



Antibody-dependent cell-mediated cytotoxicity induced by active immunotherapy based on racotumomab in non-small cell lung cancer patients

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Abstract

Antitumor strategies based on positive modulation of the immune system currently represent therapeutic options with prominent acceptance for cancer patients' treatment due to its selectivity and higher tolerance compared to chemotherapy. Racotumomab is an anti-idiotypic (anti-Id) monoclonal antibody (mAb) directed to NeuGc-containing gangliosides such as NeuGcGM3, a widely reported tumor-specific neoantigen in many human cancers. Racotumomab has been approved in Latin American countries as an active immunotherapy for advanced non-small cell lung cancer (NSCLC) treatment. In this work, we evaluated the induction of Ab-dependent cell-mediated cytotoxicity (ADCC) in NSCLC patients included in a phase III clinical trial, in response to vaccination with racotumomab. The development of anti-NeuGcGM3 antibodies (Abs) in serum samples of immunized patients was first evaluated using the NeuGcGM3-expressing X63 cells, showing that racotumomab vaccination developed antigen-specific Abs that are able to recognize NeuGcGM3 expressed in tumor cell membranes. ADCC response against NeuGcGM3-expressing X63 (target) was observed in racotumomab-treated- but not in control group patients. When target cells were depleted of gangliosides by treatment with a glucosylceramide synthase inhibitor, we observed a significant reduction of the ADCC activity developed by sera from racotumomab-vaccinated patients, suggesting a target-specific response. Our data demonstrate that anti-NeuGcGM3 Abs induced by racotumomab vaccination are able to mediate an antigen-specific ADCC response against tumor cells in NSCLC patients.

Keywords Racotumomab · Active immunotherapy · ADCC · Non-small cell lung cancer · NeuGcGM3 ganglioside

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
Anti-id	Anti-idiotypic
PDMP	D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

Introduction

Lung cancer is one of the most frequently diagnosed malignancies and also the leading cause of cancer deaths among males and females [1]. In particular, NSCLC represents 85% of total lung cancer and is usually diagnosed in an advanced or metastatic stage [2]. Therapeutic options for NSCLC treatment include surgical removal of the primary tumor, chemo- and radiotherapy [3] and molecular-targeted therapies [4]. Most recently, immunotherapy has also become a standard of care therapy for a selected group of NSCLC patients [5]. However, lung cancer prognosis remains poor and overall survival rate is still less than 20%. In this scenario, where new, safe and specific therapeutic approaches are needed, immunotherapeutic agents are promising drugs that overcome the limitations of conventional strategies.

Immunotherapy has been used for the treatment of cancer patients for many years, with clinically proven benefits. The development of successful immunotherapeutic products is

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the result of decades of scientific research in tumor antigens and Abs, tumor-relevant signaling pathways and the interaction between cancer cells and the immune system [6–9]. Nonetheless, one of the main challenges in this field is the improvement in the efficacy of Ab-based therapies, such as tumor-specific Ab (passive immunotherapy) and cancer vaccines (active immunotherapy) [10, 11].

Immunotherapeutic strategies are based on the modulation of the immune system, by activating either humoral or cellular immunity, or both. Cellular immune response quantification is mainly focused on the evaluation of cytotoxic T lymphocytes, cytokine production, macrophages and natural killer (NK) cell activation. While antitumor activity of antigen-specific T lymphocytes and inflammatory cytokine production have been extensively reported, the role of NK cells in cancer immunotherapy has been just recently highlighted [12]. Ab-dependent cell-mediated cytotoxicity (ADCC) is believed to play an important role in antitumor immunotherapy-based treatments. Both monocytes/macrophages and NK cells are described as the main effector cells in this mechanism [12–14]. In particular, NK cells play a key role in the induction of ADCC since they highly express the activating receptors FcγIIIa [15] and FcγIIa/c [16], and they do not co-express the inhibitory receptor FcγRIIb [17]. ADCC mechanism is triggered when target cells are first coated with specific Abs that are then recognized by Fc receptors present on cytotoxic effector cells. The killing of Ab-coated cells occurs through a non-phagocytic process, characterized by granule exocytosis containing perforin and granzymes, activation of TNF family death receptors signaling and recruitment of nearby immune cells by INFγ release [12]. Clinical efficacy of many tumor antigen-directed mAbs used in cancer treatment as passive immunotherapy is mediated by NK cells through ADCC induction [18, 19]. However, there are only a few reports describing the induction of ADCC as an antitumor mechanism in active immunotherapies.

Active immunotherapies are therapeutic strategies intended to activate the endogenous immune system against cancer antigens. Anti-Id mAbs are defined as the mirror image of an Ab that specifically reacts with an antigen. Anti-Id mAbs directed to tumor immunogens are able to induce an antitumor response against the molecule they mimic acting as active immunotherapy effectors. Racotumomab-alum (Vaxira®) is an anti-NeuGc-containing gangliosides anti-Id mAb [20] that has been approved in Latin American countries as active immunotherapy for advanced NSCLC treatment [21, 22]. In particular, NeuGcGM3 ganglioside has been described as a tumor neoantigen in NSCLC in humans [23–25]. Preclinical studies have demonstrated that racotumomab exerts its antitumor activity by increasing CD8⁺ tumor-infiltrating T lymphocytes and tumor apoptosis [26]. In addition, Diaz and colleagues also reported that racotumomab reduced the number of tumor blood vessels [27].

Moreover, we demonstrated the induction of NeuGcGM3-specific Abs in the sera of tumor-bearing mice vaccinated with racotumomab [28] and Hernandez and colleagues showed the presence of anti-NeuGcGM3 cytotoxic Abs induced by racotumomab in NSCLC patients [29].

Despite these studies regarding the mechanism of action of racotumomab, the induction of cell-mediated immunity based on ADCC has not been described for this active immunotherapy. Considering the growing evidence that supports ADCC as one of the most successful approaches for induction of tumor cell killing by Ab-based therapies, the purpose of our study was to evaluate whether racotumomab vaccination is able to induce an ADCC response against NeuGcGM3-expressing cells using serum samples from NSCLC patients included in a phase III study for racotumomab.

Materials and methods

Cell lines

NeuGcGM3-positive P3 × 63 Ag8.653 (X63) murine myeloma cell line was obtained from the American Type Culture Collection (CRL-1580). Tumor cells were grown in Dulbecco's Modified Eagle Media (DMEM) culture medium (Gibco by Life Technologies, California, United States) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco by Life Technologies, California, United States). Cells were subcultured three times a week, in suspension culture, at 37 °C in a humidified atmosphere of 5% CO₂. Cell viability was assessed using the trypan blue exclusion technique.

Monoclonal antibodies

We used the specific anti-NeuGcGM3 mouse IgG1 mAb14F7 [30], produced and kindly provided by the Center of Molecular Immunology, La Habana, Cuba. Mouse and human IgG1 Ab (Dako by Agilent, Santa Clara, United States) were used as isotype controls. Polyclonal R-phycoerythrin-conjugated goat anti-mouse Igs, polyclonal rabbit anti-mouse Igs fluorescein isothiocyanate (FITC)-conjugated (Dako by Agilent, Santa Clara, United States) and polyclonal goat anti-human IgG, IgM, IgA FITC-conjugated (Abcam, Cambridge, United Kingdom) were utilized as secondary Ab in flow cytometry assays.

Immune response evaluation

Humoral immune response against NeuGcGM3 induced by racotumomab was evaluated in serum samples from advanced NSCLC patients included in a prospective, randomized, multicenter, open label phase III study of active specific immunotherapy with racotumomab plus best supportive

care vs best support care (NCT01460472). Patients were 18 years or older, with histologically or cytologically confirmed stage IIIB–IV NSCLC according to the American Joint Committee on Cancer (AJCC). All patients in the study received best supportive care according to institutional standards including any subsequent onco-specific therapies. Vaccinated patients were immunized intradermally and received 15 doses of racotumomab-alum (1 mg). Immunization protocol consists of an induction phase of five doses administered every 2 weeks, followed by a maintenance phase consisting of ten more doses of racotumomab injected every 4 weeks. Patients in the best supportive care (control group) did not receive racotumomab. Serum samples were collected previously (basal samples) and at the third and sixth months post vaccine treatment (hyperimmune samples). For patients in the control group, serum samples were obtained previous (basal samples) and at the third month post treatment.

The presence of anti-NeuGcGM3 IgG and IgM Abs in serum samples from racotumomab-treated patients was first determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated with 20 ng of NeuGcGM3 per well in methanol high performance liquid chromatography grade. Control wells treated with NeuAcGM3 were included. After solvent evaporation, plates were blocked with 4% phosphate-buffered saline (PBS) human serum albumin. Serum samples were used in two-fold dilution series starting at 1:200 for titration. Alkaline phosphatase-conjugated goat anti-human IgG or IgM (Jackson ImmunoResearch Laboratories) were used to detect bound Abs. Specific anti-NeuGcGM3 reactivity was calculated by subtraction of the absorbance of the control wells. Ab titers were defined as the inverse of the dilution yielding an absorbance of 0.25.

Antibody-binding assay

The detection of anti-NeuGcGM3 Abs present in serum samples from vaccinated patients was confirmed using X63 cells. Briefly, 5×10^5 cells were incubated with serum samples diluted 1:20 with PBS for 30 min at room temperature. After washing with PBS, cells were incubated for 30 min on ice with a goat FITC-labeled anti-human Igs (Dako by Agilent, Santa Clara, United States). Acquisition was achieved by a FACSCalibur flow cytometer (Becton Dickinson, New Jersey, United States) and data were analyzed by the FlowJo software. 14F7 mAb was used as positive NeuGcGM3 staining control.

ADCC assay

Cytotoxic activity induced by anti-NeuGcGM3 Abs was analyzed by a colorimetric lactate dehydrogenase (LDH)-release assay. For 30 min, 8×10^4 X63 cells (target) were incubated

with a 1:50 dilution of serum samples from patients in DMEM medium supplemented with 2% FBS. Then, 2×10^6 peripheral blood mononuclear cells (PBMCs) from healthy donors (effector cells) were co-incubated (test cell mix) in DMEM 1% FBS for 4 h. Both incubations were done at 37 °C and 5% CO₂. PBMC were isolated using Ficoll density centrifugation (Lobov, Buenos Aires, Argentina), according to standard procedures. After co-incubation, 50 µl of the supernatant was collected and cell lysis was detected by Cyto-Tox 96 kit (Promega, Wisconsin, United States) measuring absorbance at 490 nm in an ELISA reader (Asys VVM340), according to manufacturer's instructions. Maximum LDH release was determined in target cells treated with 1% Triton X-100 (Max lysis), while minimum release (low control) was determined for each patient incubating target cells with serum samples, without adding effector cells. Spontaneous release control was determined in target cells incubated with effector cells without serum samples. The percentage of specific lysis was calculated according to the following formula:

$$\begin{aligned} \text{Specific lysis (\%)} &= \frac{(\text{Test cell mix} - \text{Spontaneous release control})}{(\text{Max Lysis} - \text{Low control})} \\ &\times 100. \end{aligned}$$

To make a comparison between ADCC activity from various patients and their own responses observed after racotumomab therapy, specific lysis data were expressed in terms of percentage of basal sample, considering that 100% of the value is obtained with the basal sample from each patient.

D-threo-PDMP treatment

X63 cells with impaired ganglioside synthesis were obtained by adding D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) to the culture medium at 10 µM for 4 days. X63-treated cells were used as negative controls in ADCC assays as previously described.

Evaluation of NeuGcGM3 specificity

Serum samples were diluted in PBS 1% BSA (the working dilution used in the ADCC assay) and incubated in coated-NeuGcGM3 during 2 h at 37 °C. Coating with NeuGcGM3 and plate blocking were done as previously described (see Immune response evaluation). Pre-incubated sera were used in ADCC assays to evaluate NeuGcGM3-dependent cytotoxic response. Control samples were equally diluted and incubated in a non-coated plate under the same conditions. Pre-absorbed and control serum samples were then used in ADCC assay as described.

Statistical analysis

Results described in this work are expressed as mean values \pm standard deviation. Statistical analyses were carried out using Prism 6.0 statistical software (GraphPad, Inc., CA, United States). The normal distribution of data was evaluated using the D'Agostino and Pearson omnibus normality test. For multiple comparisons between experimental groups, we used one-way ANOVA or Kruskal Wallis followed by Dunnett post hoc test, and two-way ANOVA followed by mean 95% confidence interval comparison post hoc test. For repeated measures ANOVA, the Geisser–Greenhouse correction was used for assumption of sphericity. Correlation of data was evaluated using Pearson r test. Significant levels were defined as $p < 0.05$.

Results

Racotumomab induces anti-NeuGcGM3 antibodies

To evaluate if vaccination with racotumomab was able to induce specific anti-NeuGcGM3 Ig Abs, serum samples

were collected as previously described and analyzed by ELISA. Figure 1 shows that racotumomab vaccination induced a significant Ab response against NeuGcGM3, reaching the maximum IgG titer at the end of the induction phase (third month samples) and sustaining it during the maintenance phase (sixth month samples) (Fig. 1a). Patients showed a two-fold increase in the median IgG titer against NeuGcGM3 at the third month post vaccination compared to basal samples, with titers that increased up to six-fold. NeuGcGM3-specific IgM Abs were also induced after vaccination. Similar to the IgG response, the maximum title was obtained at the third month samples when the induction phase was completed (Fig. 1b). Figure 1c, d represent the development of anti-NeuGcGM3 IgG and IgM Abs, respectively, after vaccination with racotumomab for all analyzed patients.

We then evaluated whether Abs induced by racotumomab were able to recognize the antigen as part of the tumor cell membrane. Reactivity of Abs present in basal and hyper-immune samples to X63 myeloma cell line was evaluated by flow cytometry. These cells are known to have a predominant expression of NeuGcGM3 ganglioside and, to a much lesser extent, its precursor NeuAcGM3 [31]. As shown

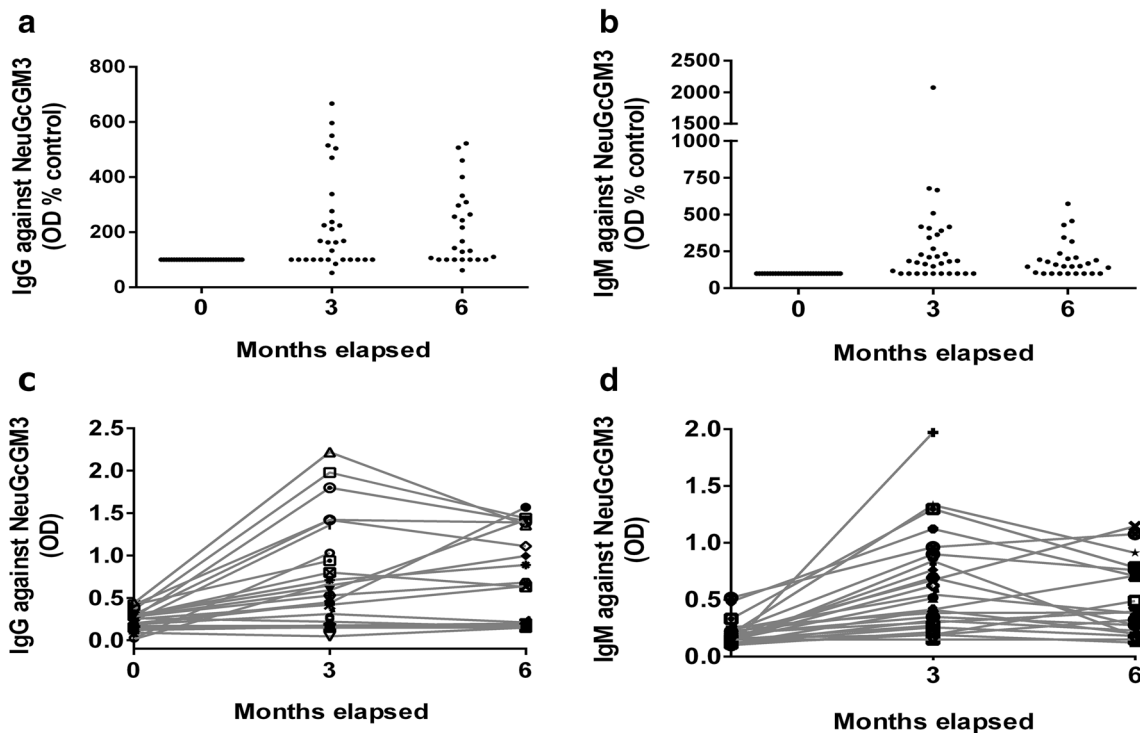


Fig. 1 IgG and IgM Abs against NeuGcGM3 in NSCLC patients vaccinated with racotumomab. ELISA assays were performed to identify anti-NeuGcGM3 Abs induced by racotumomab treatment in hyper-immune serum samples. Optical density (OD) values were used to follow Ab levels. IgG (a) and IgM (b) titers against the ganglioside are depicted as percentage of OD values obtained for basal samples

(indicated as 0 month). Kruskal–Wallis followed by Dunnett's multiple comparison test was used to analyze differences between groups; $p < 0.001$ basal vs month 3 and basal vs month 6; $p > 0.05$ month 3 vs month 6, for both IgG and IgM titers. IgG (c) and IgM (d) humoral immune response against NeuGcGM3 developed in vaccinated patients

in Fig. 2a, the binding of Abs present in the third month post-vaccination sera to X63 cells was significantly higher in comparison with basal sera, with a median fluorescence intensity value (MFI) that reaches twice the value of the basal samples. No significant binding differences were found between hyperimmune serum samples, as it was observed in the ELISA assay. Data analysis demonstrated a moderate but

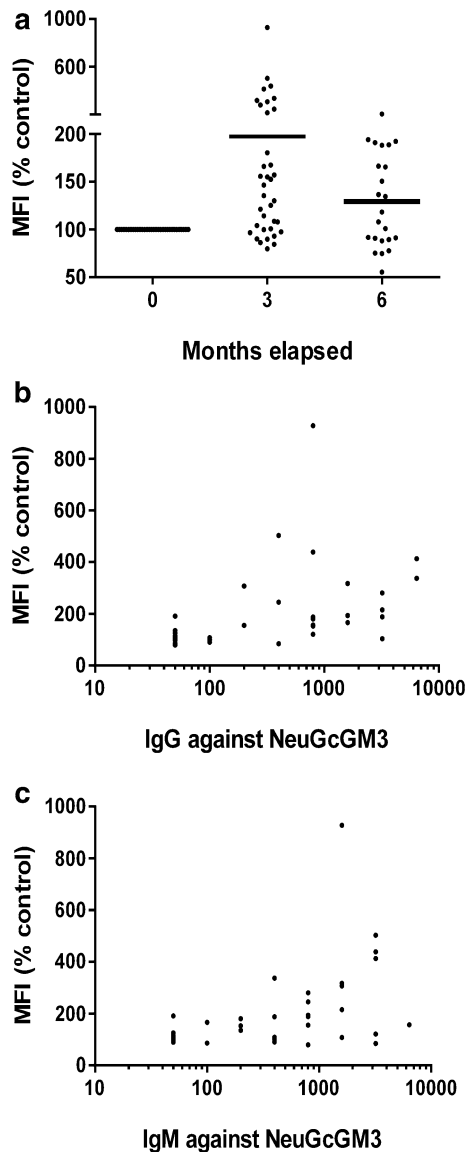


Fig. 2 Binding of basal and hyperimmune sera from vaccinated patients to NeuGcGM3-expressing X63 cells. Serum samples from vaccinated patients were incubated with target cells and analyzed by flow cytometry. **a** Binding to X63 cells are expressed as percentage of MFI obtained with basal samples for each patient. Kruskal–Wallis followed by Dunnnett’s multiple comparison test was used to analyze differences between groups; $p < 0.001$ basal vs month 3. **b**, **c** Direct correlation exhibited between MFI, depicted as percentage of basal samples, and anti-NeuGcGM3 IgG titer (**b**) or and anti-NeuGcGM3 IgM titer (**c**). Pearson r test was used to analyze the results; $p < 0.01$, $r = 0.4339$, MFI vs log IgG titer; $r = 0.4454$, MFI vs log IgM titer

still significant positive correlation between NeuGcGM3-specific IgG (Fig. 2b) and IgM Abs (Fig. 2c) and the binding to tumor cells. These results confirm that racotumomab vaccination develops antigen-specific Abs that are able to recognize purified NeuGcGM3s as well as NeuGcGM3 expressed in tumor cell membranes, measured by ELISA and flow cytometry, respectively.

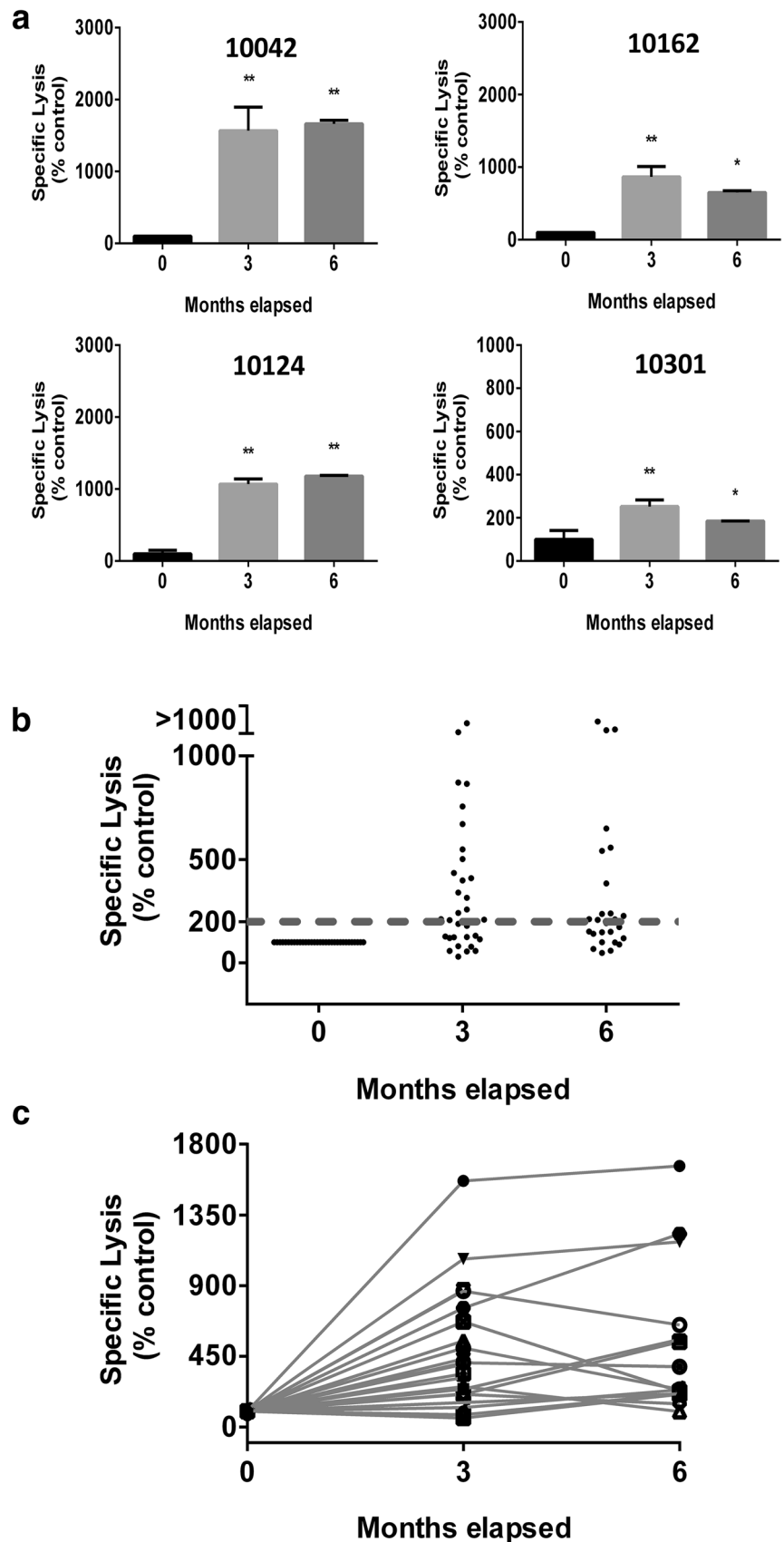
Antibodies induced by racotumomab mediate a cytotoxic activity based on ADCC

To assess if anti-NeuGcGM3 Abs induced by racotumomab were able to mediate an ADCC activity against X63 cells, we performed a LDH-release assay using serum samples from 36 patients treated with racotumomab and PMNC from healthy donors. Figure 3a shows the ADCC response obtained when serum samples from four selected patients were analyzed. The development of specific ADCC response was then evaluated in all racotumomab-treated patients (Fig. 3b). ADCC response obtained with hyperimmune samples showed a significant increase compared to basal samples. No significant differences were observed between ADCC responses obtained with hyperimmune sera corresponding to the third or the sixth month post vaccination. We considered that a patient develops a positive ADCC response when a two-fold increase of the cytotoxic activity obtained with its own basal sample was reached. The development of ADCC was demonstrated in 22 out of the 36 evaluated patients (61%) (Fig. 3c). As expected, a moderate but still significant correlation was found between ADCC response and anti-NeuGcGM3 IgG titer (Fig. 4). Serum samples from seven control group patients were also analyzed, showing no differences between the binding to X63 cells obtained with basal and third month post-treatment samples (Fig. 5a). As shown in Fig. 5b, specific lysis values exhibited no significant differences between samples collected at the beginning of the clinical study and at the third month, demonstrating that the ADCC response observed in racotumomab-treated patients is related to vaccination.

ADCC induced by racotumomab depends on NeuGcGM3 expression

Finally, we explored whether the induction of ADCC is dependent on the expression of NeuGcGM3 in target cells. ADCC activity was evaluated on X63 cells incubated with PDMP, a well-described inhibitor of the glucosylceramide synthase that blocks the formation of glucosylceramide from ceramide, decreasing cell surface glycosphingolipids [32]. Ganglioside expression was significantly reduced in PDMP-treated X63 cells, showing a decrease of 65% in comparison to non-treated cells ($p < 0.01$, Unpaired T test; data not shown). X63 cells with impaired ganglioside synthesis

Fig. 3 ADCC induced by anti-NeuGcGM3 Ab present in serum samples from racotumomab-vaccinated patients. Specific lysis against X63 cells was analyzed using a LDH-release assay and effector cells from healthy donors. **a** ADCC activity (indicated as specific lysis) obtained with hyperimmune serum samples corresponding to the third and the sixth months post-vaccination from four representative patients. Bars indicate mean values \pm standard deviation. Patients' identification numbers are indicated on each graph. ANOVA followed by Dunnett's multiple comparison test was used to analyze differences between groups; * $p < 0.05$, ** $p < 0.01$. **b** ADCC response obtained for 36 racotumomab vaccinated NSCLC patients using basal and hyperimmune serum samples. Results are expressed as percentage respect to basal samples (0 month). Dotted line indicates the minimum specific lysis value to be considered a positive ADCC response (threshold value). Kruskal–Wallis followed by Dunnett's multiple comparison test was used to analyze differences between groups; $p < 0.001$ basal vs month 3 and basal vs month 6. **c** ADCC activity developed by anti-NeuGcGM3 Ab present in basal and hyper-immune samples



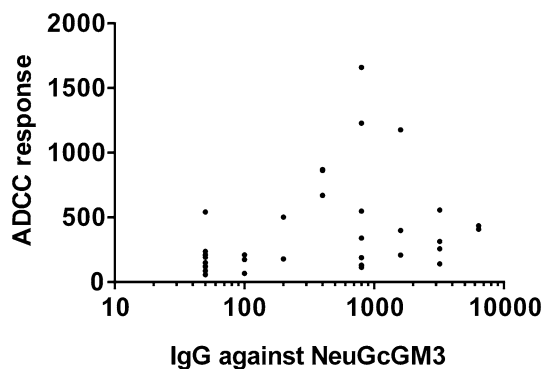


Fig. 4 Direct correlation exhibited between ADCC response and anti-NeuGcGM3 IgG titer. Pearson *r* test was used to analyze the results; $p < 0.05$, $r = 0.3424$, ADCC response vs log IgG titer

were then used as target cells in ADCC assay using the same hyperimmune sera. A significant reduced ADCC response against PDMP-treated X63 cells was observed in this set of experiments (Fig. 6a). To confirm ADCC specificity, we also evaluated the cytotoxic response developed against X63 using serum samples pre-absorbed by NeuGcGM3. ADCC

was significantly reduced when pre-absorbed sera was used, in contrast to nonabsorbed samples. As expected, there was no difference between the responses developed by pre- and nonabsorbed basal samples (Fig. 6b). These results confirm the participation of NeuGcGM3 as target ganglioside in the cellular antitumor response developed by racotumomab vaccination.

Discussion

Lung tumor microenvironment has been traditionally characterized as highly immunosuppressive. Nevertheless, the understanding of tumor immunosurveillance and the recent approval of several immune checkpoints inhibitors as therapeutic options for NSCLC, some of them as first-line treatments, has led to a new era in the treatment of this pathology. In this scenario, immunotherapy for NSCLC has recently become an accurate treatment modality for this type of tumor. Cancer vaccines have demonstrated the induction of an enhanced immune response, evolving into promising therapeutic alternatives characterized by the ability to elicit

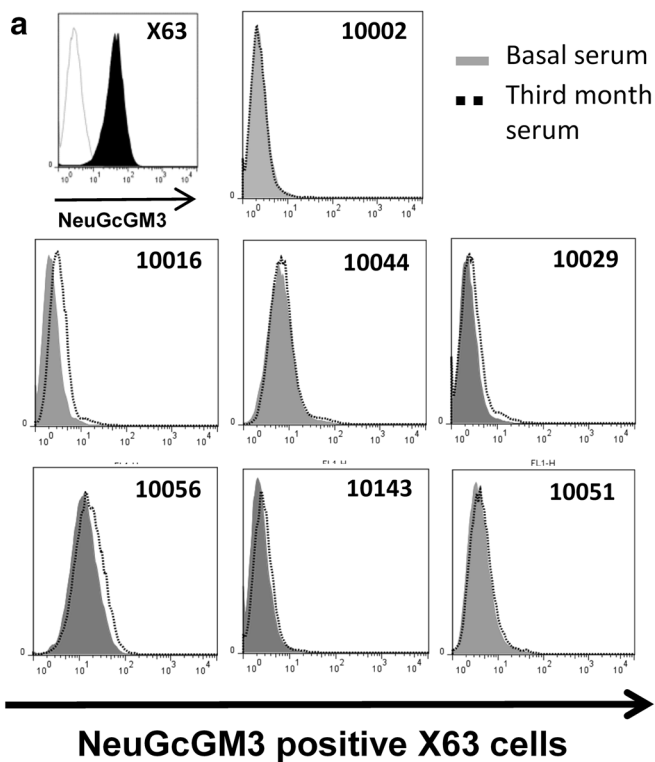
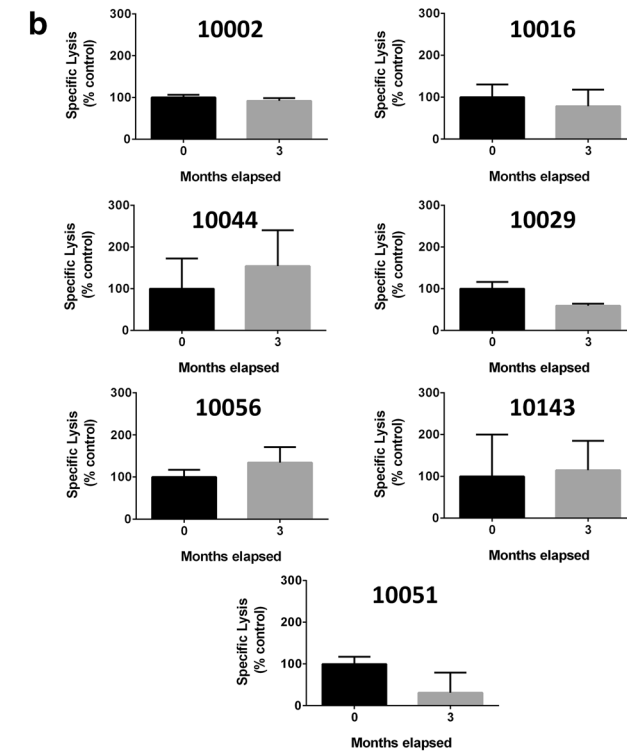


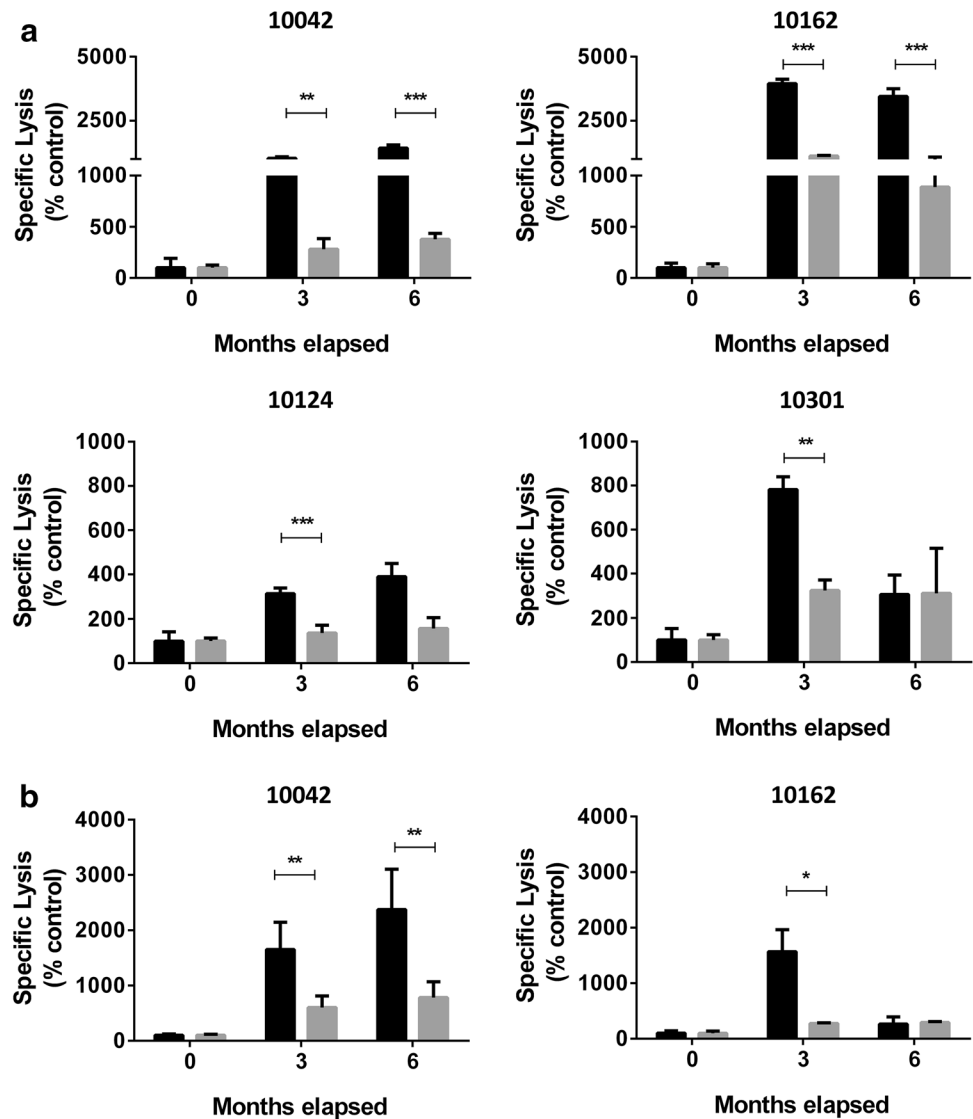
Fig. 5 Binding to NeuGcGM3-expressing X63 cells and cell-mediated cytotoxicity developed by serum samples from control group patients. Binding and ADCC response were measured as previously described. **a** Histograms of the flow cytometric analysis showing post-treatment (3 month) sera binding to X63 cells. Positive NeuGcGM3 staining control using 14F7 mAb was included. **b** ADCC



response (shown as specific lysis values) obtained with post-treatment serum samples for each control patient. Results are depicted as percentage respect to basal samples (0 month) and bars indicate mean values \pm standard deviation. Patients' identification numbers are indicated on each individual graph

Fig. 6 NeuGcGM3-specific ADCC response. **a** ADCC against NeuGcGM3-expressing (black bars) or NeuGcGM3-depleted (gray bars) X63 cells was evaluated as previously described. Two-way ANOVA followed by mean 95% confidence interval comparison post hoc test was used to analyze differences between groups; $**p < 0.01$, $***p < 0.001$.

Results are shown as percentage respect to the control and bars indicate mean values \pm standard deviation. **b** Evaluation of ADCC response using pre-absorbed with NeuGcGM3 serum samples (gray bars). Controls indicate ADCC response induced by nonabsorbed sera (black bars). Paired *T* test was used to analyze differences between non and pre-absorbed sera for the same month; $**p < 0.01$; $*p < 0.05$. Results are shown as percentage respect to the control and bars indicate mean values \pm standard error. ADCC responses are expressed as specific lysis. Basal samples are indicated as 0 month. Patients' identification numbers are indicated on each graph



an immune response against specific tumor antigens and showing minor adverse effects than other strategies. Combination strategies based on checkpoint inhibitors and cancer vaccines are currently being evaluated in several clinical trials [33–35].

Normal human tissues do not express NeuGc-containing gangliosides due to a deletion in the CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) gene. CMAH catalyzes NeuGc synthesis [36]. In particular, NeuGcGM3 ganglioside has been widely reported as a specific tumor antigen not only in NSCLC [23, 25] but also in many other human cancers such as melanoma [30], breast [37], retinoblastoma [38] and neuroectodermal tumors [39] among others. Although there are several theories that have been postulated to explain the expression of NeuGcGM3 in tumor cells [40, 41], the most accepted mechanism proposes a metabolic incorporation from dietary sources [42]. In fact, studies with tumor cells demonstrate that sialin, a sialic

acid transporter, is overexpressed under hypoxic conditions promoting the incorporation of NeuGc from culture medium [43]. The upregulation of sialin allows tumor cells to increase the amount of NeuGcGM3 on their membranes, which confers advantages over cells that do not express it. In line with this evidence, we have previously reported that murine B16 melanoma cells with NeuGcGM3 expression exhibit an increased proliferation and adhesion in vitro [44]. Moreover, tumor cells can uptake NeuGc from culture medium increasing their ability to disseminate when they are injected in mice [28, 45]. In addition, the presence of NeuGcGM3 has been associated with a down-modulation of CD4 molecule in tumor infiltrating T cells [46] without altering CD4⁺CD25⁺ regulatory T-cell inhibitory functions. Tumor-associated NeuGcGM3 also reduces the antigen-presenting function of dendritic cells [47]. These experimental evidences support the relevance of NeuGcGM3 ganglioside as an important target for lung cancer immunotherapy.

Despite the differential expression observed in tumors over normal tissues, NeuGcGM3 as well as glycosphingolipids in general are poorly immunogenic due to their carbohydrate nature. However, an anti-id mAb that acts as NeuGcGM3 surrogate was developed to overcome the immune tolerance and to elicit an effective immune response against this tumor antigen. Racotumomab is an anti-NeuGc-containing gangliosides anti-id mAb, approved in Latin American countries as active immunotherapy for NSCLC treatment. Racotumomab exerts its mechanism of action by inducing tumor-infiltrating CD8⁺T lymphocytes and tumor apoptosis. Moreover, it reduces the number of tumor blood vessels and augments the presence of anti-NeuGcGM3 cytotoxic Abs in vaccinated NSCLC patients. Hernández and co-workers also described that the cytotoxic activity developed by antigen-specific Abs involves a mechanism independent of complement activation and the formation of membrane lesions that resembles an oncotic necrosis process [29]. In the present work, we demonstrate for the first time the induction of ADCC in NSCLC patients vaccinated with the active immunotherapy racotumomab.

Serum samples from 36 racotumomab-vaccinated patients were first analyzed to confirm the presence of NeuGcGM3 specific Abs. As it was previously reported in different clinical trials of racotumomab, we were able to confirm the induction of an Ab response of both IgG and IgM isotypes against the ganglioside using hyperimmune sera corresponding to either the third or the sixth month post vaccination. Those patients who completed the induction phase of the immunization achieve the highest Ab response against NeuGcGM3 ganglioside, maintaining the Ab titer after 6 months of racotumomab treatment. Same results were also described in breast and melanoma cancer patients included in two phase I clinical trials for racotumomab. In both clinical studies, evaluation of the IgG subclasses represented in the humoral response showed the induction of Abs predominantly of IgG2 subclass, with very low representation of other IgG subclasses in most patients [48, 49]. On the contrary, basal samples show no reaction in the ELISA assay, confirming the immunogenicity of racotumomab in this group of patients as it was reported [48–52]. In addition, anti-NeuGcGM3 Abs present in hyperimmune serum samples show a significant binding to X63 myeloma cells demonstrating they can recognize the ganglioside when it is expressed in tumor cell membranes. This result confirms findings previously reported by Alfonso and co-workers, who evaluated the binding of patients' sera to a different cell line, the murine lymphocytic leukemia L1210, using serum samples from patients enrolled in a phase II/III clinical trial of racotumomab vaccine [21].

Abs mediate tumor cell killing by inducing one of these three mechanisms: direct action on tumor cells, immune-mediated cell killing and specific action on tumor

microenvironment cells. An increasing body of evidence suggests that immune-mediated cell killing mechanisms are important contributors to the anti-tumor activity of many Ab-based therapies. In particular, the induction of ADCC activity has been extensively reported in response to the treatment with passive immunotherapies based on the administration of rituximab and trastuzumab among others mAbs mainly directed against protein antigens [12, 53]. With the approval of dinutuximab, a chimeric anti-GD2 therapeutic Ab, the ganglioside GD2 became the first glycosphingolipid proven as an effective target antigen for passive immunotherapy against neuroblastoma [54]. Dinutuximab induces ADCC response against GD2-expressing cell lines [55]. On the other hand, the induction of ADCC in cancer patients treated with active immunotherapies has been less described. To our knowledge, Snijderwint and co-workers described for the first time the induction of ADCC response in breast cancer patients vaccinated with MUC1 peptide in a phase I clinical trial [56]. Even though two anti-Id mAbs, mimicking carcinoembryonic antigen (3H1) and GD2 (gangliodimab), have demonstrated the induction of ADCC in murine tumor models [57, 58], no further clinical trials reporting cell cytotoxic response in cancer patients have been described for these Abs. Moreover, the induction of ADCC in response to immunotherapies directed against glycan antigens has not been reported so far.

ADCC against target cells is triggered when tumor antigen-specific Abs belonging to IgG, IgA and IgE classes bind to Fc receptors present on effector cells. Both therapeutic Abs themselves (passive immunotherapy) and Abs induced in response to active immunotherapies are able to bind to Fc receptors and trigger ADCC against tumor cells. It was previously reported that racotumomab induces a ganglioside-specific cellular response in breast cancer patients that were included in a year-long vaccination scheme. PBMCs from these vaccinated patients secreted INF γ when incubated with dendritic cells and NeuGcGM3 [50]. In addition, Abs elicited by vaccination can directly kill target cells with NeuGcGM3 expression [21]. However, the evaluation of cell cytotoxic activity developed by racotumomab-induced Abs has not been demonstrated to date. Here, we describe that anti-NeuGcGM3 Abs present in serum samples from NSCLC-vaccinated patients are able to mediate the lysis of NeuGcGM3-expressing X63 cells in the presence of PBMCs from healthy donors by an ADCC mechanism. This Ab-induced cytotoxic response is antigen dependent because specific lysis was significantly reduced when both, X63 cells lacking ganglioside expression and sera pre-absorbed with purified NeuGcGM3, were used. In agreement with previous clinical studies, NSCLC patients analyzed in this work developed specific IgG Abs against NeuGcGM3 in response to the vaccination with racotumomab. Furthermore, our data

indicate that there is a correlation between anti-ganglioside IgG titer and the ADCC activity against X63cells.

In summary, we report here that therapeutic active immunization with racotumomab induces anti-NeuGcGM3-specific Abs capable of mediating a cell-mediated cytotoxic response based on ADCC against tumor cells in NSCLC patients.

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Author contributions Valeria I. Segatori and Mariano R. Gabri participated in the conception and design of the experiments, the analysis and interpretation of data and in the process of manuscript writing. Héctor A. Cuello, Cynthia A. Gulino and Marina Albertó also contributed to the final version of the manuscript. Valeria I. Segatori, Héctor A. Cuello, Cynthia A. Gulino and Marina Albertó worked in development of methodology and acquisition of data. Cecilia Venier was involved in the ELISA assays. Marcelo D. Guthmann and Ignacio A. Demarco were responsible for patients' samples. Review of the manuscript was done by Daniel F. Alonso and Mariano R. Gabri.

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Compliance with ethical standards

Conflict of interest Marcelo D. Guthmann and Ignacio A. Demarco are full employees of Elea Laboratories. They have no conflict of interest to declare. All other authors declare that they have no potential conflict of interest.

Ethical approval and ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

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