mutation that results in inactive cytidine-5′-monophosphate-NAc hydroxylase (CMP-NAc hydroxylase) [2]. However, altered glycosylation is a common feature of cancer [3], and one of these tumor-specific alterations is the metabolic incorporation of diet-derived NGc [4] that generates NGcGM3, which is in turn present in several human cancers [5–8] as a xeno-autoantigen. The humoral response against xeno-autoantigens has a polyclonal profile of xeno-autoantibodies in human serum [9].

Abs are produced by Ag-activated Bc as they turn into plasma blasts and terminally differentiated plasma cells. The present consensus is that B cell receptor engagement by Ag, followed by cognate T cell help, drives the proliferation of Ag-specific naive Bc and their differentiation into memory Bc and plasma cells [10, 11]. It has been described that iNKT can facilitate Bc proliferation and Ab production in vitro and in vivo [12]. iNKTs are a unique subset of T cells that express both T cell receptors (TCRs) and C-type lectin receptors that are characteristic of NK cells. In humans, they express a TCR that consists of a conserved invariant Vα chain (Vα24-JαQ) paired with a limited TCRβ chain repertoire [13, 14]. iNKT recognize synthetic, self, and microbial glycolipids, as well as self-phospholipid Ags that are presented by CD1d [15]. Considering maturation of iNKT depends on the thymus, Ab responses triggered by interactions between iNKT and Bc upon recognition of the CD1d–(glycolipid or phospholipid) complex on the Bc were recently classified as innate cell-driven thymus-dependent type 2 Ab responses [16].

In a previous approach to identify lipid Ags that are presented by CD1d, the authors eluted and subsequently characterized the lipids bound to human CD1d molecules. Two main classes of lipids were identified: glycerophospholipids and sphingolipids. Among the latter, glycosphingolipids like GM3 were identified [17]. These findings and the fact that some kind of T cell help could explain the presence of aNGcGM3 in human serum led us to hypothesize that NGcGM3 could be presented on the surface of Bc by CD1d to human iNKT.

In this study, we demonstrated binding of NGcGM3 to human CD1d and explored the outcome of a cross talk between human Bc and iNKT, mediated by NGcGM3.

Materials and methods

Antibodies and flow cytometry

Fluorochrome-conjugated mAbs specific for human CD1d, CD3, CD56, CD19, Ki-67, and CDR3 of the iNKT TCR (6B11), mouse IgG1 (to detect 14F7 binding), and isotype control mAbs were purchased from BD Biosciences and Dako. 14F7 Ab, against NGcGM3, a highly specific mAb able to discriminate between the almost identical molecular structure of the NGcGM3, and the NAcGM3 was a kind gift from L. E. Fernández at the Centro de Inmunología Molecular (CIM), Cuba. To evaluate the Ki-67 Ag by intracellular staining, cells were incubated with Cytofix/Cytoperm (BD PharMingen) for 20 min in the dark and washed with Perm/Wash solution (BD PharMingen). Following permeabilization, the cells were stained with anti-Ki-67 (aKi-67). Cells were acquired using FACSAria II (BD Biosciences) and analyzed with FlowJo software (Treestar). Single stained controls were used to set compensation parameters. Fluorescence minus one and isotype-matched Ab controls were used to set analysis gates.

Cell lines

The B lymphoblastoid cell line, C1R [18], stably expressing full-length CD1d, designed as C1RCD1d, was used in this study. Transfection details and characteristics of the cell line had been already described [19]. The C1R line and its CD1d transfectant were maintained in IMDM (Life Technologies) containing 10 % heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37 °C. The murine cell line X63 (myeloma) was purchased from the American Type Culture Collection.

Cell-free NGcGM3 presentation assay

Commercial NGcGM3 (Enzo Life Sciences, catalogue number ALX-302-019, purity ≥98 % according to the product data sheet) was adsorbed by solvent methanol (MeOH) evaporation on the surfaces of 96-well microtiter plates (PolySorp, Nunc) and then washed for removal of unbound ganglioside. After BSA blocking, either recombinant

CD1d-IgG1 fusion (CD1d-DimerX, BD Biosciences) or IgG1 isotype (Dako) was incubated for 2 hs at 37 °C. Detection of immobilized CD1d by NGcGM3-coated surfaces was performed with a biotinylated anti-IgG1 (aIgG1, BD Biosciences), extravidin alkaline phosphatase (Sigma), and p-nitrophenyl phosphate (Sigma) with absorbance detected using a spectrophotometer at a wavelength of 405 nm.

Competition with NGcGM3 pre-incubated in solution (Fig. 1b) was performed by preparing mixture solutions combining CD1d and IgG1 (5 μg/mL) and increasing amounts of NGcGM3 (0, 5, 10, 50, 250, and 500 μg/mL). Mixtures were incubated overnight (ON) at 37 °C and subsequently added to the NGcGM3-adsorbed plates for 2 hs at 37 °C and developed as above.

The competitive assay with 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-*N*-(Biotinyl) (18:1 Biotinyl PE, Avanti Polar Lipids) has been already described in [20]. All lipid stock solutions were performed in MeOH. The IC_{50} value was calculated using GraphPad Prism 5.0 (GraphPad Software).

NGcGM3 loading of cells

Cells were incubated ON at 37 °C and 5 % CO_2 with either 50 μg/mL NGcGM3 liposomes or empty liposomes in Bc medium as described below, but they were supplemented with 10 % human serum instead of 10 % FCS. The NGcGM3 liposomes and control empty liposomes were a kind gift from L. E. Fernández at the CIM, Cuba. Mouse anti-human CD1d (10 μg/mL; clone CD1d 42, BD Biosciences) was used to block CD1d.

Isolation of $CD19^+$ and $CD3^+$ cells from healthy donors

Primary human mononuclear cells were isolated from either buffy coats taken from healthy adult donors or fresh peripheral blood (100 mL) drawn from healthy volunteers in tubes (containing EDTA K_2), yielding PBMCs isolated by density gradient centrifugation with Ficoll-Hypaque (GE Healthcare). The viability of primary cells determined by trypan blue exclusion was >99 % in all preparations. Informed consent was obtained from subjects before the study. The institutional ethics committee (Clinical Hospital, School of Medicine, Buenos Aires) approved the collection and use of clinical material, which conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Further separation into autologous B and T cells was performed by cell sorting with a FACSAria II flow cytometer (BD Biosciences) after CD19/CD3 staining. The cell populations were purified at >95 % for use in the experiments. Experiments were performed with freshly isolated cells (no freezing steps). Primary human Bc were cultured in IMDM medium (Life Technologies) containing 10 % heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, 1 mM sodium pyruvate, and 50 μM 2-ME (all from Invitrogen). Human IL2 (20 ng/ml; R&D Systems) and human IL4 (20 ng/ml; R&D Systems) were added immediately before the experiments as supplements.

In vitro expansion of human iNKT

Sorted $CD3^+$ cells (not more than 95 % purity) from PBMCs were grown in 24-well plates in IMDM medium (Life Technologies) supplemented with 10 % human serum, 100 ng/ml α-galactosylceramide (α-GalCer, Avanti Polar Lipids, Inc.) and human IL2 (20 ng/ml; R&D Systems). After 5 days of culture, the presence and functional response of iNKT were monitored by FACS, as described in "Expansion of iNKT from PBMCs" section. The staining of these cultures with CD1d-IgG1 was performed by incubating ON at 37 °C, 10 μg CD1d-IgG1 with 50 μg NGcGM3 liposomes (or empty liposomes as control), in 250 μl of PBS and subsequently used to stain $2x10^6$ expanded iNKT (after thorough washing previously to eliminate α-GalCer) on ice for 30 min. Then, the cells were washed twice with PBS containing 10 % human serum and incubated with labeled anti-mouse IgG1 antibody and anti-human CD3 on ice for 30 min. After washing, the stained cells were acquired using FACSAria II (BD Biosciences) and analyzed with FlowJo software (Treestar).

Isolation of $CD19^+$ from tonsils

Primary human mononuclear cells were isolated from tonsils obtained from healthy adults undergoing routine tonsillectomy. Tonsillar mononuclear cells (TMC) were prepared as follows. Briefly, tonsils were collected in PBS buffer containing 50 μg/ml amphotericin B (Richet). Tissues were chopped with a scalpel in complete medium and passed through a 70-μm pore-size cell strainer (Falcon). To isolate Bc, TMC were purified by density gradient centrifugation with Ficoll-Hypaque (GE Healthcare). The viability of primary cells, determined by trypan blue exclusion, was greater than 99 % in all preparations. Informed consent was obtained from subjects before the study. The institutional ethics committee (Clinical Hospital, School of Medicine, Buenos Aires) approved the collection and use of clinical material according to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Bc were isolated using the Bc isolation kit II according to the instructions of the manufacturer (Miltenyi). The purity of isolated Bc subsets was determined by flow cytometry using a mAb against human CD19. Primary human Bc were cultured in IMDM medium (Life Technologies) supplemented as described above for Bc isolated from blood.

Cytokine production by iNKT

Supernatants from in vitro expanded iNKT were assayed by ELISA for IFNγ secretion with a human IFNγ OptEIA kit (BD Bioscience Pharmingen) according to the manufacturer's instructions.

Co-culture of Bc with iNKT

Autologous Bc (loaded with NGcGM3 or vehicle only) and expanded iNKT were co-cultured for 3 days at 1:3 ratios in 96-well round-bottom plates (Corning Life Sciences) at cell densities of 5×10^5 cells/well. Murine anti-human CD1d (10 μg/mL; clone CD1d 42, BD Biosciences) was used to prevent Bc–iNKT interaction.

Statistical analyses

The results were analyzed using GraphPad Prism 5.0 software. The statistical analysis of the results was performed using the unpaired *t* test, and a *p* value of <0.05 was considered significant unless indicated otherwise.

Results

NGcGM3 binds CD1d

To test the CD1d and NGcGM3 interaction in vitro, we set up a cell-free Ag presentation assay, which uses immobilized NGcGM3 to assay CD1d binding. This format eliminates some of the complexities of interpreting results when whole APCs are used. However, this approach cannot be used to detect Ags that require extensive processing. We incubated a commercial recombinant CD1d-IgG1 fusion on NGcGM3-coated plates and tested whether there were differences in the binding of the fusion and a mouse IgG1 isotype control. Differences between them were reproducibly significant ($p < 0.01$, unpaired *t* test), which was also true for differences with control wells that were not coated with NGcGM3 (Fig. 1a).

To assay the response against different concentrations of NGcGM3, we performed a competitive test. We evaluated whether pre-incubating the CD1d-IgG1 fusion with increasing amounts of NGcGM3 in solution would titrate the CD1d that could subsequently bind NGcGM3 on plates. Indeed, at a fixed amount of CD1d-IgG1 fusion, we observed that preloading the sample with increasing amounts of soluble NGcGM3 resulted in less binding to NGcGM3-coated plates until complete abrogation (Fig. 1b). We then sought to evaluate the ability of NGcGM3 to compete with a well-characterized CD1d ligand using a competitive assay that was previously described [20]. We observed a dose-dependent competition by NGcGM3 to the tagged lipid 18:1 Biotinyl PE (a pollen derived, phosphatidylethanolamine known as a CD1d ligand) for CD1d-IgG1 fusion (Fig. 1c). The extrapolated IC_{50} value (1.2 μmol/L) indicated a lower affinity than the one reported for α-GalCer using the same assay (84 nmol/L) [21] and within the same range of affinity, as that reported for 18:1 Biotinyl PE (1 μmol/L) under the same conditions [22].

Next, we used a murine myeloma cell line X63, in which NGcGM3 is expressed on the cell membranes (Fig. 1d top panel). In this system, NGcGM3 is displayed on a cellular membrane, and we used a similar strategy to assess CD1d binding to NGcGM3. Thus, we incubated the X63 cells with either CD1d-IgG1 fusion or IgG1 isotype control and examined the binding differences through a biotinylated aIgG1. In this case, detection of immobilized aIgG1 was achieved by flow cytometry using labeled streptavidin. A complete fluorescence bias that was not detected in control tubes evidenced CD1d binding to the surface of X63 cells, which overexpressed NGcGM3 (Fig. 1d lower panels).

NGcGM3 is a CD1d ligand

To further evaluate the ability of NGcGM3 to bind CD1d, we took advantage of the B lymphoblastoid cell line (C1R) that was transfected with CD1d (C1RCD1d). We tested the expression of CD1d by flow cytometry (Suppl. Figure 1). For these cellular assays, we had to incorporate NGcGM3 into liposomes because of its insolubility in an aqueous environment. The use of liposomes promoted solubility in cell medium and also provided a native environment for this lipophilic ligand, which is essential to mimic physiological conditions [23].

C1RCD1d cells were cultured with either vehicle alone (empty liposomes) or with NGcGM3-loaded liposomes, harvested, stained with a mAb (14F7) specific for NGcGM3 and analyzed by flow cytometry. Positive staining on NGcGM3-loaded cells compared to vehicle-treated cells (Fig. 2a) indicated that CD1d could present NGcGM3. Of note, we detected some non-specific NGcGM3 binding on the untransfected C1R cell line, which can be clearly discriminated from the specific interaction (Fig. 2b) and is likely to represent NGcGM3 delivered by fusion of NGcGM3-loaded liposomes to the plasma membrane.

To test whether cell metabolism is required for surface NGcGM3 presentation, we pretreated the cells with PFA. Then, we measured NGcGM3 binding in treated cells by flow cytometry, as described above. Ligand binding was reduced compared to untreated cells, but the levels were compatible with unspecific binding (Fig. 2c). As the cell surface expression of CD1d was not affected by PFA treatment (Fig. 2d), it would be tempting to speculate that NGcGM3 presentation requires viable cells. This was unexpected in light of the results shown in Fig. 1. Because

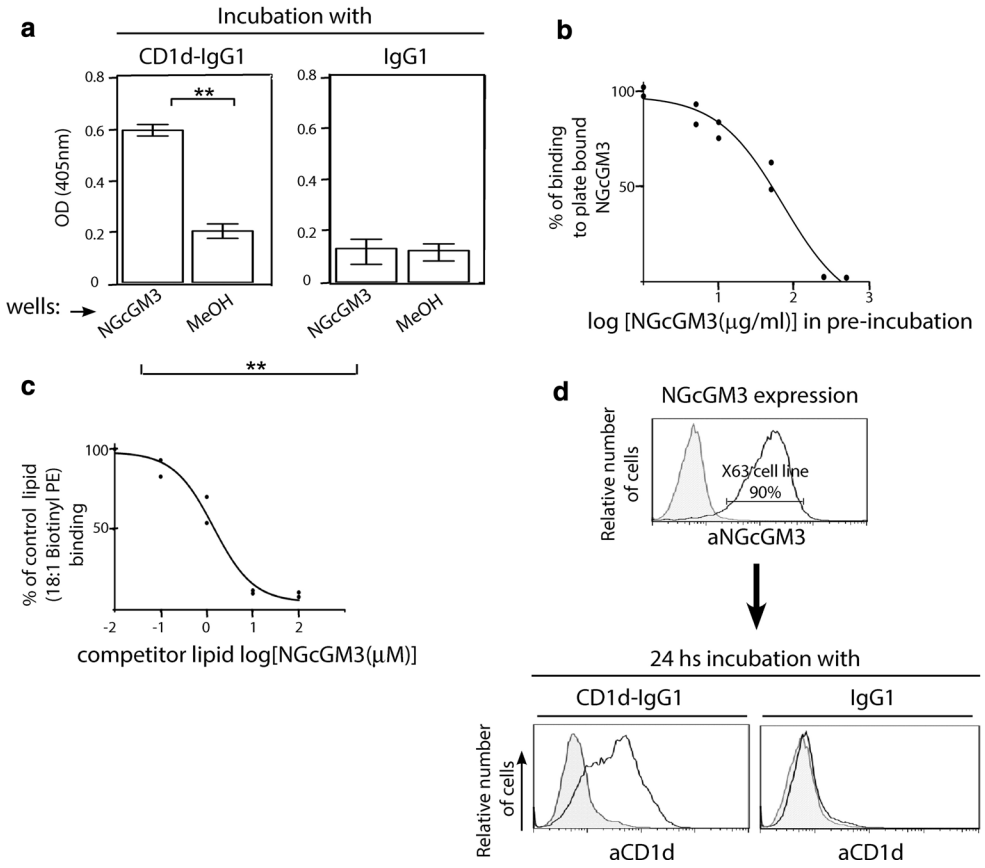

Fig. 1 CD1d interacts with NGcGM3. **a** NGcGM3 was adsorbed on 96-well microtiter plates that were incubated with either recombinant CD1d-IgG1 fusion or IgG1 isotype control. Detection of immobilized CD1d by NGcGM3-coated surfaces was performed with a biotinylated aIgG1, extravidin alkaline phosphatase, and p-nitrophenyl phosphate. *Bars* show the mean absorbance at 405 nm ± standard error of the mean (SEM) of 10 wells per treatment. For a control, we used wells that were not coated with NGcGM3 and were only coated with MeOH. $^{**}p < 0.01$, *t* test. **b** Competitive ELISA assays with CD1d-IgG1 fusion that was pre-incubated ON with increasing amounts of NGcGM3 in solution (0, 5, 10, 50, 250 and 500 µg/mL) and subsequently added to the NGcGM3 adsorbed plates. The assays were performed in duplicate. **c** Competitive ELISA assays with 18:1 Biotinyl PE (2 µg/mL). Plates were coated with goat anti-mouse IgG1 Ab, and CD1d-IgG1 fusion was pre-incubated ON in mixtures containing control lipid (2 µg/mL) in the presence of NGcGM3 (competitor lipid) at the indicated concentrations; the mixtures were subsequently added to the anti-mouse IgG1 adsorbed plates. The assays were performed in duplicate. **d** *Top panel* flow cytometry analysis of NGcGM3 expression on the murine myeloma cell line X63. The cell line was stained with isotype-matched control mAb (*gray-filled histogram*) or 14F7 mAb (*black line*) followed by PE-conjugated aIgG1. The numbers represent the percentages of NGcGM3-expressing cells. Data are representative of three independent experiments. *Lower panels* flow cytometry analysis of recombinant CD1d-IgG1 fusion binding to the surface of the murine myeloma cell line X63. The cell line was incubated for 24 hs with isotype-matched control mAb (*right panel*, IgG1 *black line*) or CD1d-IgG1 (*left panel*, *black line*). Detection of CD1d bound to the X63 cells surface was evidenced by PE-conjugated aIgG1 staining (referred to as aCD1d in the figure). The staining controls were fresh cells stained with PE-conjugated aIgG1 (*gray-filled histogram*). Data are representative of three independent experiments

we could not rule out subtle alterations on the CD1d-binding pocket due to PFA treatment, the issue requires further investigation into CD1d functionality in response to treatment and putative presentation pathways through different experimental approaches.

NGcGM3 is presented by primary Bc

To extend our observations to a more physiological model, we wanted to replicate the assays described above for primary human Bc cultures. As we have previously reported on functional differences between tonsillar and circulating Bc [24], we tested whether CD1d was differentially expressed in these populations. Thus, PBMCs and TMC from ten healthy donors were labeled with mAbs specific for CD19 and CD1d. Cell surface expression of CD1d resulted in a similar proportion (60 ± 5 %) for tonsillar and peripheral Bc (Fig. 3a, b). We found no significant difference amid the mean percentages of CD1d expression on each B cell population (Fig. 3c).

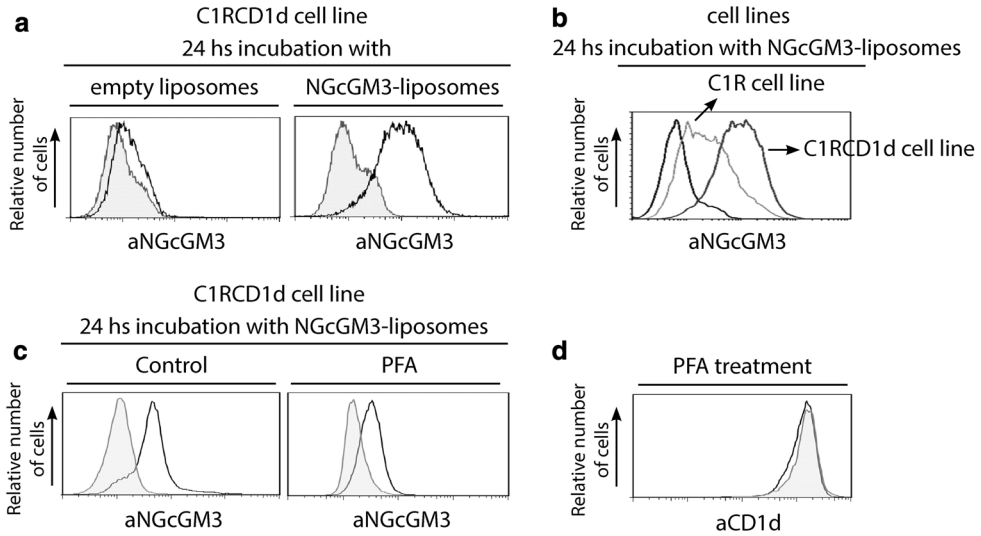

Fig. 2 Cells overexpressing CD1d bind to NGcGM3. **a** Flow cytometry analysis of NGcGM3 binding to the surface of the C1RCD1d cell line. Cells were incubated for 24 hs with empty liposomes (*left panel*) or with NGcGM3-loaded liposomes (*right panel*) followed by staining with a mAb (14F7, referred as aNGcGM3 in the figure, *black line*) or isotype-matched control mAb (*gray-filled histogram*). Detection of 14F7 bound to C1RCD1d cell surface was evidenced by PE-conjugated aIgG1 staining. Data are representative of three independent experiments. **b** Flow cytometry analysis of NGcGM3 binding to the surface of the C1RCD1d and C1R cell lines. Staining as in **a** with an untransfected C1R cell line. **c** Effect of PFA treatment on NGcGM3 binding to C1RCD1d cells. Control (untreated, *left panel*) and PFA-treated cells (*right panel*) were incubated with NGcGM3-loaded liposomes, which was followed by staining, as in **a**, for either NGcGM3 binding (*black line*) or isotype-matched controls (*gray-filled histogram*). Data are representative of three independent experiments. **d** Effect of PFA treatment on CD1d expression evaluated by C1RCD1d. The *black line* indicates cells that were fixed with 4 % PFA for 20 min at 4 °C and washed with PBS with 10 % FCS. Finally, they were stained with PE-conjugated aCD1d (clone CD1d42). The *gray-filled histogram* indicates untreated cells. Data are representative of three independent experiments

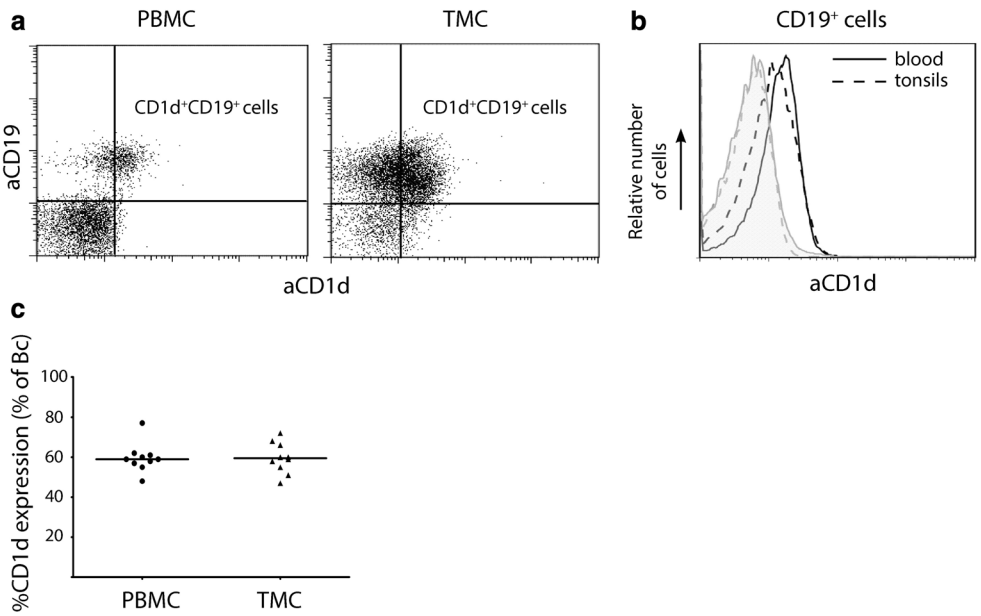

Fig. 3 CD1d is uniformly expressed in human Bc from blood and tonsils. **a** *Left and right panels* representative flow cytometry *dot plot* showing the identification of the B cell population expressing CD1d in PBMCs and TMC. **b** Representative histograms of CD1d expression by $CD19^+$ cells from blood (*full line*) and tonsils (*dashed line*). Data in **a** and **b** are representative of ten independent experiments. **c** Proportion of Bc that express cell surface CD1d. Horizontal lines represent the means from ten donors

We next checked their ability to present NGcGM3. Sorted Bc from PBMCs and TMC were cultured with either NGcGM3-containing or NGcGM3-free liposomes, harvested, stained with 14F7, and analyzed by flow cytometry. Positive staining on the cultures incubated with NGcGM3-loaded liposomes compared to those grown with empty liposomes (Fig. 4a) indicated that human primary Bc from both tissues present NGcGM3 on their cell surface to a similar degree as for their CD1d expression (65 ± 5 %). The addition of aCD1d-blocking antibody significantly reduced NGcGM3 binding by approximately 50 ± 5 % (Fig. 4b), demonstrating that its surface presentation was at least partially dependent on CD1d because the CD1d-specific antibody sterically hampered interaction between CD1d and NGcGM3.

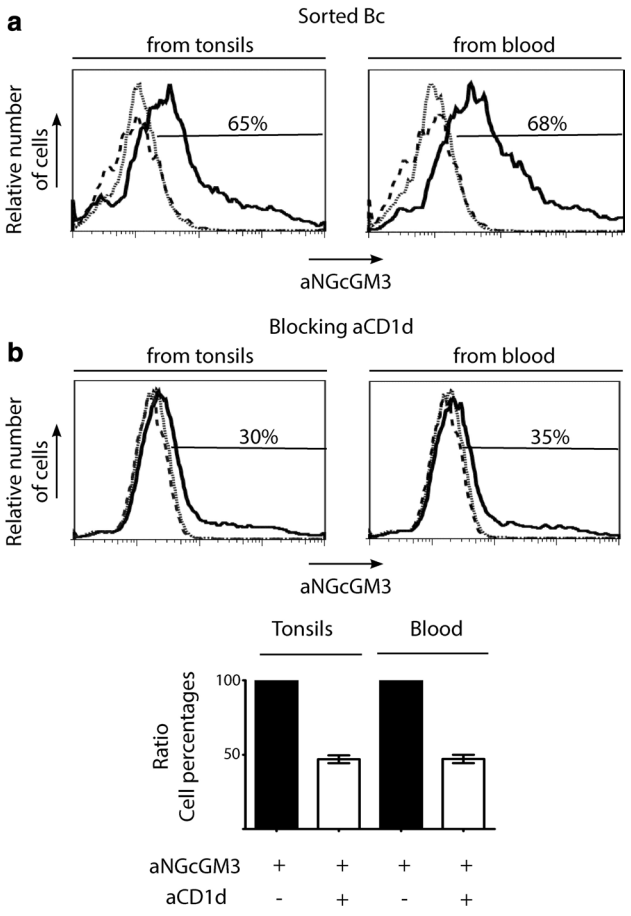

Fig. 4 Primary Bc bind NGcGM3 and binding can be inhibited by anti-CD1d. **a** Tonsillar (*left*) and peripheral blood (*right*) sorted Bc were incubated for 24 hs with empty liposomes (*gray line histogram*) or with NGcGM3-loaded liposomes (*black line histogram*), followed by staining with a mAb (14F7, referred as aNGcGM3 in the figure) or isotype-matched control mAb (*dashed histogram*). The numbers represent the percentages of NGcGM3-binding Bc. Data are representative of three independent experiments. **b** *Upper panels* tonsillar (*left*) and peripheral blood (*right*) sorted Bc were pretreated for 2 hs at 37 °C with aCD1d mAb (clone CD1d42) prior treatment as in **a**. The numbers represent the percentages of NGcGM3-binding Bc. Data are representative of three independent experiments. *Lower panel*: *bars* show the ratio between the percentage of cells binding NGcGM3 when CD1d is blocked and when it is not (mean of three independent experiments ± SEM)

Expansion of iNKT from PBMCs

We next determined the proportion of iNKT in the PBMCs of healthy donors by three-color flow cytometry using CD3, CD56, and 6B11 mAbs. NKT are thus called because they simultaneously express surface receptors from the T cell lineage (as CD3) and NK cells (as CD56). The 6B11 mAb was used for specifically detecting iNKT because it recognizes a unique determinant in the CDR3 region of the invariant (Vα24-JαQ) TCR chain of iNKT, being comparable to the CD1d tetramer [25]. The fraction of $CD3^+CD56^+$ cells (NKT) in a string of healthy donors varied from 2 and 13 %, and $CD3^+6B11^+$(iNKT) accounted for approximately half of such a population (Fig. 5a, upper panel). In view of the relatively small proportion of iNKT in PBMCs, we needed to enrich and expand iNKT from PBMCs ex vivo to acquire sufficient cells to perform co-culture experiments with autologous Bc. To achieve this, we sorted autologous $CD19^+$ and $CD3^+$ cells from PBMCs. We maintained $CD19^+$ cells in culture with IL2 and IL4, allowing for cell survival with minimal stimulation, albeit it was limited to 5–7 days. We cultured sorted $CD3^+$ cells (95 % purity) on medium supplemented with α-GalCer, assuming it could be presented by the 5 % of non-CD3 cells remaining in culture, and IL2, which induces iNKT survival and proliferation. Following 5–7 days, the phenotype of the α-GalCer-expanded cells was assessed by flow cytometry (Fig. 5a, lower panel). The method resulted in a selective enrichment of the $CD3^+CD56^+$ population of 100–200 % over the initial proportion. Importantly, all of the expanded $CD3^+CD56^+$ population resulted in $6B11^+$ cells, as previously reported for α-GalCer-expanded NKT [25]. To test the function of these α-GalCer-expanded cells, we measured IFNγ on culture supernatant. IFNγ secretion for this group was significantly higher than in non-secreting controls (autologous sorted Bc), indicating that the in vitro expanded iNKT modeled the functional status of the cells in vivo (Fig. 5b). Considering that our strategy leads to an enrichment in the iNKT population without establishing iNKT clones, lower IFNγ levels than those previously reported for iNKT clones established from healthy donors [19, 26] were expected.

Finally, before assessing the functional responses to NGcGM3 presentation, we wanted to determine whether we could visualize NGcGM3 binding to such expanded autologous iNKT population. Hence, we stained them with the NGcGM3-loaded CD1d-IgG1 fusion. Human iNKT were indeed stained by the NGcGM3/CD1d-IgG1 complex (Fig. 5c).

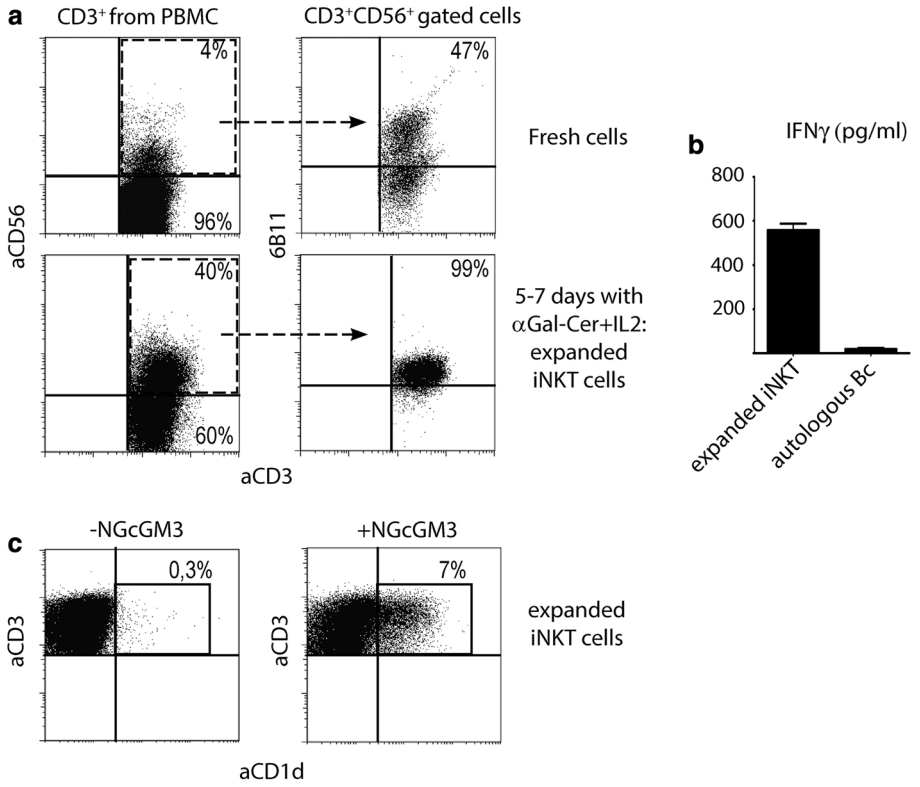

Fig. 5 Strategy used to enrich iNKT population from PBMCs. **a** *Upper panels* on the left, representative flow cytometry *dot plot* showing CD3 and CD56 expression by freshly isolated PBMCs gated on the CD3 population. The *numbers* indicate the percentages of $CD3^+CD56^+$ (NKT, *dashed square*) and $CD3^+CD56^-$ (T cells) in an average donor. *Right* flow cytometry *dot plot* showing the percentages of iNKT ($CD3^+CD56^+V\alpha24\text{-}J\alpha Q^+TCR$) within the $CD3^+CD56^+$ population (NKT). The numbers indicate the percentages of iNKT within the NKT population in fresh PBMCs from an average donor. The *dashed arrow* indicates gated cells as indicated on the top of the panel. *Lower panels* same as above using α-GalCer-expanded cells, as indicated on the right, instead of fresh cells. **b** IFNγ secretion measured by ELISA on the sorted $CD19^+$ and $CD3^+$ (95 %) culture supernatant (5 days). **c** CD1d-IgG1 was incubated ON with NGcGM3-loaded liposomes (*right*) or empty liposomes (*left*) and then used to stain human expanded iNKT. Detection of CD1d-IgG1 bound to the cell surface, as evidenced by PE-conjugated aIgG1 staining (referred to as aCD1d in the figure). Data are representative of four independent experiments

NGcGM3 promotes proliferation of in vitro expanded human iNKT in co-cultures with autologous primary Bc

The ability of human Bc to present NGcGM3 to iNKT was investigated by co-culturing peripheral Bc with autologous expanded iNKT in the absence or presence of NGcGM3. We used aKi-67 mAb and flow cytometry to identify and quantify the proliferating cell populations. Ki-67 antigen expression is strictly associated with cell proliferation because it is expressed in the nucleus of dividing cells but not during the G0 phase [27]. A four-color multiparametric assay was performed to distinguish between the populations in culture (CD19 for Bc and CD3 for NKT) and to exclude dead cells. Flow cytometry analysis revealed an average 2.5-fold increase in the expression of Ki-67 on the expanded iNKT (CD3 population) compared to vehicle-treated controls (Fig. 6). As expected, either expanded iNKT cultured in presence of NGcGM3 (Suppl. Figure 2) or those co-cultured with peripheral Bc in the absence of NGcGM3 exhibited some degree of proliferation due to treatment with α-GalCer (to achieve enrichment). The percentage of proliferating Ag Ki-67^+ iNKT on both controls was consistently comparable (Fig. 6a; Suppl. Figure 2). However, the actual proliferating fraction on such controls was quite variable between experiments compared to the fold increment of proliferating Ag Ki-67-expressing $CD3^+$ cells on co-cultures supplemented with NGcGM3, which was normalized to those controls (Fig. 6b). In the representative experiment shown in Fig. 6a, the frequency of proliferating Ag Ki-67-expressing $CD3^+$ cells increased from 6 to 14 % of the total $CD3^+$ cells. Importantly, when a blocking Ab against CD1d was added to iNKT-Bc co-cultures, the increment in the cell proliferation induced by Bc in the presence of NGcGM3 was significantly abrogated. Figure 6b quantitatively summarizes four experiments and provides statistical analysis. An unpaired

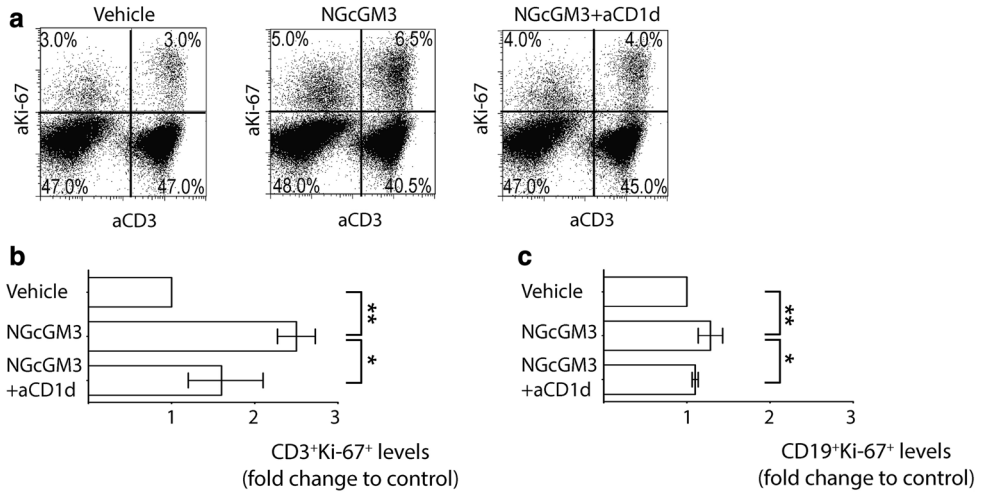

Fig. 6 Addition of NGcGM3 to co-cultures of Bc and iNKT induces proliferation that can be inhibited by aCD1d. **a** Representative flow cytometry *dot plots* showing expression of CD3 and Ki-67 in co-cultures of Bc and expanded iNKT in the presence of empty liposomes (*left*, referred as vehicle in the figure), NGcGM3-loaded liposomes (*middle*) and NGcGM3-loaded liposomes plus blocking aCD1d mAb (*right*). *Numbers* indicate the percentages of cells in the quadrants. **b** *Histogram* presenting the proportions of $CD3^+Ki\text{-}67^+$ cells in co-cultures, as in **a**, normalized to those of the empty liposomes (referred as vehicle in the figure) supplemented cultures. **c** *Histogram* presenting the proportions of $CD19^+Ki\text{-}67^+$ cells in co-cultures, as in **a**, normalized to those of the empty liposome (referred as vehicle in the figure) supplemented cultures. Data in **b** and **c** show the mean of the pool ratios ± SEM from four independent experiments. $*p < 0.05$ and $**p < 0.01$, unpaired *t* test

two-tailed *t* test was applied; the null hypothesis was that there were no differences in the Ki-67 expression of $CD3^+$ cells between NGcGM3-supplemented cultures and vehicle-supplemented cultures. The same test was applied between NGcGM3-supplemented cultures and aCD1d + NGcGM3-supplemented cultures. The null hypothesis was disproven for the first case, as well as for the latter ($p < 0.01$ and $p < 0.05$, respectively). Considering all of these results, we concluded that in vitro expanded iNKT are activated by CD1d-expressing Bc that are loaded with NGcGM3.

It would subsequently be expected that activated iNKT communicate with the CD1d-expressing Bc to provide helper signals. Hence, in the same assay, we analyzed Ki-67-expressing $CD19^+$ cells. We found a slight but reproducible tendency toward an increased frequency of Ki-67-expressing $CD19^+$ cells in NGcGM3-supplemented co-cultures compared to vehicle (Fig. 6c), which was significantly abrogated by CD1d treatment. Our results suggest that NGcGM3-activated iNKT help Bc, but they are less efficient than α-GalCer in stimulating proliferation. Notably, the fold increment of proliferating Ki-67-expressing $CD19^+$ cells over vehicle control resulted quite marginal if compared with the fold change detected for the iNKT population. Considering we used α-GalCer-expanded iNKT for the co-cultures, this was not unexpected. It is well known, at least for murine cells, that α-GalCer treatment of iNKT biases their cytokine profile toward a Th2 phenotype [28–30], stimulating Bc proliferation.

Discussion

NGcGM3 is a ganglioside that is abundantly expressed on human cancers, such as melanoma, breast carcinoma, neuroectodermal tumors, and non-small cell lung cancer cells. Because NGcGM3 is not expressed on normal human tissues, it has been a target of choice for immunotherapy. Antibodies against NGcGM3 were found to be more prominent in patients with carcinomas than other diseases [31]. The immune mechanism that generates such xeno-autoantibodies has not been described thus far. In this paper, we demonstrate that CD1d can bind NGcGM3 and activate iNKT. Although the molecular principles that rule the variety of glycolipids presented by human CD1d are not yet fully understood, there have been various attempts to identify those molecules [17, 32]. It is clear from such studies that most lipids presented by CD1d molecules do not activate iNKT.

In fact, it has been shown that synthetic preparations of β-glucosylceramide (β-GluCer, a glycosylceramide present in all cell types) result strong activators of iNKT [33]. However, recently, Kain et al. [34] discovered mammalian α-linked monoglycosylceramides (such as α-GalCer) as contaminants of β-GluCer commercial stocks and demonstrated that such tracing compounds were essentially the ones triggering activation of the iNKT exposed to β-glucosylceramides. The authors also outlined the putative cellular catabolic processes that can generate mammalian

α-GalCer. We cannot rule out contaminating traces of those metabolites in the commercial NGcGM3 that we used because it was purified from mouse hybridoma cells, and it is nearly impossible to detect contaminations below 0.5–1.0 % in glycolipid preparations. Notably, β-GluCer is structurally similar to α-GalCer; hence, they are likely to be co-purified. On the other hand, considering the chemical structure of NGcGM3, it would be expected that putative impurities contaminating preparations would be a mixture of sialyllactosylceramides (such as NAcGM3 and other GM3 of higher molecular mass) instead of monoglycosylceramides [35]. Furthermore, we used an alternative source of NGcGM3 (a kind gift from the CIM, Havana, Cuba) and obtained the same results as with the commercial stock.

While others have reported on the binding of GM3 to human CD1d [17] and murine CD1d [36, 37], we are the first to report the activating effect of NGcGM3 on human iNKT. Interestingly, Park et al. [37] found that NAcGM3 suppresses the T helper-like response of murine NKT. This is consistent with our findings; NAcGM3 does not constitute a xeno-autoantigen because it is normally expressed by nearly all tissues.

Circulating and tonsillar Bc represent different Bc pools with different functional responses [24]. However, we found that CD1d is not only expressed at comparable levels by both B cell populations, but NGcGM3 also specifically binds at comparable levels on CD1d expressed on the two B cell subsets, as confirmed through an aCD1d.

In the present study, we developed a culture system in which a population of cells, enriched with $CD3^+CD56^+$ lymphocytes, was obtained by ex vivo growth of practically sorted $CD3^+$ cells (95 %) from PBMCs from healthy donors in medium supplemented with α-GalCer and IL2. It is important to note that the relatively short time of culture interfered with the generation of a pure, homogenous, $CD3^+CD56^+$ cell population (Fig. 5a). Importantly, the brief scheme allowed us to perform co-cultures with Bc that were simultaneously isolated from the same patient.

Although other authors have reported the expansion of NKT in long-term PBMC cultures supplemented with INFγ, IL2, and anti-CD3 mAb [38, 39], the NKT expanded under these conditions, also called NKT-like, are not restricted to CD1d and do not express the invariant TCR. The culture scheme we used allowed for collection of NKT that can bind 6B11 (Fig. 5a), thus making them CD1d restricted.

An earlier in vitro co-culture study of Bc with expanded human iNKT [12] showed that iNKT can induce human Bc proliferation in vitro in the presence and absence of α-GalCer. Our findings further confirm those observations and extend them by demonstrating that NGcGM3 mediates productive cross talk between autologous human iNKT and Bc, which requires CD1d molecules on the Bc surface and induces a significant increase in the percentage of proliferating cells on the NKT population compared to cultures that were not supplemented with ganglioside. Further work into improving the culture system is required to elucidate whether the fold increase in proliferating Ki-67-expressing $CD19^+$ cells over vehicle control is of physiological relevance.

It has already been reported that CD1d-restricted iNKT–Bc interactions match MHC class II-restricted T-Bc cognate interactions [16]. What is the significance of the NGcGM3-mediated B/iNKT interaction that we describe? It is tempting to speculate that human ganglioside-specific Bc receive cognate help by iNKT in vivo and produce Abs against glycosphingolipid xeno-autoantigens like NGcGM3, as detected in human serum. Notably, such aNGcGM3 xeno-autoantibodies can mediate tumor growth inhibition and serve as biomarkers for early carcinoma detection [31]. Immune responses can either support tumor development or destruction, which depends on the fine balance between the induction of general chronic inflammation and immune attack that is specifically directed to abnormal cells. Therapeutic interventions that bias this balance require full comprehension of the immune interactions involved. In any case, this issue requires further investigation. For instance, it would be interesting to investigate the cytokines involved in the NGcGM3-mediated B/iNKT cross talk in the future, as well as to fully characterize its functional consequences.

In conclusion, we characterized the tumor Ag NGcGM3 as a novel endogenous CD1d ligand that can mediate a productive interaction between human iNKT and Bc. Our results advance our understanding of the immune interactions involved in tumor immunology and they may have implications for cancer immune therapeutics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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