Breast Cancer Res Treat
DOI 10.1007/s10549-009-0399-9

PRECLINICAL STUDY

Combined therapeutic effect of a monoclonal anti-idiotype tumor vaccine against NeuGc-containing gangliosides with chemotherapy in a breast carcinoma model

D. Fuentes · J. Avellanet · A. Garcia · N. Iglesias · M. R. Gabri · D. F. Alonso · A. M. Vazquez · R. Perez · E. Montero

Received: 15 December 2008 / Accepted: 2 April 2009

Abstract Anti-idiotypic monoclonal antibodies (mAb) have been evaluated for actively induced immunotherapy with encouraging results. However, rational combination of cancer vaccines with chemotherapy may improve the therapeutic efficacy of these two approaches used separately. The main objective of this study was to evaluate the antitumor effect of the co-administration of 1E10 (Racotumomab), a monoclonal anti-idiotype tumor vaccine against an IgM mAb, named P3 that reacts specifically with NeuGc-containing gangliosides and low-dose Cyclophosphamide in a mammary carcinoma model. F3II tumor-bearing mice were immunized subcutaneously with 100 μg of 1E10 mAb in Alum or with 150 mg/m^2 of Cyclophosphamide intravenously 7 days after the tumor inoculation. While a limited antitumor effect was induced by a single 1E10 mAb immunization; its co-administration with low-dose Cyclophosphamide reduced significantly the F3II mammary carcinoma growth. That response was comparable with the co-administration of the standard high-dose chemotherapy for breast cancer based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide, without toxicity signs. Combinatorial chemo-immunotherapy promoted the CD8$^+$ lymphocytes tumor infiltration and enhanced tumor apoptosis. Furthermore, 1E10 mAb immunization potentiated the antiangiogenic effect of low-dose Cyclophosphamide. Additionally, splenic myeloid cells Gr1$^+$/CD11b$^+$ associated with a suppressor phenotype were significantly reduced in F3II tumor-bearing mice immunized with 1E10 mAb alone or in combination with low-dose Cyclophosphamide. This data may provide a rational for chemo-immunotherapy combinations with potential medical implications in breast cancer.

Keywords Breast cancer · Chemotherapy · Cancer vaccine · Combinatorial therapy · Anti-idiotype monoclonal antibody · NeuGc-containing ganglioside · Myeloid cells Gr1$^+$/CD11b$^+$ · Apoptosis · Angiogenesis

Abbreviations

Ab	Antibody
Cy	Cyclophosphamide
Cy^{Hi}	600 mg/m^2
Cy^{Lo}	150 mg/m^2
Dox	Doxorubicin
ELISA	Enzyme-linked immunosorbent assay
Id	Idiotype
mAb	Monoclonal antibody
NeuGc	Neu-Glycolyl
Treg	Regulatory T cells
s.c.	Subcutaneous
i.p.	Intraperitoneal
i.v.	Intravenous

D. Fuentes · A. Garcia
National Center for Laboratory Animal Breeding, Havana, Cuba

J. Avellanet · A. M. Vazquez · R. Perez · E. Montero (✉)
Experimental Immunotherapy Department, Center of Molecular Immunology, P.O. Box 16040, 11600 Havana, Cuba
e-mail: enrique@cim.sld.cu

N. Iglesias
Pathology Department, Hermanos Ameijeiras Hospital, Havana, Cuba

M. R. Gabri · D. F. Alonso
Laboratory of Molecular Oncology, Quilmes National University, Buenos Aires, Argentina

Published online: 18 April 2009

Introduction

Actively induced immunotherapy is one of the most promising fields in cancer research and numerous approaches are being studied to design effective cancer vaccines [1]. Immunization with anti-idiotype (Id) antibodies represents an attractive approach for tumor immunotherapy. Extensive studies in animal models have demonstrated the efficacy of these vaccines for triggering the immune system to induce specific and protective immunity against tumors of different origin [2, 3].

The 1E10 (Racotumomab) is an anti-idiotype vaccine designed to mimic the NeuGc-GM3 ganglioside. This monoclonal antibody (mAb) is an Ab2-type-antibody which recognizes the Ab1 antibody called P3, the latter is a monoclonal antibody that reacts specifically with gangliosides and also recognizes antigens in human breast and melanoma tumors [4]. Vaccination with 1E10 anti-Id mAb has resulted in suppression of tumor growth in mouse systems; in BALB/c mice, vaccination with several i.p. doses at 14-day intervals of 50 μg of 1E10 coupled to keyhole limpet hemocyanin in Freund's adjuvant, significantly reduced s.c. tumor growth of F3II carcinoma cells and the number of spontaneous lung metastases [5]. Moreover, 1E10 immunization induces tumor apoptosis and antiangiogenic effects in a metastatic lung carcinoma [6].

The antitumor effects of immunotherapy may be further enhanced by its combination with other therapies [7, 8]. Conventional chemotherapy has been combined with immunotherapy [9]; however, chemotherapeutic drugs have been designed to kill tumor cells and side effects such as neurotoxicity and damage to proliferating cells in healthy tissues pose serious constraints on their use. For these reasons, low-dose chemotherapy has been evaluated in combination with other anti-cancer agents to minimize toxic side effects and provide additive or synergistic benefits [10].

High-doses of anthracyclines and alkylating agents are standard chemotherapeutic drugs extensively applied in clinical oncology currently. Combination of Doxorubicin (60 mg/m^2) and Cyclophosphamide (600 mg/m^2) is used as a first-line breast cancer treatment [11]. In addition, low-dose Cyclophosphamide has also been applied as either an antiangiogenic/antivasculogenic or an immunostimulatory agent in combination with cancer immunotherapies [12, 13] demonstrating to be safe, reasonably efficacious, and potentially applicable for chronic treatments. However, there have been only limited preclinical studies exploring low-dose Cyclophosphamide combined with anti-idiotype vaccines.

The main objective of this study was to explore the concurrent antitumor effect of 1E10 anti-idiotype vaccine co-administered with low-dose Cyclophosphamide in a mammary carcinoma model, based on their potentialy shared antiangiogenic properties and/or a complementary proapoptotic effect induced by 1E10 vaccine. That combination enhanced the efficacy of chemotherapy or immunotherapy alone in the F3II mice carcinoma model.

Materials and methods

Mice and tumor cell line

Ten week old female BALB/c/cenp mice were obtained from the National Center for Laboratory Animal Breeding (CENPALAB, Havana, Cuba). Food and water were administered ad libitum. All animal studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

The sarcomatoid mammary carcinoma cell line F3II is a highly invasive and metastatic variant established from a clone of a spontaneous BALB/c mouse mammary tumor. F3II cells grow as spindle-cell carcinoma tumors with a high local invasiveness and a 90–100% incidence of lung metastases [14]. Stock F3II cells were maintained in minimal essential medium (MEM 41500, Gibco, BRL) supplemented with 10% fetal bovine serum, 2 mM glutamine, 80 mg/ml Gentamycin, and 20 mg/ml tetracycline in monolayer culture. For harvesting, cells were trypsinized using standard procedures.

Cytostatics

Cyclophosphamide and Doxorubicin (Lemery, Mexico) were diluted in water for injection and injected i.v. in mice.

mAb

1E10 (Racotumomab) is an anti-idiotype mAb (IgG1, k) generated against the murine anti-NeuGc-containing gangliosides P3 mAb (IgM) [4]. 1E10 mAb was purified from ascitic fluid by affinity chromatography using a Protein A-Sepharose column (Amersham Pharmacia Biotech). Briefly, sterile purified 1E10 mAb was aseptically mixed at a final concentration of 1 with 5 mg/ml of Aluminum hydroxide (Alum) as adjuvant (Superfos Biosector, Frederikssund, Denmark). The mixture was gently stirred for 3 h at room temperature. The Aluminum hydroxide-precipitated mAb (1E10/Alum) was aliquoted into pyrogen-free, sterile glass vials and stored at 4°C until use.

Antitumor experiments

All mice received 2 × 10^5 F3II cells in the subcutis of the right flank. Seven days after tumor inoculation, the animals

were separated in different groups and treated as follows: subcutaneously with normal saline; subcutaneously with 100 μg of 1E10/Alum; intravenously with 150 mg/m^2 of Cyclophosphamide (Cy^{Lo}); intravenously with 150 mg/m^2 of Cyclophosphamide and subcutaneously with 100 μg of 1E10/Alum or intravenously with 60 mg/m^2 of Doxorubicin (Dox) and 600 mg/m^2 of Cyclophosphamide (Cy^{Hi}).

The time of appearance of local tumors was monitored by palpation and further confirmed by histopathology. In all cases, tumors were diagnosed as spindle-cell carcinomas. Tumor size was measured with a caliper twice a week.

Immunocytochemical detection of gangliosides

The expression of target ganglioside antigens in F3II mammary tumor cells was confirmed by indirect immunoperoxidase staining using the P3 (Ab1) mAb. F3II cell monolayers were grown in Chamber Slides Systems (Lab-Tek, Nalgene Nunc International, Naperville, IL), and fixed with formalin for 4 h. Slides were then incubated for 1 h with the P3 anti-ganglioside mAb at a concentration of 20 μg/ml in PBS or the 1E10 anti-idiotype mAb as a control. Bound antibodies were detected by incubation with biotinylated horse anti-mouse immunoglobulins, followed by peroxidase-conjugated avidin-biotin complex using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). After incubation with the peroxidase substrate diaminobenzidine for 2–4 min, slides were counterstained with hematoxylin. Pertinent specificity tests were performed, including blocking of endogenous peroxidase, omission of the first antibody and utilization of isotype control antibodies.

Antibody binding assay

An ELISA assay was used to measure Ab3 reactivity in mouse sera. Solid-phase ELISA was performed using 96-well polystyrene microtiter plates (High binding, Costar), which were coated with 10 μg/ml of 1E10 F(ab′)2 fragments and incubated overnight at 4°C. Absorbance was measured at 405 nm in an ELISA reader (Organon Teknika, Salzburgo, Austria). ior C5 F(ab′)2 fragments were used as controls. Three samples of each experiment were tested and the standard deviation was less than 10% for all values. Background values of absorbance were less than 0.1.

Pathology

Animals were killed and necropsied on day 35. Spleen, thymus, lymph node, liver, lungs and tumors were removed and fixed in 10% formalin and paraffin-embedded sections were stained with hematoxylin/eosin. To investigate the presence of spontaneous metastases, lungs were removed, fixed in Bouin's solution and the number of surface lung nodules was determined by two independent observers under a dissecting microscope [15].

Apoptotic and mitotic cells were defined in ten high-power fields from tumor paraffin sections stained with hematoxylin and eosin and avoiding necrotic zones [16]. The percentage of apoptotic and mitotic cells in 1,000 tumor cells examined was designated as the apoptotic and mitotic index, respectively.

Immunohistochemistry

Immunostaining was performed using 4 μm cryo sections obtained from each study block of tumor tissue and spleen. The slides were air dried and fixed with pre-cooled acetone for 10 min. Endogenous peroxidase was blocked by immersing the sections in 0.03% hydrogen peroxide for 10 min. Tumor cell mitosis was corroborated by Ki-67 analysis (DAKO Corporation, CA) by immunohistochemistry (IHC). Apoptosis was determined in tumor sections by the APOPTAG® test (Oncor, Argentina), following the instructions given by the manufacturer. Tumor infiltrating lymphocytes $CD4^+$ (Sigma), $CD8^+$ (Sigma) and $CD19^+$ (BD Biosciences PharMingen, CA) were also detected by immunohistochemistry. $Gr1^+$ (BD BioScience Pharmingen) and $CD11b^+$ (Caltag Laboratories) cells were determined in spleen cryo-sections by immunohistochemical techniques. Angiogenesis was analyzed in frozen sections immunostained with an anti-CD31 monoclonal antibody (BD BioScience Pharmingen). Sections were visualized with 3,3′-diaminobenzidine as a chromogen for 5 min and counterstained with Mayer's hematoxylin.

Morphometry

The quantitative image morphometric analysis was carried out on spleen sections (3 sections/animal) after being stained with hematoxylin and eosin. The images data for morphometry was treated with the DIGIPAT IBM/PC computer system [17]. Follicles were evaluated in absolute numbers, follicle areas and whole spleen areas were also determined. An estimate of the follicle areas to whole spleen areas ratio (%) was derived from these two values.

Flow cytometry

Spleen samples were washed and resuspended in 50 μl of FACS buffer for staining with CD11b-FITC (Caltag Laboratories) and Gr1-FITC (BD Biosciences PharMingen). After 30 min, cells were washed, resuspended in FACS buffer and analyzed with a FACSCalibur flow cytometer (BD).

Statistical analysis

All statistical analyses were carried out using SPSS Data Analysis Program version 10.0 (SPSS, Inc, Chicago, IL). Statistical evaluation was performed by a randomized complete analysis of variance design with significance assessed at $P < 0.05$ level (ANOVA). When data did not have a normal distribution, the Kruskall–Wallis test and the two-tailed Mann Whitney test were used. The effect of treatment on the local invasiveness of F3II primary tumors was compared using X^2 test.

Results

Ganglioside expression in F3II sarcomatoid mammary carcinoma

To prove the expression of target ganglioside antigens in F3II mouse mammary carcinoma cells, a sensitive immunoperoxidase assay was employed. As shown in Fig. 1a, the P3 (Ab1) mAb reacted brightly against formalin-fixed F3II monolayers. A cytoplasmatic staining pattern was observed, with Golgi accentuation in some tumor cells (inset). No direct reactivity against F3II cells was obtained using the anti-idiotype 1E10 mAb as expected (Fig. 1b). These results confirm the presence of NeuGc-containing gangliosides in the F3II mammary tumor cell line.

Enhancement of 1E10 mAb antitumor response by co-administration of low-dose Cyclophosphamide in a mammary carcinoma model

As shown in Fig. 2, no antitumor effect was evidenced when a low-dose Cyclophosphamide was administered. Moreover, 1E10/Alum immunization at the evaluated dose only affected slightly the subcutaneous tumor growth; however, its co-administration with low-dose Cyclophosphamide significantly reduced the F3II mammary carcinoma growth. Interestingly, that response is comparable with the co-administration of the standard high-dose chemotherapy for breast cancer based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide, although the latter was associated with a significant body weight loss 7 days after the treatment (Data not shown).

A statistically significant anti-invasive effect was induced by the combinatorial treatment with 1E10 mAb immunization and low-dose Cyclophosphamide in the F3II mammary carcinoma (Table 1). Some tumors from animals of this group grew by filling the subcutis, without signs of active invasion of neighboring tissues, such as muscle and dermis.

Extrapulmonary tumor colonies were not found in any of the control or treated mice. The number of lung metastases analysis revealed no significant differences ($P > 0.05$) between control and treated mice.

Enhancement of 1E10 mAb induced apoptosis by co-administration of low-dose Cyclophosphamide in a mammary carcinoma model

Mitotic index and Ki-67 expression did not show any variation between tumors from control and chemo-immunotherapy treated animals, but a statistically significant antiproliferative effect was confirmed by morphological analysis and immunohistochemistry in mice treated with high-dose Doxorubicin and Cyclophosphamide. In contrast, as shown in Fig. 3, the immunization with 100 μg of 1E10 mAb in Alum induces some proapoptotic effect that was significantly enhanced in mice co-administered with 150 mg/m^2 of Cyclophosphamide, compared with that treatment alone or high-dose chemotherapy.

1E10 mAb combined with low-dose Cyclophosphamide reduces tumor-associated angiogenesis in a mammary carcinoma model

F3II mammary carcinoma angiogenesis was analyzed in frozen sections immunostained with an anti-CD31 specific antibody. A high microvessel density was seen in control

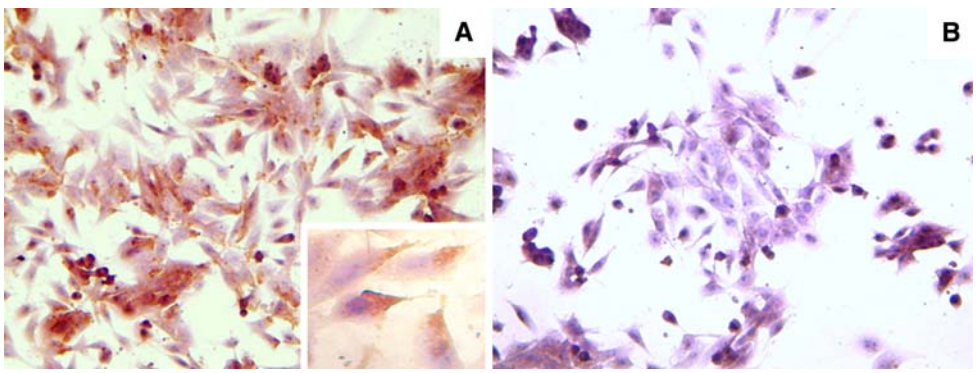

Fig. 1 Ganglioside expression in the F3II sarcomatoid mammary carcinoma. **a** Detection of NeuGc-containing gangliosides by immunocytochemistry using the P3 (Ab1) mAb in formalin-fixed tumor cell monolayers (×400; *inset* ×1,000). **b** No direct reactivity using the 1E10 anti-idiotype mAb (×400)

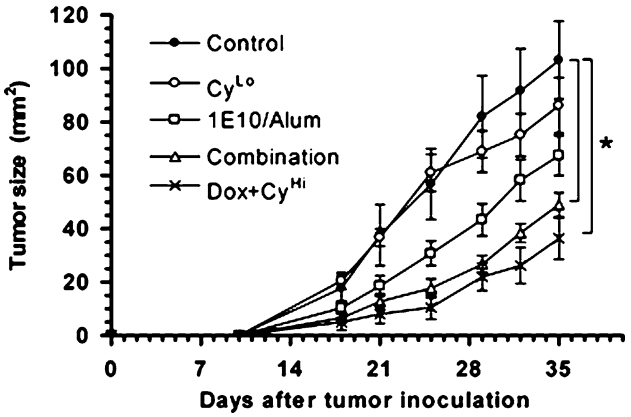

Fig. 2 Enhancement of 1E10 mAb antitumor response by co-administration of low-dose Cyclophosphamide in the F3II mammary carcinoma model. F3II tumor-bearing mice were immunized subcutaneously with 100 μg of 1E10 mAb in Alum (1E10/Alum) or with 150 mg/m^2 of Cyclophosphamide i.v. (Cy^{Lo}) 7 days after the tumor inoculation. Despite some antitumor effect induced by 1E10 mAb immunization; its co-administration with Cyclophosphamide (Combination) reduced significantly the F3II mammary carcinoma growth. This response is comparable with the administration of high-dose chemotherapy based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide (Dox + Cy^{Hi}). * Significant differences ($P < 0.05$). Kruskal–Wallis Test and Tukey's multiple comparison test

Table 1 Treatment effect on the local invasiveness of the sarcomatoid mammary carcinoma F3II

Treatment	Tumor localization positive/total animals (%)	
	Subcutis	Neighboring tissues
Control	15/15 (100)	12/15 (80)
Cy^{Lo}	15/15 (100)	10/15 (66.6)
1E10/Alum	14/14 (100)	7/14 (50)
Combination	15/15 (100)	3/15 (20)*
Dox + Cy^{Hi}	11/11 (100)	3/11 (33.3)*

Mice received 2 × 10^5 F3II cells in the subcutis of the right flank. Seven days after the tumor inoculation mice were immunized with 100 μg of 1E10 mAb in Alum (1E10/Alum), administered with 150 mg/m^2 of Cyclophosphamide (Cy^{Lo}) or both (Combination). The effect of the latter was comparable to the administration of high-dose chemotherapy based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide (Dox + Cy^{Hi})

* Significative differences ($P < 0.05$) X^2 test

mice, those immunized with 100 μg of 1E10 mAb in Alum or those that received high-dose chemotherapy (Fig. 4). On the contrary, the reduced microvessel density observed in mice treated with 150 mg/m^2 of Cyclophosphamide significantly increased in mice co-immunized with 1E10/Alum. Quantification of the number of blood microvessels per fields evidenced that chemo-immunotherapy combination correlates with a reduction in the tumor-associated angiogenesis.

1E10 mAb co-administrated with low-dose Cyclophosphamide increases the number of CD8$^+$ cells surrounding the F3II tumor

Histopathological examination of the tumor stroma (Fig. 5) evidenced a minimal number of mononuclear cells with some neutrophils and abundant fibroblasts indistinctly in the control group or in mice immunized with 100 μg of 1E10 mAb in Alum, Cyclophosphamide at 150 mg/m^2 or receiving high-dose chemotherapy based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide. However, a significant increment of lymphocytes surrounding the tumor cells were detected in animals co-administered with 1E10/Alum and low-dose Cyclophosphamide. Immunohistochemical analysis of the tumor-surrounding leukocyte population demonstrated that chemo-immunotherapy combination increased the number of CD8$^+$ T lymphocytes around the F3II tumor cells without affecting the CD4$^+$ T or B cell populations.

Effect of 1E10 mAb co-administrated with low-dose Cyclophosphamide in the immune response

On day 35, no significant differences were observed in the kidney, heart, lung and brain weights of the treated animals, while a significant increase in liver and spleen weights were observed in F3II tumor-bearing mice, with the lowest values for mice treated with chemo-immunotherapy or with high-dose chemotherapy (Data not shown).

Ab3 antibodies were not detected in the sera of mice treated with one dose of 100 μg of 1E10 mAb in Alum; on the other hand, no changes in size of the compartments and germinal centre formation were noted in spleen (Table 2). These results suggest that Ab3 antibodies do not seem to be responsible for the antitumor effect observed in mice immunized with 1E10/Alum, as previously reported [6].

1E10 mAb combined with low-dose Cyclophosphamide reduces the number of Gr1$^+$/CD11b$^+$ splenic cells in a mammary carcinoma model

Spleen and hepatic histological examination evidenced extramedullary myelopoiesis and megakaryopoiesis in mice inoculated with the sarcomatoid mammary carcinoma F3II, as previously reported [18]. Because these changes were less intense in animals treated with chemo-immunotherapy or with high-dose chemotherapy than in mice injected with low-dose Cyclophosphamide or control, we determined if these treatments reduced the number of Gr1$^+$/CD11b$^+$ splenic cells using immunohistochemistry techniques in frozen sections from treated animals. As shown in Fig. 6, splenic myeloid cells Gr1$^+$/CD11b$^+$ associated with a

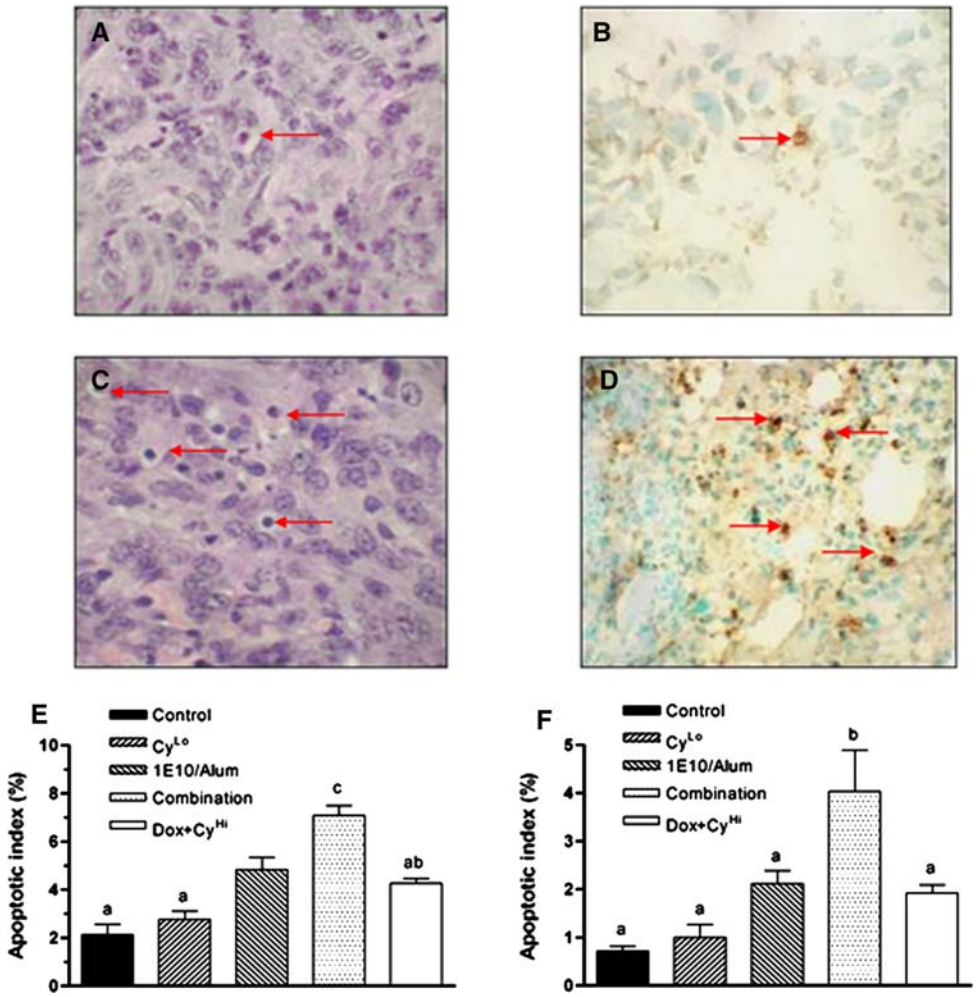

Fig. 3 Tumor apoptosis is enhanced by 1E10 mAb co-administration with low-dose Cyclophosphamide. Apoptosis was analyzed in tumor paraffin sections by hematoxylin and eosin staining (**a**, **c**) and by immunohistochemical study using the APOPTAG® tests (**b**, **d**). Subcutaneous immunization with 100 µg of 1E10 mAb in Alum (1E10/Alum) induces some proapoptotic effect that is enhanced significantly in mice co-administered with 150 mg/m^2 of Cyclophosphamide i.v. (Combination), compared with that treatment alone (Cy^{Lo}) or high-dose chemotherapy based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide (Dox + Cy^{Hi}). **a**, **b** Minimal apoptotic cells per field in control animals or treated with Cy^{Lo} (400×). **c**, **d** Abundant apoptotic cells per field in animals co-administered with 1E10 mAb and low-dose Cyclophosphamide (400×). **e**, **f** Apoptotic index was obtained by counting the apoptotic cells per 1,000 tumor cells. *Uneven letters* represent groups with statistically significant difference $P < 0.05$. Kruskal Wallis and Tukey's Multiple Comparison Tests

suppressor phenotype [19], are significantly reduced in F3II tumor-bearing mice immunized with 100 µg of 1E10 mAb in Alum alone or in combination with 150 mg/m^2 of Cyclophosphamide. High-doses of Cyclophosphamide (600 mg/m^2) co-administered with Doxorubicin (60 mg/m^2) 7 days after tumor inoculation also reduced those cells.

Discussion

Malignant growth is dictated from essential alterations in cell physiology acting collectively [20]. Consequently, cancer therapies may increase their efficacy potentiating a single antitumor mechanism they may exert; i.e. antiangiogenesis [10]. Alternatively, targeting simultaneously different of those acquired capabilities may overcome tumor escape mechanisms.

Cyclophosphamide may promote distinct antitumor effects associated with the administered dose. Low-dose Cyclophosphamide reduces angiogenesis and may enhance the antitumor effect of other therapeutic approaches displaying a similar mechanism of action [21].

The contribution of gangliosides to cell biology together with the NeuGc-GM3 ganglioside expression as tumor-associated antigen [22], and its limited toxicity as a targeted immunotherapy molecule [23, 24], support its relevance for cancer control. Moreover, targeting the NeuGc-GM3 ganglioside by immunotherapeutic approaches may promote

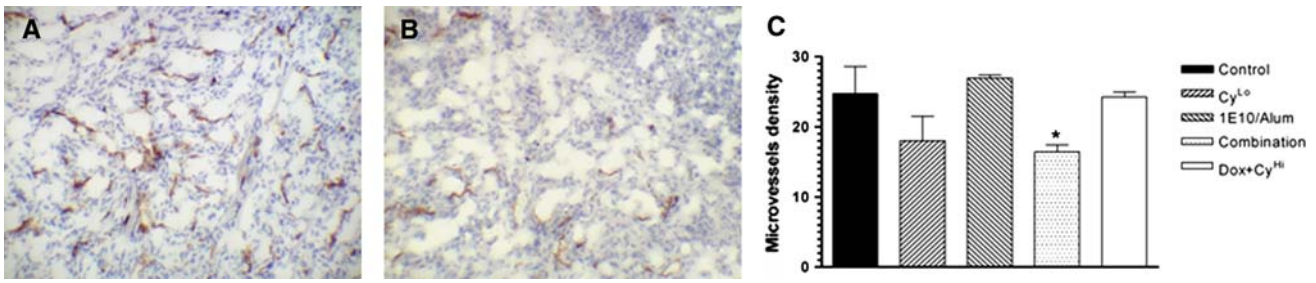

Fig. 4 1E10 mAb immunization enhances the antiangiogenic effect of low-dose Cyclophosphamide. F3II mammary carcinoma angiogenesis was analyzed in frozen sections immunostained with an anti-CD31 monoclonal antibody. **a** A high microvessels density is observed in control mice, immunized with 100 μg of 1E10 mAb in Alum (1E10/Alum) or receiving high-dose chemotherapy based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide (Dox + Cy^{Hi}) (100×). **b** The microvessel density reduction in mice treated with 150 mg/m^2 of Cyclophosphamide (Cy^{Lo}) is potentiated with the co-immunization of 1E10 mAb (100×). **c** Blood microvessels quantification evidenced a significant density reduction in the combination treated group. * $P < 0.05$. Kruskal Wallis and Tukey's Multiple Comparison Tests

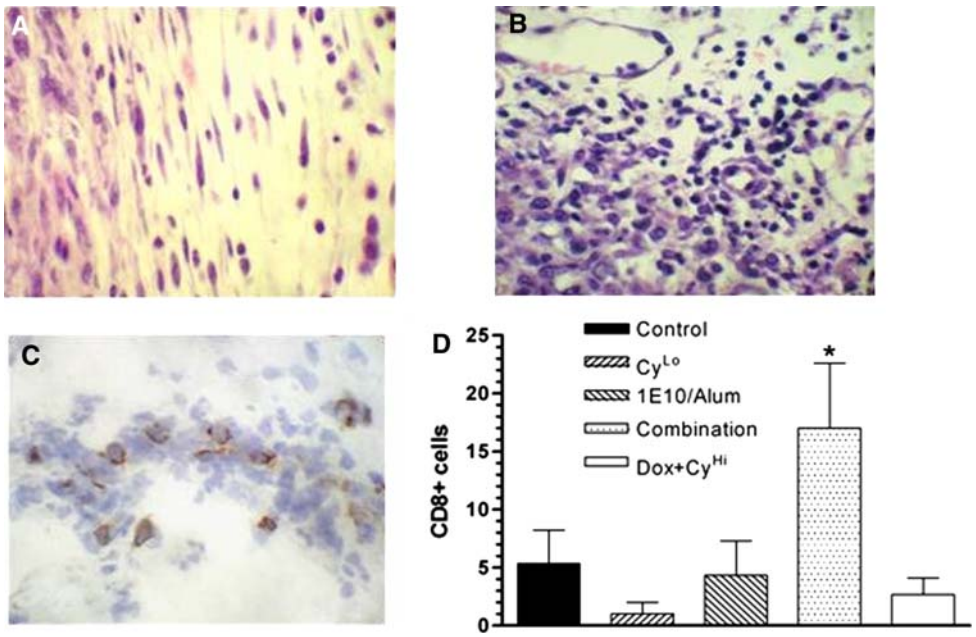

Fig. 5 Combinatorial therapy of 1E10 mAb with low-dose Cyclophosphamide promotes the CD8$^+$ lymphocytes tumor infiltration. Tumor infiltrating lymphocytes were analyzed in frozen sections. **a** A minimal number of lymphocytes with some neutrophils and abundant fibroblasts is observed indistinctly in the control group or in mice immunized subcutaneously with 100 μg of 1E10 mAb in Alum (1E10/Alum), Cyclophosphamide at 150 mg/m^2 (Cy^{Lo}) or receiving high-dose chemotherapy based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide (Dox + Cy^{Hi}) (400×). **b** A significant increment of tumor infiltrating lymphocytes was detected in animals co-administered with 1E10 mAb and low-dose Cyclophosphamide (Combination) (400×). **c** Tumor infiltrating CD8$^+$ lymphocytes evidenced by immunohistochemistry (400×). **d** Tumor infiltrating CD8$^+$ lymphocytes quantification evidenced a significant increment in the combination treated group. * $P < 0.05$ Kruskal Wallis and Tukey's Multiple Comparison Tests

tumor cell death through different mechanisms [6, 25]. It makes them suitable to be explored in combination with conventional drugs as a way to improve the antitumor efficacy of the latter. The development of new protocols combining chemotherapy with immunotherapy indicates an exciting potential for therapeutic synergy with general applicability to many cancer types [26, 27].

Here we provide evidence that 1E10/Alum vaccine in combination with low-dose Cyclophosphamide induced antitumor response in the F3II model, which expresses the NeuGc-GM3 ganglioside (Fig. 1). This spontaneous mouse sarcomatoid mammary carcinoma model is a highly aggressive neoplasm with a high local invasiveness, similar to the spindle cell/sarcomatoid carcinoma of the breast in humans [14, 28]. Interestingly, the chemo-immunotherapeutic approach significantly reduced the tumor growth (Fig. 2), and its local invasiveness (Table 1). That therapeutic response was associated with the induction of

Table 2 Morphometric assessment of the splenic compartments

Treatment	Follicle number		Follicle area (mm^2)	
	X	SD	*X*	SD
Control	5.6	0.55	806.88	367.50
Cy^{Lo}	6.0	1.00	996.40	307.07
1E10/Alum	5.2	0.80	876.57	323.61
Combination	6.0	1.50	857.71	273.07
Dox + Cy^{Hi}	5.5	1.29	1,109.62	331.52

F3II tumor-bearing mice were immunized at day 7 with 100 μg of 1E10 mAb in Alum (1E10/Alum), administered with 150 mg/m^2 of Cyclophosphamide (Cy^{Lo}) or both (Combination). An additional group of animals was administered with high-dose chemotherapy based on 60 mg/m^2 of Doxorubicin and 600 mg/m^2 of Cyclophosphamide (Dox + Cy^{Hi}). Spleen follicles were characterized 35 days after the tumor inoculation

programmed cell death (Fig. 3), an antiangiogenic effect (Fig. 4), as well as reduction of $Gr1^+/CD11b^+$ cells (Fig. 6).

In a previous report was demonstrated the antitumor activity of 1E10 coupled to keyhole limpet hemocyanin in Freund's adjuvant in the F3II model [5]. In the present work, mice treated with a single injection of aluminum hydroxide-precipitated 1E10 anti-Id mAb 7 days after the tumor inoculation displayed a discrete antitumor effect which permitted to evidence a substantial therapeutic response after a low-dose Cyclophosphamide co-administration. These data corroborate that the antitumor effects of conventional chemotherapy may be enhanced by combining with immunotherapy used in a therapeutic scenario [7]. Therefore, these results may have implications for the development of new protocols combining chemotherapy with immunotherapy, indicating an exciting potential for therapeutic convergence with general applicability to different cancer types.

The immunopotentiating effects of Cyclophosphamide, using the adequate dosage and timing for drug administration, have been extensively studied [29]. It has been established that low-dose chemotherapy can function as an angiogenesis inhibitor [10, 12, 30]. In our study, it was corroborated that low-dose Cyclophosphamide promoted an antiangiogenic effect, which is enhanced with the 1E10/Alum immunization in the sarcomatoid mammary carcinoma F3II (Fig. 4).

On the other side, the capability of 1E10 vaccine to induce apoptosis in NeuGc-GM3 ganglioside expressing tumor cells [6] was corroborated in this experimental conditions (Fig. 3). Despite moderate tumor apoptosis was observed after high-dose Doxorubicin and Cyclophosphamide treatment, 1E10/Alum immunization combined with low-dose Cyclophosphamide increased significantly the tumor apoptotic index. Consequently, the combinatorial therapeutic effect based on the complementary antiangiogenic effect of low-dose Cyclophosphamide with the tumor apoptosis induced by 1E10/Alum immunization resulted in significant growth retardation of F3II tumors. These results strongly suggest that enhanced apoptosis can contribute to tumor control [26]. Moreover, it may support that antitumor angiogenesis efficacy can be increased with combinatorial therapies, wherein novel tumor apoptosis promoting and/or cytotoxic dosing schedules of chemotherapeutic drugs or radiation are used concurrently [10].

In 1E10 vaccine clinical trials, most patients with advanced malignant breast cancer have been able to generate a specific antibody response against the 1E10 mAb and the Neu-Glycolyl-GM3 ganglioside [31]. In contrast, an Ab3 antibody response has not been possible to be

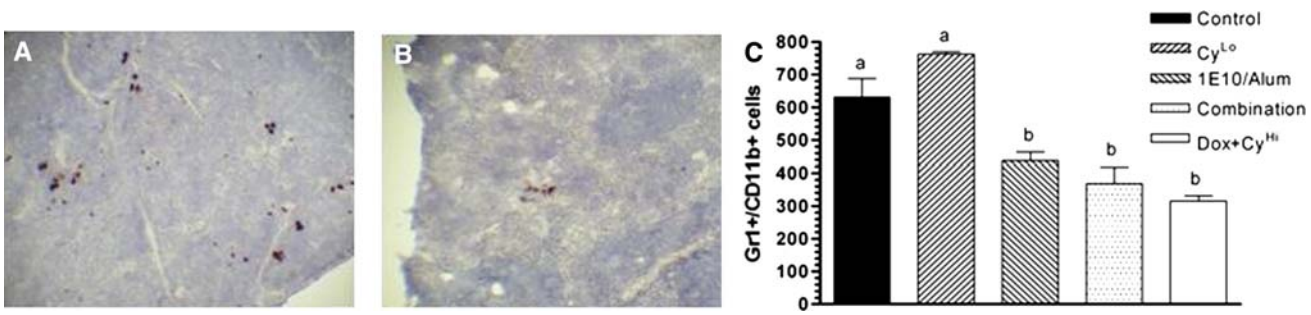

Fig. 6 1E10 mAb induced antitumor response is associated with a reduction in extramedullary myelopoiesis. Splenic myeloid cells $Gr1^+/CD11b^+$ associated to a suppressor phenotype are significantly reduced in F3II tumor-bearing mice immunized with 100 μg of 1E10 mAb in Alum (1E10/Alum) alone or co-administered with low-dose Cyclophosphamide (Combination). High-doses of Doxorubicin (60 mg/m^2) co-administered with Cyclophosphamide (600 mg/m^2) 7 days after tumor inoculation also reduced those cells (Dox + Cy^{Hi}). **a** High frequency of splenic myeloid cells $Gr1^+/CD11b^+$ foci from F3II tumor-bearing mice in the control group or inoculated with 150 mg/m^2 of Cyclophosphamide (Cy^{Lo}) alone (100×). **b** Reduction of splenic myeloid cells $Gr1^+/CD11b^+$ foci in mice immunized with 1E10 mAb, co-administered with low-dose Cyclophosphamide or receiving high-dose chemotherapy (100×). **c** Quantification of $Gr1^+/CD11b^+$ cells. *Uneven letters* represent groups with statistically significant difference $P < 0.05$. Kruskal Wallis and Tukey's Multiple Comparison Tests

evidenced in 1E10/Alum immunized mice [6]. However, a marked mononuclear cell reaction including macrophages and lymphocytes surrounding the tumor masses mainly of $CD8^+$ phenotype were demonstrated in animals immunized with 1E10/Alum in combination with 150 mg/m^2 of Cyclophosphamide (Fig. 5). Cellular immunity induction in cancer patients by the anti-idiotypic 1E10 vaccine has been reported previously in metastatic breast cancer patients with a year long vaccination scheme [32]. Our results may suggest that 1E10 vaccine combinatorial therapy can stimulate cellular and immune mechanisms for host protection against tumor formation and dissemination.

Mechanism of actions dissected from combined chemo-immunotherapy approaches, based on histological studies, suggest that inflammatory as well as T cell mechanisms can be involved in the antitumor response [33]. Preclinical and clinical studies have evidenced that combined therapies contribute to recruit $CD8^+$ T cells and natural killer cells, accompanied by an enhanced and prolonged antitumor cytolytic activity [34–38]. On the other hand, it has been demonstrated that low-dose of Cyclophosphamide may selectively deplete $CD4^+$ $CD25^+$ regulatory T cells (Treg) [27, 39]. Treg cells are part of physiological mechanisms controlling the emergence of pathological autoimmunity and the supervisors of tolerance maintenance toward 'self', who may in turn protect the tumor against the host immunoresponse [1, 40]. Therefore, it cannot rule out the potential effect of chemotherapeutic drugs on Treg depletion, contributing to enhance the cellular antitumor effects induced by the 1E10/Alum vaccine, which has to be defined.

Sarcomatoid mammary carcinoma F3II progression in vivo has been circumstantially associated with extramedullary myelopoiesis [18]. Interestingly, we found that myeloid $Gr1^+/CD11b^+$ cell population is represented in the liver and spleen of F3II tumor-bearing mice (Fig. 6). Furthermore, their reduction was associated with the tumor growth inhibition independently to the therapeutic approach employed. High-doses of Doxorubicin (60 mg/m^2) co-administered with Cyclophosphamide (600 mg/m^2) chemotherapy, but not Cyclophosphamide alone at the dose of 150 mg/m^2, were able to extensively decrease the number of myeloid $Gr1^+/CD11b^+$ cells. Unexpectedly, 1E10/Alum alone or in combination with low-dose Cyclophosphamide also significantly reduced those cells in tumor-bearing mice. $Gr1^+/CD11b^+$ cells, which include neutrophils and macrophages, have been shown to contribute to tumor angiogenesis. It was recently found that tumor accumulation of $Gr1^+/CD11b^+$ renders them refractory to angiogenic blockade by anti-vascular endothelial growth factor (VEGF) antibodies [41]. Elimination of these myeloid cells also allowed T cells to remain active, prevented neovascularization and prevented tumor resorption, so that tumor size remained stationary [42].

Myeloid $Gr1^+/CD11b^+$ cells have been associated with a suppressor cell phenotype [19] and they would act in concert with other lymphoid cells (e.g. Treg), hampering the host antitumor immunoresponse [1, 40]. Our results may suggest that differing from the documented effect of low-dose Cyclophosphamide selectively reducing Treg in vivo [43], that result was not documented on myeloid $Gr1^+/CD11b^+$ cells under current experimental conditions. Furthermore, these results may support the contribution of multiple tumor escape mechanisms which also involve immunoregulatory pathways [44, 45]. Therefore, the elimination of myeloid suppressor cells will likely be a valuable additional strategy to lessen tumor-induced immunosuppression contributing to augment immunotherapy efficacy [46–48].

Cancer vaccines represent an attractive approach for tumor control due to their limited toxicity and diversity of mechanisms exerted [49]; however, it is necessary to improve their therapeutic efficacy [50]. Manipulating immunopharmacological variables [51] or their rational combination with chemotherapeutic agents may induce a rapid and persistent immune response [52]. In addition, concurrent mechanisms of antitumor effects between chemotherapy and immunotherapeutic approaches could be exploited. Cancer vaccines would be combined with low-dose chemotherapy resulting at least in an equivalent antitumor effect, when compared with standard high-dose protocols, which was evidenced in our experimental setting. This data may provide a rational for chemo-immunotherapy combinations with potential medical implications in breast cancer.

References

1. Montero E, Amador JF, Perez R, Lage A (2000) Tumor-specific immunotherapy based on dominant models of natural tolerance. Med Hypotheses 54:531–536. doi:10.1054/mehy.1999.0892
2. Bhattacharya-Chatterjee M, Chatterjee SK, Foon KA (2001) The anti-idiotype vaccines for immunotherapy. Curr Opin Mol Ther 3:63–69
3. Mohanty K, Saha A, Pal S, Mallick P, Chatterjee SK, Foon KA, Bhattacharya-Chatterjee M (2007) Anti-tumor immunity induced by an anti-idiotype antibody mimicking human Her-2/neu. Breast Cancer Res Treat 104:1–11. doi:10.1007/s10549-006-9391-9
4. Vazquez AM, Perez A, Hernandez AM, Macias A, Alfonso M, Bombino G, Perez R (1998) Syngeneic anti-idiotypic monoclonal antibodies to an anti-NeuGc-containing ganglioside monoclonal antibody. Hybridoma 17:527–534
5. Vazquez AM, Gabri MR, Hernandez AM, Alonso DF, Beausoleil I, Gomez DE, Perez R (2000) Antitumor properties of an anti-idiotypic monoclonal antibody in relation to *N*-glycolyl-containing gangliosides. Oncol Rep 7:751–756

6. Diaz Y, Gonzalez A, Lopez A, Perez R, Vazquez AM, Montero E (2008) Anti-ganglioside anti-idiotypic monoclonal antibody-based cancer vaccine induces apoptosis and antiangiogenic effect in a metastatic lung carcinoma. Cancer Immunol Immunother
7. Honeychurch J, Glennie MJ, Illidge TM (2005) Cyclophosphamide inhibition of anti-CD40 monoclonal antibody-based therapy of B cell lymphoma is dependent on $CD11b^{+}$ cells. Cancer Res 65:7493–7501. doi:10.1158/0008-5472.CAN-04-3808
8. Reece DE, Foon KA, Bhattarcharya-Chatterjee M, Adkins D, Broun ER, Connaghan DG, Dipersio JF, Holland HK, Howard DA, Hale GA et al (2003) Use of the anti-idiotype breast cancer vaccine 11D10 in conjunction with autologous stem cell transplantation in patients with metastatic breast cancer. Clin Breast Cancer 3(Suppl 4):S152–S157. doi:10.3816/CBC.2003.s.005
9. Sinkovics JG, Horvath JC (2006) Evidence accumulating in support of cancer vaccines combined with chemotherapy: a pragmatic review of past and present efforts. Int J Oncol 29: 765–777
10. Hanahan D, Bergers G, Bergsland E (2000) Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 105:1045–1047. doi:10.1172/JCI9872
11. Fisher B, Brown AM, Dimitrov NV, Poisson R, Redmond C, Margolese RG, Bowman D, Wolmark N, Wickerham DL, Kardinal CG et al (1990) Two months of doxorubicin-cyclophosphamide with and without interval reinduction therapy compared with 6 months of cyclophosphamide, methotrexate, and fluorouracil in positive-node breast cancer patients with tamoxifen-nonresponsive tumors: results from the National Surgical Adjuvant Breast and Bowel Project B-15. J Clin Oncol 8:1483–1496
12. Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, Folkman J (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 60:1878–1886
13. Loeffler M, Kruger JA, Reisfeld RA (2005) Immunostimulatory effects of low-dose cyclophosphamide are controlled by inducible nitric oxide synthase. Cancer Res 65:5027–5030. doi:10.1158/0008-5472.CAN-05-0646
14. Alonso DF, Farias EF, Urtreger A, Ladeda V, Vidal MC, Bal De Kier Joffe E (1996) Characterization of F3II, a sarcomatoid mammary carcinoma cell line originated from a clonal subpopulation of a mouse adenocarcinoma. J Surg Oncol 62:288–297. doi:10.1002/(SICI)1096-9098(199608)62:4<288::AID-JSO14>3.0.CO;2-1
15. Alonso DF, Farina HG, Skilton G, Gabri MR, De Lorenzo MS, Gomez DE (1998) Reduction of mouse mammary tumor formation and metastasis by lovastatin, an inhibitor of the mevalonate pathway of cholesterol synthesis. Breast Cancer Res Treat 50: 83–93. doi:10.1023/A:1006058409974
16. Trump BF, Berezesky IK, Chang SH, Phelps PC (1997) The pathways of cell death: oncosis, apoptosis, and necrosis. Toxicol Pathol 25:82–88
17. Coro RM, Borrajero I (1996) DIGIPAT. Un sistema cubano para morfometría de imágenes. Rev Latinoam Patol 34:9–10
18. Gabri MR, Menna PL, Scursoni AM, Gomez DE, Alonso DF (1999) Role of tumor-derived granulocyte-macrophage colony-stimulating factor in mice bearing a highly invasive and metastatic mammary carcinoma. Pathobiology 67:180–185. doi:10.1159/000028070
19. Bronte V, Apolloni E, Cabrelle A, Ronca R, Serafini P, Zamboni P, Restifo NP, Zanovello P (2000) Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. Blood 96:3838–3846
20. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70. doi:10.1016/S0092-8674(00)81683-9
21. du Manoir JM, Francia G, Man S, Mossoba M, Medin JA, Viloria-Petit A, Hicklin DJ, Emmenegger U, Kerbel RS (2006) Strategies for delaying or treating in vivo acquired resistance to trastuzumab in human breast cancer xenografts. Clin Cancer Res 12:904–916. doi:10.1158/1078-0432.CCR-05-1109
22. Marquina G, Waki H, Fernandez LE, Kon K, Carr A, Valiente O, Perez R, Ando S (1996) Gangliosides expressed in human breast cancer. Cancer Res 56:5165–5171
23. Bada A, Casaco Parada A, Arteaga M, Martinez J, Leon A, Santana E, Hernandez O, Orphee R, Gonzalez A, Mesa C et al (2002) Toxicity of a GM3 cancer vaccine in Macaca fascicularis monkey: a 12-month study. Hum Exp Toxicol 21:263–267. doi:10.1191/0960327102ht248oa
24. Oliva JP, Valdes Z, Casaco A, Pimentel G, Gonzalez J, Alvarez I, Osorio M, Velazco M, Figueroa M, Ortiz R et al (2006) Clinical evidences of GM3 (NeuGc) ganglioside expression in human breast cancer using the 14F7 monoclonal antibody labelled with (99 m)Tc. Breast Cancer Res Treat 96:115–121. doi:10.1007/s10549-005-9064-0
25. Roque-Navarro L, Chakrabandhu K, de Leon J, Rodriguez S, Toledo C, Carr A, de Acosta CM, Hueber AO, Perez R (2008) Anti-ganglioside antibody-induced tumor cell death by loss of membrane integrity. Mol Cancer Ther 7:2033–2041. doi:10.1158/1535-7163.MCT-08-0222
26. Lake RA, Robinson BW (2005) Immunotherapy and chemotherapy—a practical partnership. Nat Rev Cancer 5:397–405. doi:10.1038/nrc1613
27. Taieb J, Chaput N, Schartz N, Roux S, Novault S, Menard C, Ghiringhelli F, Terme M, Carpentier AF, Darrasse-Jeze G et al (2006) Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. J Immunol 176:2722–2729
28. Carter MR, Hornick JL, Lester S, Fletcher CD (2006) Spindle cell (sarcomatoid) carcinoma of the breast: a clinicopathologic and immunohistochemical analysis of 29 cases. Am J Surg Pathol 30:300–309
29. Nowak AK, Lake RA, Robinson BW (2006) Combined chemoimmunotherapy of solid tumours: improving vaccines? Adv Drug Deliv Rev 58:975–990. doi:10.1016/j.addr.2006.04.002
30. Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Kerbel RS (2000) Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 105:R15–R24. doi:10.1172/JCI8829
31. Diaz A, Alfonso M, Alonso R, Saurez G, Troche M, Catala M, Diaz RM, Perez R, Vazquez AM (2003) Immune responses in breast cancer patients immunized with an anti-idiotype antibody mimicking NeuGc-containing gangliosides. Clin Immunol 107:80–89. doi:10.1016/S1521-6616(03)00036-6
32. Guthmann MD, Castro MA, Cinat G, Venier C, Koliren L, Bitton RJ, Vazquez AM, Fainboim L (2006) Cellular and humoral immune response to *N*-glycolyl-GM3 elicited by prolonged immunotherapy with an anti-idiotypic vaccine in high-risk and metastatic breast cancer patients. J Immunother 29:215–223. doi:10.1097/01.cji.0000188502.11348.34
33. Zhou H, Sequeira M, Goad ME, Erickson J, Wong A, Clark E, Dunussi-Joannopoulos K, Li RC, Friedrich S, Hayes LL et al (2001) Efficacy and mechanisms of action of rmB7.2-Ig as an antitumor agent in combination with adriamycin and cytoxan chemotherapy. Clin Immunol 101:303–314. doi:10.1006/clim.2001.5123
34. Kanwar JR, Kanwar RK, Pandey S, Ching LM, Krissansen GW (2001) Vascular attack by 5, 6-dimethylxanthenone-4-acetic acid combined with B7.1 (CD80)-mediated immunotherapy overcomes immune resistance and leads to the eradication of large tumors and multiple tumor foci. Cancer Res 61:1948–1956

35. Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D, Okoye FI, Jaffee EM (2001) Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. Cancer Res 61:3689–3697
36. Eralp Y, Wang X, Wang JP, Maughan MF, Polo JM, Lachman LB (2004) Doxorubicin and paclitaxel enhance the antitumor efficacy of vaccines directed against HER 2/neu in a murine mammary carcinoma model. Breast Cancer Res 6:R275–R283. doi:10.1186/bcr787
37. Monzavi-Karbassi B, Pashov A, Jousheghany F, Artaud C, Kieber-Emmons T (2006) Evaluating strategies to enhance the anti-tumor immune response to a carbohydrate mimetic peptide vaccine. Int J Mol Med 17:1045–1052
38. Rice J, Dunn S, Piper K, Buchan SL, Moss PA, Stevenson FK (2006) DNA fusion vaccines induce epitope-specific cytotoxic CD8(+) T cells against human leukemia-associated minor histocompatibility antigens. Cancer Res 66:5436–5442. doi:10.1158/0008-5472.CAN-05-3130
39. Emens LA, Jaffee EM (2005) Leveraging the activity of tumor vaccines with cytotoxic chemotherapy. Cancer Res 65:8059–8064. doi:10.1158/0008-5472.CAN-05-1797
40. Montero E, Alonso L, Perez R, Lage A (2007) Interleukin-2 mastering regulation in cancer and autoimmunity. Ann N Y Acad Sci 1107:239–250. doi:10.1196/annals.1381.026
41. Shojaei F, Ferrara N (2008) Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells. Cancer Res 68:5501–5504. doi:10.1158/0008-5472.CAN-08-0925
42. Zhang B, Zhang Y, Bowerman NA, Schietinger A, Fu YX, Kranz DM, Rowley DA, Schreiber H (2008) Equilibrium between host and cancer caused by effector T cells killing tumor stroma. Cancer Res 68:1563–1571. doi:10.1158/0008-5472.CAN-07-5324
43. Ercolini AM, Ladle BH, Manning EA, Pfannenstiel LW, Armstrong TD, Machiels JP, Bieler JG, Emens LA, Reilly RT, Jaffee EM (2005) Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. J Exp Med 201:1591–1602. doi:10.1084/jem.20042167
44. Kroemer G, Pouyssegur J (2008) Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 13:472–482. doi:10.1016/j.ccr.2008.05.005
45. Zitvogel L, Apetoh L, Ghiringhelli F, Andre F, Tesniere A, Kroemer G (2008) The anticancer immune response: indispensable for therapeutic success? J Clin Invest 118:1991–2001. doi:10.1172/JCI35180
46. Bronte V, Serafini P, Apolloni E, Zanovello P (2001) Tumor-induced immune dysfunctions caused by myeloid suppressor cells. J Immunother 24:431–446
47. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM (2005) Gemcitabine selectively eliminates splenic Gr-1^{+}/CD11b^{+} myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res 11:6713–6721. doi:10.1158/1078-0432.CCR-05-0883
48. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, Dolcetti L, Bronte V, Borrello I (2006) Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med 203:2691–2702. doi:10.1084/jem.20061104
49. Finn OJ (2008) Cancer immunology. N Engl J Med 358:2704–2715. doi:10.1056/NEJMra072739
50. Finn OJ (2008) Tumor immunology top 10 list. Immunol Rev 222:5–8. doi:10.1111/j.1600-065X.2008.00623.x
51. Rodriguez PC, Gonzalez I, Gonzalez A, Avellanet J, Lopez A, Perez R, Lage A, Montero E (2008) Priming and boosting determinants on the antibody response to an epidermal growth factor-based cancer vaccine. Vaccine 26:4647–4654. doi:10.1016/j.vaccine.2008.07.003
52. Montero E, Valdes M, Avellanet J, Lopez A, Perez R, Lage A (2009) Chemotherapy induced transient B-cell depletion boosts antibody-forming cells expansion driven by an epidermal growth factor-based cancer vaccine. Vaccine 27:2230–2239. doi:10.1016/j.vaccine.2009.02.018