A shift from *N*-glycolyl- to *N*-acetyl-sialic acid in the GM3 ganglioside impairs tumor development in mouse lymphocytic leukemia cells

Ana Victoria Casadesús · Yuniel Fernández-Marrero · Marilyn Clavell · José Alberto Gómez · Tays Hernández · Ernesto Moreno · Alejandro López-Requena

Abstract Humans, in contrast to other mammals, do not synthesize *N*-glycolyl-neuraminic acid (Neu5Gc) due to a deletion in the gene (*cmah*) encoding the enzyme responsible for this conversion, the cytidine monophospho-*N*-acetyl-neuraminic acid hydroxylase (CMP-Neu5Ac hydroxylase). The detection of considerable amounts of Neu5Gc-sialoconjugates, in particular gangliosides, in human malignancies makes these antigens attractive targets for immunotherapy, in particular with monoclonal antibodies (mAbs). We have previously described a GM3(Neu5Gc) ganglioside-specific mAb, named 14F7, with the ability to kill tumor cells in a complement-independent manner. Silencing the *cmah* gene in GM3(Neu5Gc)-expressing L1210 mouse lymphocytic leukemia B cells caused the abrogation of this cytotoxic effect. We now show that *cmah*-silenced L1210 cells (*cmah*-kd) express a high level of GM3(Neu5Ac) and have an impaired ability for anchorage-independent cell growth and tumor development *in vivo*. No evidences of increased immunogenicity of the *cmah*-kd cell line were found. These results provide new evidences on the role of GM3(Neu5Gc), or Neu5Gc-sialoconjugates in general, in tumor biology. As an important tool in this study, we used the humanized version (here referred to as 7C1 mAb) of a recently described, rationally-designed mutant of 14F7 mAb that is able to bind to both GM3(Neu5Gc) and GM3(Neu5Ac). In contrast to its parental antibody, the humanized 14F7 (14F7hT) mAb, 7C1 mAb was able to kill not only GM3(Neu5Gc)-expressing L1210 wild type cells, but also GM3(Neu5Ac)-expressing *cmah*-kd cells, which endorses this antibody as a potential agent for cancer immunotherapy.

Keywords cmah · Cytotoxic antibody · GM3 ganglioside · Neu5Gc · Tumorigenicity

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Introduction

Sialic acid-containing glycosphingolipids, known as gangliosides, have important roles in several cellular events [1, 2]. Gangliosides are essential components of the so-called lipid rafts, which are membrane microdomains where these glycolipids associate with proteins that participate in cell signaling cascades [3–5]. It has been well documented that ganglioside expression changes during malignant transformation [6, 7] and therefore these molecules are considered attractive targets for immunotherapeutic strategies [8, 9]. An intriguing feature is the overexpression by tumor cells of gangliosides containing *N*-glycolyl-neuraminic acid (Neu5Gc) [10, 11], which is absent, or detected only in trace

amounts, in human normal tissues [12]. The absence of this form of sialic acid in humans is due to the inactivation of the enzyme responsible for its synthesis, the cytidine monophospho-N-acetyl-neuraminic acid hydroxylase (CMP-Neu5Ac hydroxylase), by an exon deletion in the coding $cmah$ gene [13]. Nevertheless, Neu5Gc-sialoconjugates have been detected in human tissues, but their expression has been attributed to dietary incorporation [14–16]. The preferential expression of Neu5Gc by malignant cells would derive from their higher metabolic rate [17], as well as the induction by hypoxic conditions of a sialic acid transporter [18, 19].

The GM3(Neu5Gc) ganglioside in particular has been detected in breast cancer [11, 20–22], melanoma [20, 23–25], non-small cell lung cancer [26–28], Wilms tumors [29], neuroectodermal tumors [30–32], sarcomas and thyroid carcinomas [32] and malignancies from the digestive [33] and genitourinary [34] systems. Three different immunotherapeutic approaches have focused on this molecule as their target [35]: a molecular vaccine [36–38], an anti-idiotypic antibody-based vaccine [39] and the use of monoclonal antibodies (mAbs) able to discriminate between GM3(Neu5Gc) and GM3(Neu5Ac) [20, 40].

One of these mAbs is 14F7 [20]. An interesting property of 14F7 mAb is its ability to kill tumor cells expressing the ganglioside in a complement-independent manner [41, 42]. A humanized version of this antibody (14F7hT), obtained by modification of potential human T cell epitopes [43], retained the properties of the mouse and chimeric antibodies [22]. Clinical trials are currently being designed for the treatment of cancer patients.

The mechanism of cell death induced by 14F7 mAb is not completely understood [44]. Interestingly, 14F7 mAb readily binds to mouse normal B lymphocytes, but is unable to kill them. In contrast, the antibody is cytotoxic in GM3(NeuGc)-expressing L1210 mouse lymphocytic leukemia B cells [42]. When treated with a glucosylceramide synthase inhibitor [42, 45], or when the $cmah$ gene expression was stably silenced with a specific short hairpin RNA [45], these cells became insensitive to treatment with 14F7 mAb.

Following a structure-based rational design, it was recently isolated from a phage-displayed library a mutated version of 14F7 mAb with the ability to recognize GM3(Neu5Ac) in addition to GM3(Neu5Gc) [46]. The mutations contained in the described CR1/RRQ antibody were later transferred (unpublished data) to the humanized version of 14F7 mAb (14F7hT), resulting in a humanized antibody, named 7C1, which binds to both gangliosides. In this work, we studied two features of the $cmah$-silenced L1210 cells ($cmah$-kd): their sensitivity to 7C1 mAb direct (complement-independent) killing and their tumorigenic properties, both *in vitro* and *in vivo*. Consistently with their

inability to synthesize Neu5Gc-sialoconjugates, we demonstrated that $cmah$-kd L1210 cells express high amounts of GM3(Neu5Ac) instead of GM3(Neu5Gc). In contrast with parental 14F7hT mAb, 7C1 mAb was cytotoxic in both wild type and $cmah$-kd L1210 cells, in a complement-independent manner. The latter cells exhibited a diminished capacity for anchorage-independent growth and for developing subcutaneous tumors in syngeneic mice, without evidences of increased immunogenicity.

Materials and methods

Cells

L1210 mouse lymphocytic leukemia and HEK-293T cells (purchased from the American Type Culture Collection; ATCC, Rockville, MD); $cmah$-kd L1210 cells (L1210 cells transduced with a $cmah$-specific short hairpin RNA; [45]); L1210 mock (transduced with an empty lentiviral vector); hybridomas producing mouse 14F7 [20] or anti-colorectal cancer glycoprotein C5 [47] mAbs; NS0 transfectomas producing humanized 14F7 (14F7hT) mAb [22], chimeric C5 mAb or anti-human CD6 humanized T1 (T1h) mAb [48]; and Sp2/0 transfectoma producing 7C1 mAb (unpublished data); were cultured in Dulbecco's modified Eagles medium (DMEM) (Gibco-BRL, Paisley, UK) supplemented with 10 % heat inactivated fetal calf serum (FCS; Hyclone, Logan, UT), antibiotic mixtures of penicillin (100 U/mL) and streptomycin (100 μg/mL), and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO).

Mock L1210 cells were obtained by infection with an empty lentiviral vector (Sigma-Aldrich), according to manufacturer instructions. Briefly, a semi-confluent culture of HEK-293T cells was transfected using Lipofectamine-2000® (Invitrogen, Carlsbad, CA) with plasmids coding the lentiviral capside and an empty pLK0.1-puro plasmid. Culture medium was changed after 24 h and virions were harvested 72 h after transfection, sterile filtered, quantified and immediately used. Transduction of 3×10^6 L1210 cells was performed with 10^8 viral particles. Puromycin (Sigma-Aldrich) at 2.5 μg/mL was added as selective agent.

Mice

Female DBA/2 mice, 6 to 8 week old, were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). Animals were housed and bred in a barrier-maintained room and studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Center of Molecular Immunology.

Antibodies and gangliosides

Mouse 14F7 and C5 mAbs (IgG1, κ); chimeric C5, and humanized 14F7 (14F7hT), T1 (T1h) and 7C1 mAbs (human IgG1, κ); were purified by protein A affinity chromatography (Pharmacia, Uppsala, Sweden) and analyzed by SDS-PAGE under reducing conditions. Mouse antibodies were purified from mouse ascitic fluid, whereas chimeric and humanized antibodies were purified from transfectoma culture supernatants. The specificity of the purified antibodies was confirmed by enzyme-linked immunosorbent assay (ELISA) and protein concentration was estimated by optical density at 280 nm. Anti-GM3(Neu5Ac) GMR6 mAb was purchased from Seikagaku BioBusiness Corporation (Tokyo, Japan). Mouse total IgM were purified from serum by gel exclusion chromatography using a Sephacryl S-300 high resolution column (Pharmacia) equilibrated with PBS containing 0.5 M NaCl.

The procedures for GM3(Neu5Gc) and GM3(Neu5Ac) isolation from horse and dog erythrocytes, respectively, as well as glycolipid extraction from cells were described elsewhere [49]. Sialic acid and total proteins were quantified by a resorcinol-HCl colorimetric assay [50] and the Lowry method [51], respectively.

Thin layer chromatography

Thin layer chromatography of gangliosides was performed as previously described [52], with minor modifications. Briefly, high performance thin layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) and chloroform/methanol/0.25 % KCl in 2.5 M NH$_3$ (5:4:1, v:v:v) as solvent were used for the glycolipid fractionation. The orcinol reagent was used for chemical staining. For immunostaining, after chromatography plates were soaked for 75 s on hexane containing 0.1 % polyisobutylmethacrylate (Sigma-Aldrich). After drying and blocking with PBS-milk 4 %, plates were incubated with 5 μg/mL of antibody, washed with PBS, and an alkaline phosphatase-conjugated goat anti-human IgG antibody (Sigma-Aldrich) was added. The reaction was developed with an alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, CA).

Mass spectrometry analysis

Gangliosides were extracted from HPTLC plates using a procedure previously described [53], with minor modifications. The silica gel was scrapped off the plate with a scalpel and transferred into glass tubes. Gangliosides were extracted with 200 μL of chloroform/methanol/water (30:60:8, v:v:v) under sonication in an ultrasound bath, for 30 s. This procedure was repeated three times. The slurries were centrifuged for 2 min and the pooled supernatants were dried in a vacuum centrifuge, Proteomic CentriVap® Concentrator (Labconco, Kansas City, MO), at 20 °C and re-dissolved in 20 μL of a mixture of chloroform/methanol (50:50, v:v).

The TLC extracts were analyzed by matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI TOF/TOF) mass spectrometry, without any further purification. MALDI mass spectra were obtained using an AXIMA Performance MALDI TOF/TOF mass spectrometer (Shimadzu Kratos, Manchester, UK) with a 357 nm N$_2$ laser using delayed extraction, in negative linear mode. The glycolipid samples (0.5–1.0 μL) were deposited directly on the MALDI target plate and covered with the same amount of matrix solution. The solid ionic matrix α-cyano-4-hydroxycinnamic acid/aniline (CHCA/ANI) was prepared just before use, by adding 2.4 μL of base to a solution of 5 mg/mL CHCA (1 mL) in acetonitrile/water (1:1, v/v, 0.2 % trifluoroacetic acid) [54]. The mixture was vortexed and sonicated for 10 min before use. Two hundred laser shots were accumulated for each mass spectrum, with a 30 Hz laser repetition rate. In this analysis, signals between m/z 1,000 to 2,000 were collected. The data was processed using mMass (5.4) software [55].

The spectra were externally calibrated against five peptide ions. Then, the ganglioside abundances were normalized to the most abundant peak in the mass spectra and finally, the mass peaks were searched for matches in the mMass databases. The tolerance for the search was 0.2 Da. The peaks corresponding to GM3(Neu5Gc) species were confirmed with previous reports [11].

Flow cytometry analysis

Mean fluorescence intensity and percentage of stained cells were determined in a FACScan instrument (Becton Dickinson, NJ). The FlowJo 5.7.2 software was used to analyze a total of 10^4 cells acquired in every fluorescence-activated cell sorting assay.

Expression of GM3(Neu5Gc) or GM3(Neu5Ac) on the cell lines was detected with 10 μg/mL of anti-GM3(Neu5Gc) 14F7hT mAb or 20 μg/mL of a mouse anti-GM3(Neu5Ac) antibody, followed by a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG antibody (Dako, Denmark) or a phycoerythrin (PE)-conjugated rat anti-mouse IgM antibody (eBioscience, San Diego, CA), respectively. Binding of 7C1 mAb was revealed with the former conjugate.

Expression of the other surface molecules was determined with the following antibodies: FITC-conjugated rat anti-mouse B220, MHCII (I-A/I-E) and CD40, and hamster anti-mouse CD80 (B7-1) (BD Biosciences Pharmingen, NJ); and FITC-conjugated rat anti-mouse CD86 (eBioscience).

The presence of antibodies recognizing wild type and/or *cmah*-kd cells in sera from mice inoculated with these cells (see below) was assessed using a FITC-conjugated rabbit anti-mouse IgG+IgM (H+L) antibody (Jackson Immunoresearch, Westgrove, PA). Sera were taken before and 4 weeks after tumor challenge and were assayed at 1:50 dilution.

Cytotoxic assay

Cells in culture medium with 1 % bovine serum albumin (BSA) at 10^6 cells/mL were incubated with 100 μg/mL of antibodies in an atmosphere of 5 % CO_2 at 37 °C for 3 h. Then, they were washed, resuspended in PBS with propidium iodide (PI; Sigma-Aldrich) 10 μg/mL, and analyzed by flow cytometry. Dead cells were determined by PI internalization. Cell death percentages were considered non-spontaneous (not due to experimental manipulation) only when they were at least two-fold higher than the percentages observed for untreated cells.

Cell proliferation assay

Proliferation of wild type, *cmah*-kd and mock L1210 cells was measured using the alamarBlue® cell proliferation assay (AbD Serotec, Oxford, UK) according to manufacturer instructions. Briefly, 3×10^4 cells were seeded in 96-well flat bottom culture plates. After 42 h, the reagent was added and the absorbance was measured after 6 h incubation.

Soft agarose colony formation assay

The ability of the cell lines for anchorage-independent growth was assayed by resuspending 10^4 cells in 2 mL of medium with 0.35 % low melting point agarose (Sigma-Aldrich) and seeding in triplicate into 6-well plates containing a 2 mL layer of solidified medium with 0.6 % agarose. After 10 days, the colonies were counted under microscopy.

Subcutaneous tumor growth assay

DBA/2 mice were subcutaneously inoculated with different amounts (10^5, 5×10^5 or 10^6) of wild type, *cmah*-kd or mock L1210 cells. Tumor onset was monitored by palpation and the largest perpendicular diameters were measured with a caliper. Tumor volume was calculated using the formula: $\pi/6 \times \text{length} \times \text{width}^2$. Tumors from one mouse from each group were harvested and disrupted with cell strainers (40 μm; Bedford, MA); cells were stained with 14F7hT and 7C1 mAbs and

analyzed by flow cytometry. Sera were collected to analyze the presence of anti-cell antibodies, also by flow cytometry, as described above.

Statistical analysis

Statistical analysis was performed using the program GraphPad Prism 5.03. The one-way ANOVA test with Tukey post-test was used to analyze data from the soft agarose colony formation assay. Data from the sialic acid quantification and proliferation experiments were analyzed using the Kruskal-Wallis test with Dunn post-test. The log-rank test was used for Kaplan-Meier curves of tumor-free survival.

Results

cmah-kd L1210 cells express a high level of GM3(Neu5Ac) and are sensitive to anti-GM3(Neu5Gc/Neu5Ac) 7C1 mAb-induced cell killing

GM3(Neu5Gc) is the major ganglioside expressed by L1210 mouse lymphocytic leukemia cells [42]. In a previous work, the synthesis of Neu5Gc was impaired with a short hairpin RNA specific for the gene encoding the CMP-Neu5Ac hydroxylase (*cmah* gene), thus obtaining a *cmah*-knockdown (*cmah*-kd) cell line [45]. We now show that, in contrast to wild type cells and cells transduced with an empty lentiviral vector (mock), *cmah*-kd cells express a large amount of GM3(Neu5Ac), as proven by staining with a commercial anti-GM3(Neu5Ac) mAb (Fig. 1a). This result was further confirmed by thin layer chromatography (Fig. 1b) and mass spectrometry analysis (Fig. 1c) of glycolipids extracted from the cells. Highly abundant singly charged ions at m/z 1151.44, 1153.43, 1179.54, 1207.60, 1235.59, 1261.79, 1263.86, assigned to GM3(Neu5Ac) components with d18:1-C16:0, d18:0-C16:0, d18:1-C18:0, d18:1-C20:0, d18:1-C22:0, d18:1-C24:1 (15z) and d18:1-C24:0 [sphingosine-fatty acid (unsaturation position)], respectively, were identified for *cmah*-kd cells, while the equivalent GM3(Neu5Gc) species were detected in wild type and mock cells (Fig. 1c). As previously reported [42], and as shown in Fig. 1b, wild type cells express a small amount of GM3(Neu5Ac), whose presence was now confirmed by mass spectrometry (peaks to m/z 1151, 1261 y 1263) in both, wild type and mock cells (Fig. 1c). Thus, *cmah*-kd cells express a high level of GM3(Neu5Ac) with no GM3(Neu5Gc) being detected, whereas in wild type and mock cells the latter ganglioside clearly predominates. No differences were observed between the three cell lines regarding the total content of sialic acid (Fig. 1d), as an additional evidence that the main effect of *cmah* silencing was a shift from GM3(Neu5Gc) to GM3(Neu5Ac).
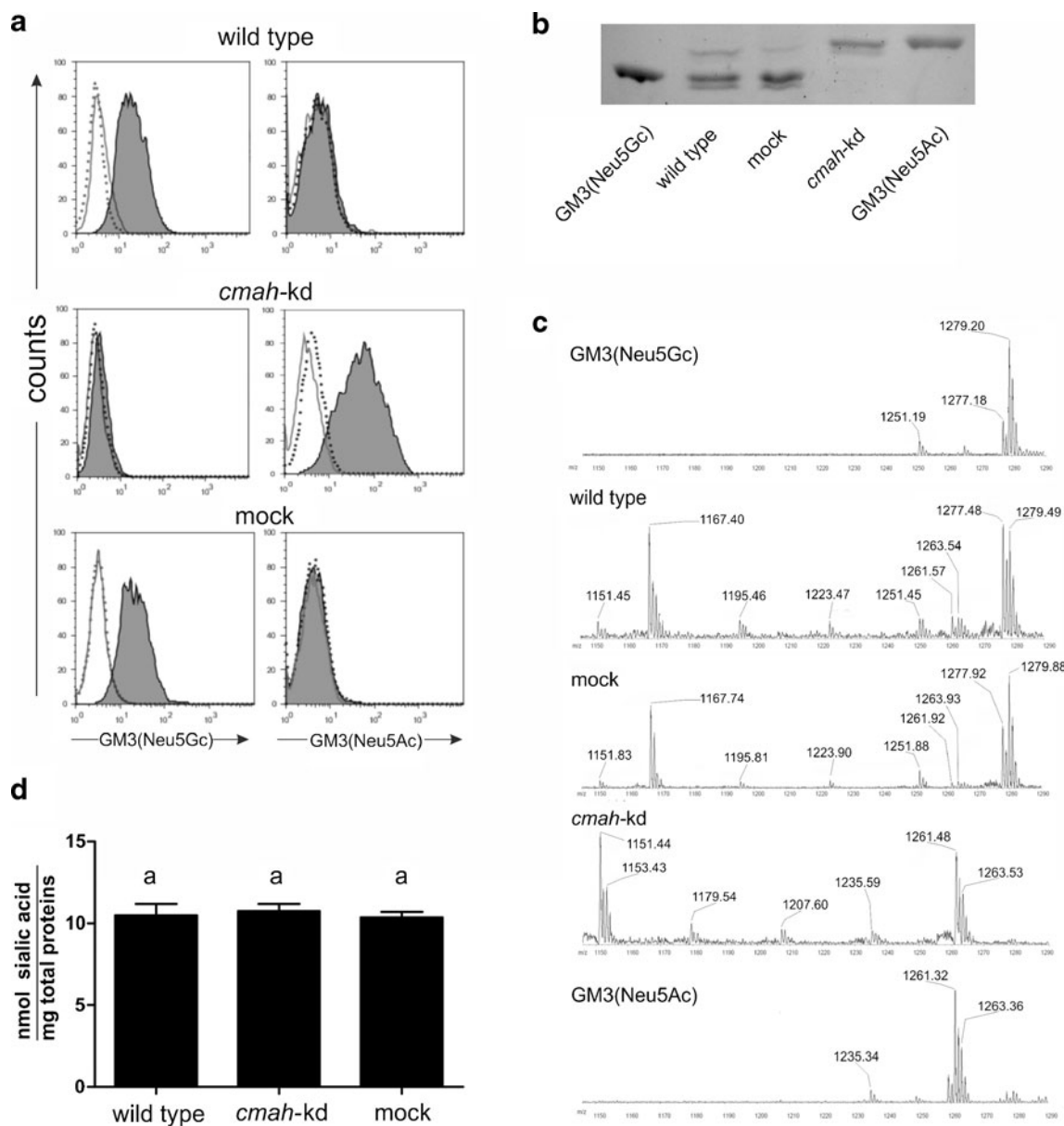
Fig. 1 *cmah*-kd L1210 cells express a high level of GM3(Neu5Ac) instead of GM3(Neu5Gc). **a** Wild type and *cmah*-kd L1210 cells were incubated with 10 μg/mL of 14F7hT mAb [anti-GM3(Neu5Gc)] or 20 μg/mL of a mouse anti-GM3(Neu5Ac) antibody (*filled histograms*). Binding was determined by flow cytometry, using a FITC-conjugated rabbit anti-human IgG antibody or a PE-conjugated rat anti-mouse IgM antibody, respectively (*empty*, *grey line histograms*: cells incubated with conjugate alone). L1210 cells transduced with an empty lentiviral vector (mock), isotype-matched chimeric C5 mAb and mouse total IgM (*empty*, *dotted line histograms*) were used as controls. **b**–**d** Total glycolipids were extracted, visualized by orcinol staining in high performance thin layer chromatography (HPTLC) plates (**b**) and analyzed by MALDI-TOF/TOF mass spectrometry (**c**). **d** Sialic acid quantification. Data correspond to three independent experiments. Statistical analysis was performed using the Kruskal-Wallis test with Dunn post-test. No significant differences were found (same letters; $P>0.05$)

7C1 mAb is a version of 14F7hT mAb containing mutations that rendered the antibody reactive to both GM3(Neu5Gc) and GM3(Neu5Ac) [46]. The binding properties of 7C1 mAb were demonstrated by thin layer chromatography-immunostaining of ganglioside standards. While the parental 14F7hT mAb recognized only GM3(Neu5Gc), 7C1 mAb reacted also with GM3(Neu5Ac) (Fig. 2a). In contrast to 14F7hT mAb, 7C1 mAb bound also to *cmah*-kd L1210 cells (Fig. 2b).

14F7 mAb is cytotoxic in GM3(Neu5Gc)-expressing tumor cells, in a complement-independent manner [42]. Taking into account the similar reactivity of 7C1 mAb with this ganglioside, we decided to investigate whether the mutated antibody also exhibited this ability. Using a
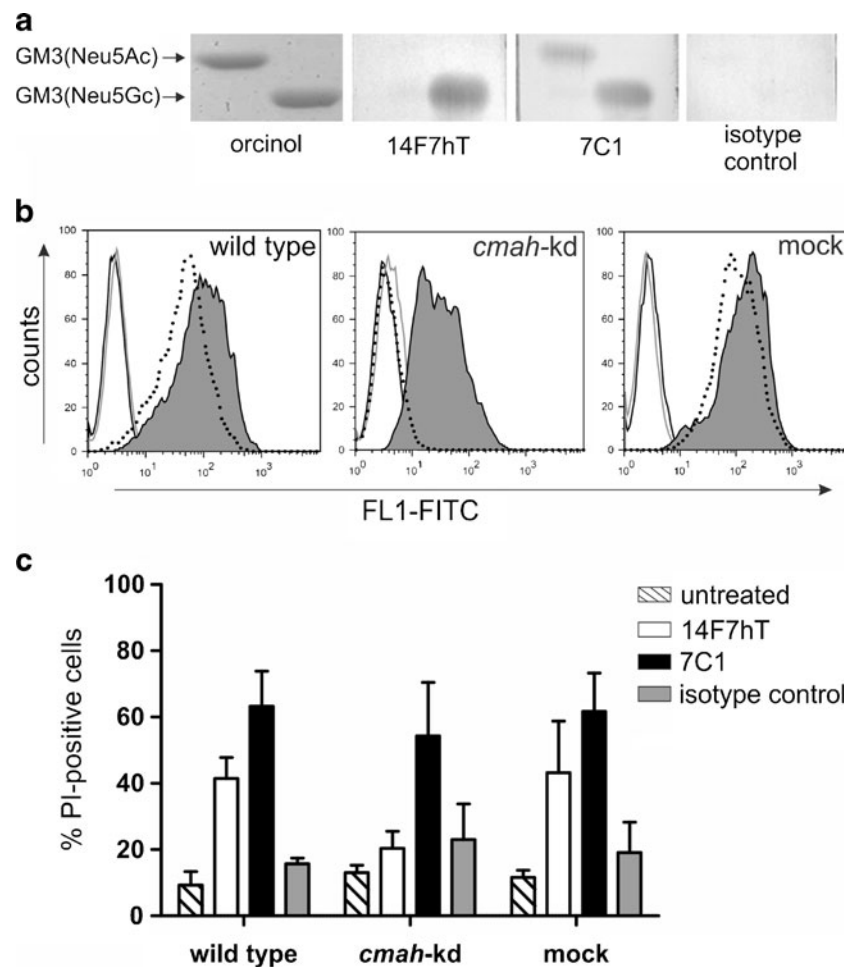
Fig. 2 Both wild type and *cmah*-kd L1210 cells are sensitive to anti-GM3(Neu5Gc/Neu5Ac) 7C1 mAb-induced cell killing. **a** Binding of 7C1 mAb to both GM3(Neu5Gc) and GM3(Neu5Ac). Ganglioside standards in HPTLC plates were stained with parental 14F7hT mAb and its mutated version 7C1 mAb. Isotype-matched T1h mAb was used as negative control. Chemical staining was done with orcinol. **b** Binding of 7C1 mAb to both wild type and *cmah*-kd L1210 cells. Cells were incubated with 10 μg/mL of 7C1 (*filled histograms*) or 14F7hT mAbs (*empty, dotted line histograms*). Binding was determined by

flow cytometry, using a FITC-conjugated rabbit anti-human IgG antibody (*empty, grey line histograms*: cells incubated with conjugate alone). Mock L1210 cells and isotype-matched chimeric C5 mAb (*empty, black line histograms*) were used as controls. **c** Cytotoxic assay. Cells were incubated with 7C1 and 14F7hT mAbs at 100 μg/mL for 3 h at 37 °C. Cell death was evaluated by propidium iodide (PI) uptake and flow cytometry analysis. *Numbers* indicate percentage of PI-incorporating cells. Chimeric C5 mAb was used as control. Data correspond to two independent experiments

cytofluorimetric propidium iodide-exclusion assay (Fig. 2c), we showed that 7C1 mAb is as much, or even more effective than the parental antibody in killing cells without the need of complement. Moreover, unlike 14F7hT mAb, it also exhibited this activity in *cmah*-kd L1210 cells, indicating that not only binding to GM3(Neu5Gc), but also to GM3(Neu5Ac) can trigger the cytotoxic mechanism. Mock cells behaved identically to wild type cells, being stained and killed by both 14F7hT and 7C1 mAbs (Fig. 2b, c).

cmah-kd L1210 cells are less tumorigenic than wild type cells

In a cell proliferation assay, no differences were found between wild type, *cmah*-kd and mock cells (Fig. 3). We
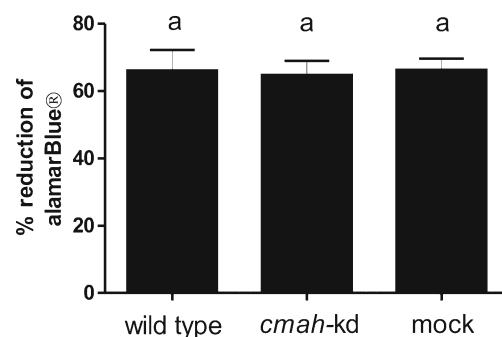

Fig. 3 *cmah*-kd L1210 cells proliferate indistinguishably from wild type cells. Cells were grown in 96-well plates and the alamarBlue® reagent was added 6 h before absorbance measurement. Data correspond to three independent experiments. Statistical analysis was performed using the Kruskal-Wallis test with Dunn post-test. No significant differences were found (same letters; *P*>0.05)

then assessed whether the modification in sialic acid type had some effect on the tumorigenic properties of *cmah*-kd L1210 cells. First, an *in vitro* assay was performed. As shown in Fig. 4, *cmah*-kd cells displayed a significantly lower capacity than wild type cells to form colonies in soft agarose. Both wild type and mock L1210 cells formed a much higher number of colonies than *cmah*-kd cells. Therefore, either the lack of expression of GM3(Neu5Gc) or other Neu5Gc-sialoconjugates in general, or the expression of GM3(Neu5Ac) or a combination of these, rendered these cells less capable of anchorage-independent growth.

Next, we assessed whether the effect of reduced tumorigenicity occurred also *in vivo*. Syngeneic DBA/2 mice were subcutaneously challenged with wild type and *cmah*-kd L1210 cells and tumor onset was monitored. We evaluated three tumor loads: 10^5 (low), 5×10^5 (medium) and 10^6 (high), for each of the two cell lines. No tumor was detected with the "low" amount of cells (data not shown). In the case of *cmah*-kd cells, no significant differences were found between the "medium" and "high" cell loads (Fig. 5a).

cmah-kd L1210 cells, however, exhibited a much lower capacity to form subcutaneous tumors than their wild type counterparts. While tumors developed in all of the animals receiving the wild type cells, the average tumor incidence for *cmah*-kd cells was less than 35 %, irrespective of tumor load ("medium": wild type 19/19, *cmah*-kd 6/18; "high": wild type 19/19, *cmah*-kd 6/19) (Fig. 5a). Moreover, we observed an increased latency for tumor onset (Fig. 5b). No differences in tumor onset or growth were found between wild type and mock cells (tumor incidence: wild type and mock 10/10, *cmah*-kd 2/10) (Fig. 5c, d), indicating that the behavior of *cmah*-kd cells was due to the introduced gene modification and not to any other alteration attributable

to the transduction process. Furthermore, *cmah* silencing was demonstrated to be stable, as cells taken from an explanted tumor remained negative to 14F7hT mAb staining while being recognized by 7C1 mAb (Fig. 5e).

It was thus demonstrated both *in vitro* and *in vivo* that *cmah*-kd L1210 cells, in which the Neu5Gc content is shifted towards the *N*-acetyl type of sialic acid, exhibit a reduced tumorigenicity.

cmah-kd L1210 cells do not appear to be more immunogenic than wild type cells

Finally, we explored the possibility that the modification in sialic acid type in the *cmah*-kd L1210 cells had an influence on the immunogenicity of the cell line. We first assessed the presence of circulating antibodies recognizing the cells in mice inoculated with wild type or *cmah*-kd cells. Sera taken 4 weeks after the challenge were evaluated by flow cytometry against both cell lines. As shown in Fig. 6a, no differences between the means of preimmune sera and sera taken from either group of animals, tested against both cell lines, were observed. Thus, no clear humoral response against either wild type or *cmah*-kd L1210 cells was detected.

The immunogenicity of L1210 subclones has been linked to the simultaneous expression of MHCII and the costimulatory molecules CD80 (B7-1), CD86 (B7-2) and CD40. Parental L1210 cells not expressing them are therefore more tumorigenic [56]. Concordantly, no expression of any of these molecules was detected in wild type cells. The same result was obtained for the *cmah*-kd cells (Fig. 6b), further suggesting that the modification in sialic acid type did not alter the immunogenicity of the cells.

Fig. 4 *cmah*-kd L1210 cells have a diminished capacity for anchorage-independent growth. Cells were grown in soft agarose and colonies counted after 10 days. **a** Statistical analysis was performed using the one-way ANOVA test with Tukey post-test. Different letters indicate significant differences ($P < 0.001$). Data correspond to three independent experiments. **b** Representative microphotographs (40× magnification)
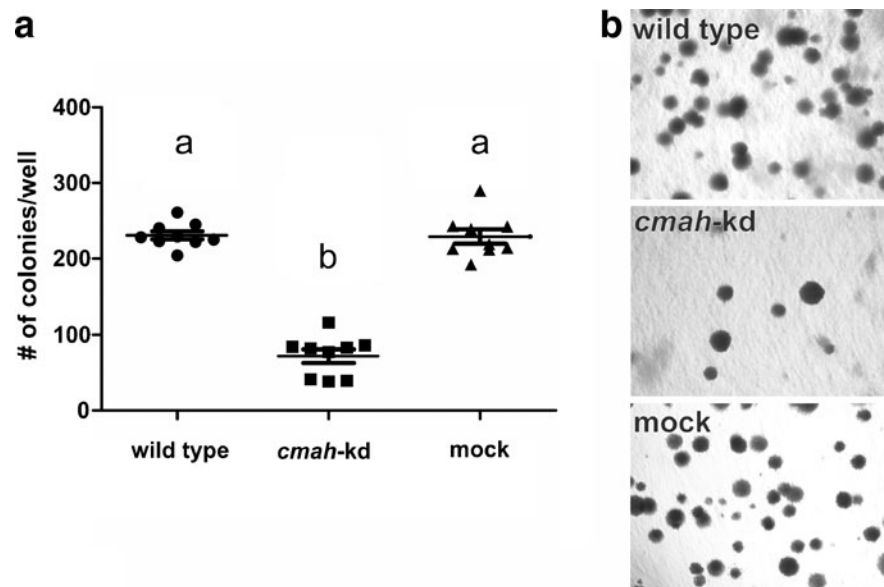
Fig. 5 *cmah*-kd L1210 cells have a diminished capacity to develop subcutaneous tumors. DBA/2 mice were inoculated subcutaneously and tumor onset (**a**, **c**) and growth (**b**, **d**) was monitored. Tumor volumes were measured with a caliper. **a**, **b** Different amounts of wild type or *cmah*-kd L1210 cells were implanted. Data correspond to two independent experiments. **c**, **d** Comparison between wild type and mock cells. Analysis of tumor-free survival was performed with the log-rank test: *$*P<0.05$; ***$P<0.001$; n.s.: non-significant. **e** Cells taken from tumor explants were stained with 14F7hT (*empty, dotted line histograms*) and 7C1 mAbs (*filled histograms*) and analyzed by flow cytometry as described in Fig. 2b. Isotype-matched chimeric C5 mAb (*empty, black line histograms*) was used as control
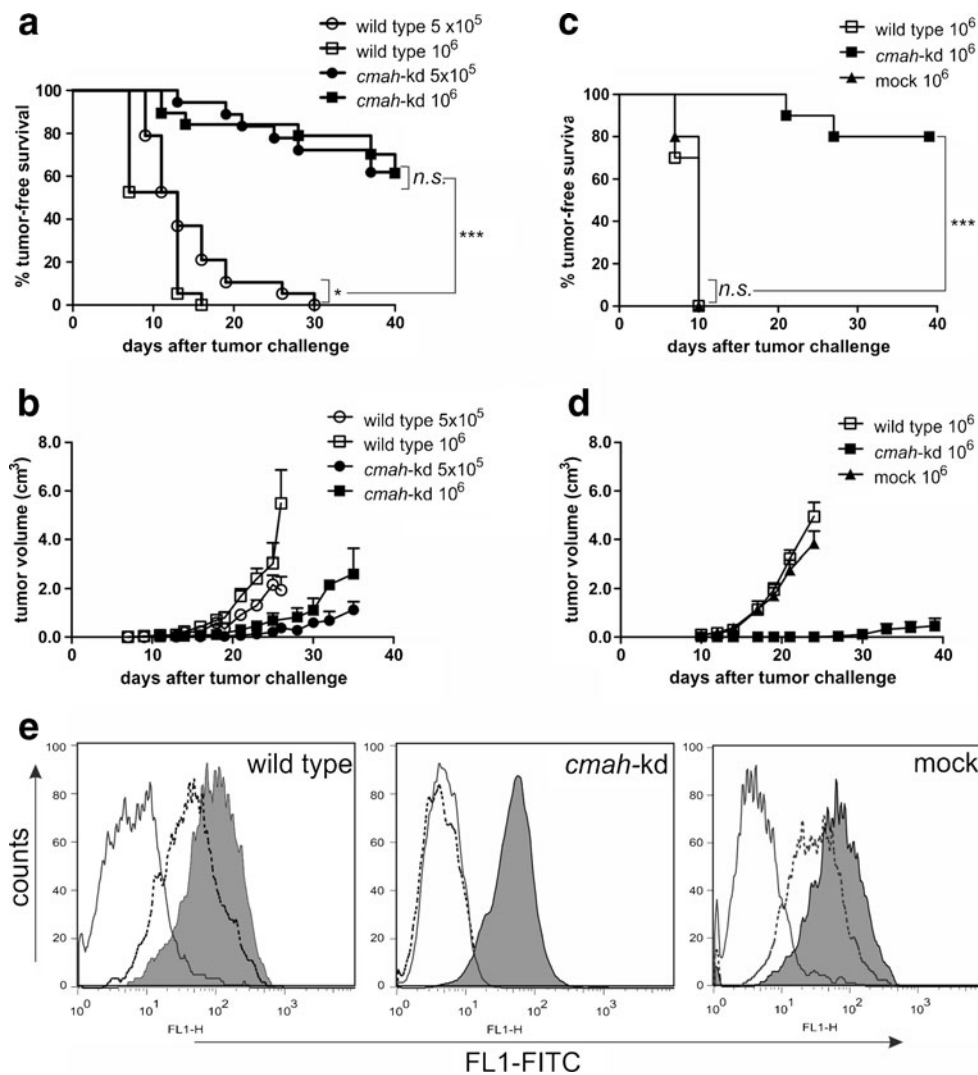
Altogether, these results suggest that the immune system does not play a fundamental role in the reduced *in vivo* tumorigenic potential of *cmah*-kd L1210 cells.

Discussion

cmah-kd L1210 cells are insensitive to 14F7 mAb-mediated killing [45]. 14F7 mAb kills tumor, but not normal cells expressing GM3(Neu5Gc), and this effect is abrogated by impairing the synthesis of gangliosides in general [42, 45] or of Neu5Gc in particular [45]. 7C1 mAb is a version of the humanized 14F7 mAb (14F7hT [22]) carrying three mutations in the binding site [46], which allow the antibody to bind not only to GM3(Neu5Gc) but also to GM3(Neu5Ac). This extended specificity rendered the antibody cytotoxic in L1210 cells expressing either ganglioside, in contrast with the parental antibody. *cmah*-kd L1210 cells were now demonstrated to express a high level of GM3(Neu5Ac), which is known to be overexpressed in some human tumors [57–59].

Among the described anti-GM3(Neu5Ac) antibodies, the mouse IgG3 DH2 mAb induced antibody-dependent cell-mediated cytotoxicity (ADCC) *in vitro* and inhibited the growth of melanoma cells *in vivo* [60]. Also, the human IgM L612 mAb [61] exhibited complement-dependent cytotoxicity (CDC) [62] and to date is the only anti-GM3(Neu5Ac) antibody that has been tested in the clinic, with no significant undesirable effects and with some clinical effectiveness in melanoma patients [63]. 7C1 mAb, on the other hand, is the first reported anti-GM3(Neu5Ac) mAb able to kill tumor cells without complement or effector cells. Further work will focus in the molecular characterization of the cell death mechanism induced by this antibody. Currently, we are also aiming to elucidate the mechanism behind the cytotoxic activity of the parental 14F7hT mAb [44].

Several works have described the role of GM3(Neu5Ac) in the biology of tumor cells [64, 65]. Expression of high levels of this ganglioside can lead to reduced cell motility [66–68] and increased propensity to undergo apoptosis [66,
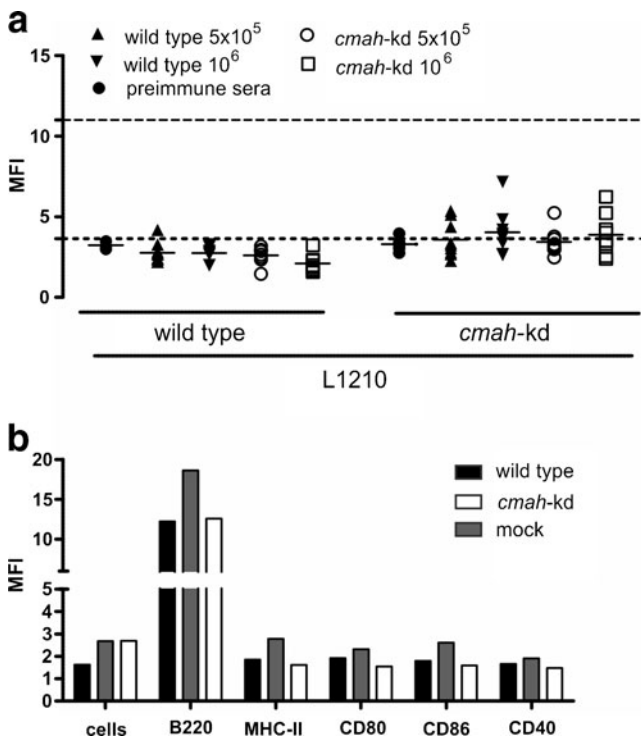
Fig. 6 *cmah*-kd L1210 cells do not appear to be more immunogenic than wild type cells. **a** Mouse sera from one of the experiments described in Fig. 5a were taken before and 4 weeks after cell implanting and were analyzed by flow cytometry for binding to wild type and *cmah*-kd cells. Binding was revealed with a FITC-conjugated rabbit anti-mouse IgG+IgM (H+L) antibody. Wild type L1210 cells were stained with mouse 14F7 (*dashed line*) and C5 (*dotted line*) mAbs at 10 μg/mL as positive and negative controls, respectively. **b** Surface molecule expression was analyzed by flow cytometry using FITC-conjugated specific antibodies

69, 70], as well as diminished ability to form tumors *in vivo* [66, 69]. Also, it is well known that GM3(Neu5Ac) interacts with the epidermal growth factor receptor (EGFR) and inhibits EGF-induced receptor activation [65, 71]. Although it is considered a relevant target for cancer immunotherapy [35], works on the relationship between GM3(Neu5Gc) and tumor development are still scarce. Mouse B16 melanoma and F3II mammary carcinoma cells incubated with a Neu5Gc-rich mucin incorporated this type of sialic acid and transiently expressed GM3(Neu5Gc) on the membrane. *In vivo*, both cell lines showed higher number of experimental lung metastasis than control cells, as well as reduced latency with increased tumor burden in subcutaneous melanoma implants [72]. Similarly, 3LL-D122 Lewis mouse lung carcinoma cells expressing this ganglioside by preincubation with purified Neu5Gc formed more experimental lung tumor nodules, particularly macronodules [73]. However, B16 melanoma cells transfected with the *cmah* gene and thus expressing GM3(Neu5Gc), despite showing increased proliferation and adhesion *in vitro*, displayed a reduced ability to develop tumors in mice as compared with

the parental cell line [74]. This observation contrasts with our present results, where parental L1210 cells, expressing a large amount of GM3(Neu5Gc), had a clear advantage for growing in animals. On the other hand, *cmah*-kd L1210 cells, expressing instead a high level of GM3(Neu5Ac) (as the more tumorigenic parental B16 melanoma cells [74]), exhibited a significantly reduced frequency of tumor development. An important distinction between the melanoma cell line transfected with the *cmah* gene and our *cmah*-kd lymphocytic leukemia cells, is that the expression of the enzyme in the former was unstable and GM3(Neu5Gc) was undetectable after some time in culture [74], while the *cmah*-kd L1210 cells remained negative to 14F7hT mAb staining irrespective of the number of passages, with no drug (selective agent for transduced cells) added to the culture (data not shown). Therefore, in the case of *cmah*-kd L1210 cells the sialic acid type modification is permanent, which allows a better characterization of their behavior both *in vitro* and *in vivo*. No GM3(Neu5Gc) was detected either in cells analyzed from tumor explants. On the other hand, 3LL-D122 lung carcinoma cells were demonstrated, using different techniques, to increase their GM3(Neu5Gc) content when analyzed from *in vitro* culture, primary subcutaneous tumor and metastatic lung lesions, in that order, suggesting that the expression of this ganglioside may be regarded as a malignancy marker in this experimental model [75].

cmah-kd L1210 cells formed a significantly lower number of colonies in soft agarose than wild type cells. The same result was obtained with viral oncoprotein-transformed mouse and chicken fibroblasts transfected with the GM3 synthase gene, as compared with transformed non-transfected cells. Oncogenic transformation led to a reduced expression of this enzyme [76]. Although in both cases the cells with reduced ability for anchorage-independent growth had a higher content of GM3(Neu5Ac), in *cmah*-kd L1210 cells the total content of GM3 appears to be the same as in wild type cells, with only a shift from Neu5Gc to Neu5Ac, while in the case of the transformed fibroblasts the comparison was made between cells with high (transfected with the GM3 synthase gene) or low levels of GM3(Neu5Ac).

Taking into account the reports cited above and the fact that L1210 cells express GM3 as their major ganglioside [42], the simplest explanation for our results would be that it is the presence of GM3(Neu5Ac) in *cmah*-kd L1210 cells what accounts for the observed tumorigenic differences with respect to the predominantly GM3(Neu5Gc)-expressing wild type cells. The small structural difference between the two gangliosides (a hydroxyl group replaces a methyl hydrogen in Neu5Gc) would hardly seem to be enough to have this influence. For instance, it has been proven that both GM3(Neu5Gc) and GM3(Neu5Ac) have the same effect on CD4 expression in T cells [49]. In contrast, in a recent work,

GM3(Neu5Gc) was less effective than GM3(Neu5Ac) in inhibiting *in vitro* EGF-induced EGFR phosphorylation, in A431 human epidermoid carcinoma cells [28]. However, the fact is that, to our knowledge, almost all the studies on the interactions of GM3 with membrane surface and signaling proteins have been done with GM3(Neu5Ac), and thus little is known about the biology of GM3(Neu5Gc). Interestingly, de-*N*-acetyl-GM3 (NeuNH_2), which is expressed in metastatic melanomas, was found to promote human melanoma cell migration and invasion [77].

Avoiding the destruction of tumor cells by the immune system is now accepted as a hallmark of cancer [78]. It is well known that tumor-associated gangliosides can act as immunosuppressors [79, 80]. GM3(Neu5Gc) in particular has been demonstrated to down-modulate the CD4 molecule in T lymphocytes [49] without modifying the inhibitory capacity of regulatory T cells, and also to affect dendritic cell differentiation and maturation [81]. *In vivo* tumor growth was reduced by treating GM3(Neu5Gc)-expressing X63 mouse myeloma cells with an inhibitor of glucosylceramide synthase [49, 81], an effect that was reversed by depleting the CD4 T cells [81]. Also, an inverse correlation between GM3(Neu5Gc) expression and infiltration of mature dendritic cells was found in tissue samples from non-small-cell lung cancer patients [26]. Although *cmah*-kd L1210 cells lack GM3(Neu5Gc), they express instead a high level of GM3(Neu5Ac), which has also been shown to down-modulate CD4 in T lymphocytes [49, 82, 83] and affect dendritic cell functionality [84, 85].

In humans, exogenous Neu5Gc incorporated by cancer cells can induce a potentially tumor-promoting xeno-autoantibody response [86, 87], as has been shown in *cmah*-null mice [88]. However, as expected, no evident antibody response against either wild type or *cmah*-kd L1210 cells was detected in the challenged mice. The immunogenicity of L1210 subclones, which inversely correlates with tumorigenicity, has been demonstrated to be related to their ability to act as antigen-presenting cells with the concomitant expression of major histocompatibility complex class II (MHCII) and the costimulatory molecules CD80 (B7-1), CD86 (B7-2) and CD40 [56]. The expression of either MHCII [56, 89] or these costimulatory molecules [56] was undetectable in parental L1210 cells. In the present study, neither wild type nor *cmah*-kd L1210 cells were found to express any of these molecules.

Altogether, these observations suggest that changes in cell biology due to impaired Neu5Gc synthesis are the leading cause of the reduced tumorigenicity of *cmah*-kd L1210 cells, both *in vitro* and *in vivo*. The results of this work contribute to validate GM3(Neu5Gc), or Neu5Gc-sialoconjugates in general, as targets for cancer immunotherapy. As we cannot rule out that other Neu5Gc-sialoconjugates different from GM3(Neu5Gc) could have a

role in the tumorigenic properties of L1210 cells, the reduced tumorigenicity of the mouse malignant B cell line devoid of Neu5Gc and expressing instead Neu5Ac deserves a deeper molecular characterization.

Finally, we showed that 7C1 mAb, a version of the anti-GM3(Neu5Gc) 14F7hT mAb that contains mutations resulting in an extended specificity towards GM3(Neu5Ac) [46], is able, unlike its parental antibody, to kill tumor cells expressing either of these gangliosides. This first evidence of antitumor activity obtained for the 7C1 mAb, as well as our recent observation that this antibody does not kill normal lymphocytes (manuscript in preparation), as has been demonstrated for the 14F7 mAb [42], reveal its potential as a cancer immunotherapeutic agent. The mechanism of cell killing without participation of complement or effector cells has not been completely elucidated [44], and therefore its characterization, together with the understanding of the role of Neu5Gc-gangliosides in tumor biology, would help to design more effective immunotherapeutic strategies against cancer cells aberrantly expressing these glycosphingolipids.

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