Cry1Ac Protoxin Confers Antitumor Adjuvant Effect in a Triple-Negative Breast Cancer Mouse Model by Improving Tumor Immunity

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ABSTRACT: The Cry1Ac protoxin from *Bacillus thuringiensis* is a systemic and mucosal adjuvant, able to confer protective immunity in different infection murine models and induce both Th1 and TCD8+ cytotoxic lymphocyte responses, which are required to induce antitumor immunity. The Cry1Ac toxin, despite having not being characterized as an adjuvant, has also proved to be immunogenic and able to activate macrophages. Here, we investigated the potential antitumor adjuvant effect conferred by the Cry1Ac protoxin and Cry1Ac toxin in a triple negative breast cancer (TNBC) murine model. First, we evaluated the ability of Cry1Ac proteins to improve dendritic cell (DC) activation and cellular response through intraperitoneal (i.p.) coadministration with the 4T1 cellular lysate. Mice coadministered with the Cry1Ac protoxin showed an increase in the number and activation of CD11c+MHCII- and CD11c+MHCII+ low in the peritoneal cavity and an increase in DC activation (CD11c+MHCII+) in the spleen. Cry1Ac protoxin increased the proliferation of TCD4+ and TCD8+ lymphocytes in the spleen and mesenteric lymph nodes (MLN), while the Cry1Ac toxin only increased the proliferation of TCD4+ and TCD8+ in the MLN. Remarkably, when tested in the in vivo TNBC mouse model, prophylactic immunizations with 4T1 lysates plus the Cry1Ac protoxin protected mice from developing tumors. The antitumor effect conferred by the Cry1Ac protoxin also increased specific cytotoxic T cell responses, and prevented the typical tumor-related decrease of T cells (TCD3+ and TCD4+) as well the increase of myeloid-derived suppressor cells (MDSC) in spleen. Also in the tumor microenvironment of mice coadministered twice with Cry1Ac protoxin immunological improvements were found such as reductions in immunosupressive populations (T regulatory lymphocytes and MDSC) along with increases in macrophages upregulating CD86. These results show a differential antitumor adjuvant capability of Cry1Ac proteins, highlighting the ability of Cry1Ac protoxin to enhance local and systemic tumor immunity in TNBC. Finally, using a therapeutic approach, we evaluated the coadministration of Cry1Ac protoxin with doxorubicin. A significant reduction in tumor volume and lung metastasis was found, with increased intratumoral levels of tumor necrosis factor-α and IL-6 with respect to the vehicle group, further supporting its antitumor applicability.

KEYWORDS: Adjuvants, breast cancer, Cry1Ac protoxin, immunotherapy, omentum, doxorubicin

Introduction

Triple-negative breast cancer (TNBC) is characterized by a lack of expression of the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2), accounting for approximately 15% to 20% of breast cancer patients.[1] The lack of specific targeted therapies and poor prognosis of patients with TNBC has fostered a major effort to discover new therapies to treat these patients.[2]

Immunotherapy has emerged as an attractive therapy for some types of cancer. Active immunotherapy broadly refers to stimulation of the immune system against cancer cells. However, a current challenge in cancer immunotherapy is the immunosuppressive tumor microenvironment (TME) and poor immunogenicity to tumor-associated antigens (TAAs).[3] One approach is tumor antigen-based vaccines administered with appropriate adjuvants that can induce specific T-cell responses against TAAs. A key component of these vaccines is an effective adjuvant to enhance the ability of dendritic cells (DC) to generate specific immune responses.[4] Nevertheless, classical approved adjuvants, such as aluminum hydroxide,

mainly induces Th2-type responses but have not been effective in producing Th1-type and TCD8+ cytotoxic responses, the kind of immune responses required for the removal of tumors. Other types of adjuvants that have been studied are TLR agonists, such as CpG (TLR9 agonist),[5,6] monophosphoryl lipid A (MPL, TLR2, and TLR4 agonists),[7] Imiquimod (TLR 7 agonist),[8-10] and poly I:C (TLR 3 agonist),[11,12] which have been shown to enhance or have antitumor effects by themselves. Nonetheless, the use of Toll ike receptor (TLR) agonists is a limitation in cancer immunotherapy because the expression of TLRs in many tumors and their stimulation might result in tumor progression.[13-15] In addition, it has been reported that some TLR agonists have toxic effects[16] and could generate chronic inflammation,[17] which is related to carcinogenesis. Therefore, it is necessary to find new, safe adjuvants capable of inducing tumor immunity.

The Cry1Ac protoxin, which is a delta-endotoxin produced during the sporulation phase of *Bacillus thuringiensis*, has been proposed as an effective and safe alternative adjuvant. When ingested by susceptible larvae, the crystal protein is solubilized

and proteolitically processed, generating the Cry1Ac toxin, which has been widely used as a bioinsecticide.[18] The recombinant Cry1Ac protoxin is immunogenic and adjuvant at the systemic and mucosal level[19-21] and is able to activate antigen presenting cells (APCs) by upregulating costimulatory molecules and increasing the production of pro-inflammatory cytokines.[22,23] It is able to confer protective immunity against distinct infection models[24-26] and remarkably in a murine brucellosis model, it showed the capacity to induce both Th1 and TCD8+ cytotoxic lymphocyte responses,[27] suggesting a potential utility to improve tumor immunity. The Cry1Ac toxin, despite having not being characterized as an adjuvant, is immunogenic[28] and able to activate macrophages, inducing upregulation of costimulatory molecules and increasing the production of pro-inflammatory cytokines.[29] The potential antitumor adjuvant effect of Cry1Ac proteins has not been explored before. Therefore, here we investigated the potential of Cry1Ac protoxin and Cry1Ac toxin to confer antitumor adjuvant effect in the TNBC murine model induced with the breast cancer cell line 4T1. To this aim, first we evaluated the capacity to induce activation of DC and to improve cellular responses. Afterward, we tested whether the coadministration of Cry1Ac proteins with 4T1 lysates in prophylactic immunization schemes could improve tumor immunity TNBC mouse model. Later, we evaluated the influence of immunization treatments on immunological populations within tumor environment. Finally, we performed a therapeutic approach, to evaluate the coadministration of Cry1Ac protoxin with doxorubicin in TNBC mouse model. The outcomes indicate a differential adjuvant capacity of Cry1Ac proteins to improve tumor immunity, being the effect of Cry1Ac protoxin outstanding compared with that of Cry1Ac toxin. In addition, Cry1Ac protoxin may confer therapeutic benefit when coadministered with doxorubicin.

Material and Methods

Materials

Fetal bovine serum (FBS), L-Glutamine, and essential amino acids were obtained from GIBCO BRL (NY14072) (Grand Island, NY). Polymyxin B resin was purchased from Bio-Rad (Hercules, CA). Sodium chloride (NaCl), sodium carbonate, sodium bicarbonate, and sodium phosphate were purchased from J.T. Baker Corp (South Plainfield, NJ). RPMI-1640 medium, E-toxate, penicillin, streptomycin, ethylenediaminetetraacetic acid (EDTA), and all other chemicals were purchased from Sigma Chemical Co (St Louis, MO).

Recombinant Cry1Ac protoxin and Cry1Ac toxin

The recombinant Cry1Ac protoxin was purified from Isopropil ß-D-1-tiogalactopiranósido (IPTG)-induced *Escherichia coli* (pOS9300) cultures as previously described.[25] The Cry1Ac protoxin was solubilized and the Cry1Ac toxin was activated with

trypsin, performed as previously described.[29] Endotoxin levels of Cry1Ac proteins were tested using the E-toxate kit and were found to be below 0.1 EU/mL. The Cry1Ac proteins were then treated with a polymyxin resin to remove any possible endotoxin remnants and finally examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and the total protein concentration was determined by the Bradford method.

Animals

Female BALB/c mice (6-8 weeks old) were used throughout this study. Animals were maintained in filter-topped cages, provided with sterile food (2019 Teklad Global 19% Protein Extruded Rodent Diet; Harlan Laboratories Inc, Indianapolis, IN) and water ad libitum and cared for according to the guidelines of the Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999; Ministry of Agriculture, Mexico). The number (n) of mice used per group is indicated in each figure legend.

Cell line and 4T1 lysate

The 4T1 mouse mammary carcinoma cells (ATCC, Manassas, VA) were cultured in RPMI-1640 medium containing penicillin (100 U/mL), streptomycin (100 μg/mL), fungicide (0.75 μg/mL), and supplemented with 10% FBS (Gibco, Gaithersburg, MD). The 4T1 cells in culture were obtained and washed by centrifugation with RPMI medium and phosphate buffered saline (PBS). The pellet containing 5×10^7 cells were suspended in 3 mL of sterile PBS containing 5 mM *p*-hydroxymercuribenzoic acid (Sigma Chemical Co) to prevent proteolysis and sonicated (Sonic Dismembrator Model 300 Fisher, Pittsburgh, PA) for 1 minute in ice. The lysates were stored in aliquots and stored at -80°C and examined by SDS-PAGE, and the protein concentration was determined by the Bradford method.

Prophylactic immunizations

Single i.p. application with the Cry1Ac protoxin or Cry1Ac toxin (at 50 μg doses each) + 4T1 cell lysate (50 μg), in 100 μL volumes were used to evaluate the activation of DC in distinct tissues which were recovered after 48 hour. For the evaluation of proliferative T-cell responses in spleen and MLN, mice were immunized at the same antigen doses twice at 1 week intervals and killed 48 hours after the second immunization. For the evaluation antitumor effects, we used 2 immunization schemes that consisted of 3 prophylactic immunizations via i.p. application with the Cry1Ac protoxin or Cry1Ac toxin (at 50 μg doses each) + 4T1 cell lysate (50 μg), in 100 μL volumes, as well as control groups administered with only the vehicle (PBS). In the first scheme, immunizations were applied every third day and in the second scheme, immunizations were applied every seventh day. Seven days after the last immunization, the tumors were

induced by subcutaneous injection, into the breast of BALB/c mice with 3×10^3 4T1 cells that were freshly obtained from cell cultures. The tumors were measured every third day with a digital caliper, and the tumor volume was obtained according to the formula V = 0.52 (A2 × L) (L = length × A = width).[30,31] All mice were killed 40 days after 4T1 cell inoculation. A similar prophylactic immunization scheme (applied every seventh day) was used to evaluate the effect on tumor growth of administration of the Cry1Ac proteins alone (at 50 μg doses each). We also evaluated a therapeutic immunization scheme at the same doses of Cry1Ac protoxin; this scheme consisted of 3 peritumoral subcutaneous injections applied every week from the second week after tumor induction with 3×10^3 4T1 cells.

Finally, to be able to evaluate immunological tumor environment, we tested a 2 subcutaneous immunization scheme, (spatiated by a week) at the same antigen doses (50 μg doses each), and then to ensure tumor induction, we injected on day 21 a larger number of 4T1 cells (1 x 10^4 cells).

Leukocyte isolation from the peritoneal cavity, omentum, mesenteric lymph nodes, and spleen

The mice i.p. injected with the 4T1 lysate alone or coadministered with the Cry1Ac protoxin or Cry1Ac toxin were killed under anesthesia 48 hours or the 10th day after the first or second injection of each treatment to collect leukocytes from distinct tissues for analysis of the activation of DC and the proliferation of lymphocytes, respectively. Peritoneal exudate cells were collected by peritoneal lavage with 5 mL of ice-cold PBS containing 5% FBS; the peritoneal cavity was gently agitated for 3 minutes before retrieval of the peritoneal fluid. The spleen and mesenteric lymph nodes (MLN) were excised and disaggregated through a sterile nylon mesh filter using 5 mL of RPMI-1640 medium supplemented with 5% FBS. Omentum was obtained as reported by Carlow et al.[32] Briefly, Omentum were mechanically excised and disaggregated through a sterile nylon mesh filter and washed twice in culture media, and finally the suspension was passed through a nylon mesh filter.

4T1 peptides

The peptides corresponding to epitopes identified in 4T1 cells were synthetized in Tmtc2 (QGVTVLAVSAVYDIFVFHRLK MKQILP), GPRC5a (FAICFSCLLAHALNLIKLVRGRK PLSW), and Qars (FPPDAINNF), in which sequences were identified as neoepitopes for CD8 by next generation sequencing of the 4T1 line[33] and surviving tumor-associated antigen (GWEPDDNPI). The pooled peptides were used to evaluate specific cellular responses in the spleen and mesenteric lymph nodes.

T cell proliferation assay

A T cell proliferation assay was performed on the 15th day (24 hours after the second immunization of mice that were weekly immunized) by labeling 4×10^6 mesenteric lymph node cells and splenocytes with carboxyfluorescein diacetate succinimidyl ester (CFSE) (5 μM) (Molecular probes, Eugene, OR, Cat#: 11524217). The cell density was adjusted to 2×10^6 cells/mL, and cells were cultivated in 12-well plates and stimulated with 10 μg/mL of a pool of synthetic peptides expressed by the tumor line 4T1 (Tmte2, Gprc5a, Qars, and surviving)[35] for 72 hours, 37°C, and 5% CO_2. Subsequently, the cells were recovered and stained for analysis by flow cytometry.

Tumor microenvironment

Tumors from mice receiving a 2 immunization scheme every seventh day, were collected 3 weeks after tumor induction. Tumors were mechanically disaggregated through a sterile nylon mesh filter and washed twice in culture media and finally the suspension was passed through a nylon mesh filter. Cell suspensions were counted and divided for the evaluation of immunological parameters on lymphocyte populations, macrophages, and myeloid-derived suppressor cells (MDSC) which had been linked with tumor immunity.

Flow cytometry analysis

Leukocytes obtained for T cell proliferation and the DC activation assay were counted by a Bio-Rad TC10 automated cell counter (Hercules, CA). Cells (1×10^6) were centrifuged at 400 g for 5 minutes at 4°C. To prevent the nonspecific binding of immunoglobulins, the pellet cells were treated for 30 minutes with 1 μg/mL of the anti-CD16/32 monoclonal antibody to the Fc γ II/III receptor (Fc block) (Biolegend, San Diego, CA, Cat#:101302) in 0.5% BSA-PBS. The cells were washed with BSA-PBS and incubated with (CD4-PeCy5, CD8-APC, and CD19-PE) for T cell proliferation and (CD11c-FITC, MHCII-APC, CD80-PeCy5, and CD86-PE) for the DC activation assay at an appropriate dilution for 30 minutes at 4°C in the dark. To evaluate the effect of immunization on immune cells in tumor environment, tumors cells were incubated with one of the following stainings: (1) CD3-APC, CD4-FITC, CD8-PE, and CD69-PeCy5; (2) CD3-APC, CD4-PeCy5, CD25-FITC, and FoxP3-PE; (3) CD11b-PeCy5, F4/80-FITC, Gr1-PE, and CD86-APC. The cells were washed with BSA-PBS and suspended in 300 μL of 1% paraformaldehyde in PBS. All dates were analyzed using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) and (BD CellQuest Pro .5.1.1 software, 2000, Becton, Dickinson and Company, San Jose, CA). Unless other thing specified in general, 100 000 events per sample were recorded.

Cell viability

To determine whether Cry1Ac protoxin and Cry1Ac toxin have cytotoxic effects on 4T1 cells, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO) was used as previously described.[34] The 4T1cells (1×10^4 cells/well) were incubated with different

concentrations of Cry1Ac proteins, washed with PBS, and incubated with MTT (0.5 mg/mL in the cell culture medium without FBS) for 4 hours at 37°C. Subsequently, isopropanol (100 µL/well) was added and mixed the plate for 2 minutes to dissolve the MTT. The optical density was read on a microplate reader at 570 nm, with a reference at 655 nm (EL800, Bio-Tek Instruments, Inc, Winooski, VT). The mitochondrial activity of treated cells to that of the negative control (cells incubated with vehicle) was normalized. To test whether Cry1Ac protoxin or Cry1Ac toxin could provoke apoptosis in 4T1 cells, the incorporation of Annexin V was determined in cells incubated for 24 hours with distinct concentrations of Cry1Ac proteins. The positive control for apoptosis was 500 µM hydrogen peroxide (H_2O_2); untreated cells served as a negative control. After 24 hours of stimulation, the cells were incubated with 25 µL of Annexin V (Sigma-Aldrich-A9460; diluted 1:50 in binding buffer: 0.1 M HEPES, 0.14 M NaCl, and 0.0025 M $CaCl_2$) for 25 minutes in the dark, then the cells were acquired (counting 30 000 events per sample) using a flow cytometer (FACSCalibur). For these assays, 3 independent experiments were performed with technical duplicates for each treatment.

In vitro cytotoxicity assay of 4T1 cells by T lymphocytes

The analysis of cell cytotoxicity mediated by T cells was assayed as described previously.[35] The spleens were collected on the 21th day post tumor induction with 4T1 cells, of animals that had received two i.p. prophylactic immunizations (applied every seventh day) with the 4T1 lysate alone or coadministered with the Cry1Ac protoxin or Cry1Ac toxin. Nonadherent cells from the spleen of immunized or healthy mice were used to isolate T lymphocytes by negative selection with MACS CD19 microbeads (Miltenyi Biotec, Bergisch, Germany, cat no 130-121-301); T cells were counted and cocultured at different ratios with 2×10^4 4T1 cells stained with CFSE for 4 hours in culture tubes. Cells were recovered, stained with Annexin V-APC, and analyzed by flow cytometry with a FACSCalibur cytometer, in which 50 000 events, from 4T1 CFSE + region, were obtained.

Doxorubicin + Cry1Ac protoxin therapy

BALB/c mice 6 to 8 weeks of age were injected with 2×10^4 4T1 cells to induce tumor growth, when tumors were palpable; the mice were separated into 3 groups: no treatment (PBS vehicle group), doxorubicin, and doxorubicin + Cry1Ac protoxin (5 mice per group). Doxorubicin was administered intravenously (i.v.) at a concentration of 5 mg/kg in 100 µL on days 9 and 17 after tumor induction. Cry1Ac protoxin was administered after doxorubicin therapy on days 10, 12, and 18 after tumor induction by intratumoral route at a concentration of 50 µg in 50 µL and intraperitoneally at a concentration of 50 µg

in 100 µL. The growth of the tumors was monitored every third day. Mice were killed on day 24 after tumor induction.

Cytokine measurements in tumor

The tumor of the mice that received the combined therapy of doxorubicin + Cry1Ac protoxin was obtained on day 24 after the induction of the tumors. The tumors were weighed and sonicated to obtain lysates with a pulse 10 s and 50% amplitude (Sonic Dismembrator Model 300 Fisher, Pittsburgh, PA). A relationship was made of the weight of the tumor and the volume of medium to be added, taking into account the heterogeneity of tumor sizes (0.58 g of tumor / 300 µL medium supplemented with cOmplete, Mini Protease Inhibitor Cocktail (Merck KGaA, Darmstadt, Germany). The tumor lysates were centrifuged for 10 minutes at 4000 g and the supernatants recovered were aliquoted and stored at -70°C until use. The levels of, MCP1, IL-12, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-10, and IL-6 were measured using the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Bioscience, San Jose, CA) cat. No. 552364 BD) according to the manufacturer's instructions: We started the standard curve at a concentration of 5 pg/mL to determine concentrations below the curve recommended by the manufacturer. Concentration of cytokines was measured using a FACSCalibur cytometer and BD CBA software (Cytometric Bead Array software © 1999–2003, Becton, Dickinson and Company, San José, CA).

Lung metastasis

For the analysis of the macro-metastases in the lungs, the therapeutic doxorubicin + Cry1Ac protoxin scheme described above was used. During killing, the lungs were filled with 1 mL of a 10% India ink solution (Winsor & Newton, London, UK), in PBS, through the trachea with the help of a cannula. The trachea was blocked by surgical thread and the lungs were extracted and washed 3 times with 10 mL of Fekete's solution (85 mL 70% ethanol, 10 mL 10% paraformaldehyde, and 5 mL acetic acid), and the lungs were fixed in the same solution overnight. The macroscopic foci were counted with a stereoscopic microscope, OLYMPUS SZ CTV (Olympus, Tokyo, Japan). Metastatic index was calculated with the number of lung metastatic nodes.

Statistical analyses

Data were analyzed with Graphpad Prism software version 7.0, San Diego, CA. One-way analysis of variance (ANOVA) was applied for multiple comparisons, and post multiple comparison Dunnett's and *t* test were used to compare all other groups, which were indicated when the *P* value *<.1, **<.05, ***<.01, ****<.001. The results are shown as mean ± standard error of the mean (SEM).

Results

Intraperitoneal immunization with the Cry1Ac protoxin increases CD80 expression in dendritic cells (CD11c+/MHCII+) of the spleen

To induce tumor antigen-specific cytotoxic T lymphocytes capable of killing tumor cells, the maturation and activation of APCs capable of activating T lymphocytes is required. Therefore, we evaluated whether the i.p. immunization of the Cry1Ac protoxin or Cry1Ac toxin as adjuvants with 4T1 lysates as a source of tumor antigens were capable of increasing the amount and activation of DC in the peritoneal cavity and spleen in BALB/c mice. The population CD11c+MHCII+ was considered mature DC

because of high levels of expression in these markers.[36] We chose 48 hours after injection as an adequate time point to analyze the activation of DC because in previous reports significant activation of peritoneal DC[37] and peritoneal cavity macrophages (PeC)[23] was detected following Cry1Ac protoxin i.p. injection.

Using CD11c and MHCII markers, 4 populations with differential expression of these markers were distinguished in peritoneal cavity cells: CD11c+MHCII+(R1), CD11c+MHCII+[low] (R3), CD11c-MHCII+(R2), and CD11c+[low]MHCII-(R4); so the expression of CD80 and CD86 activation markers was analyzed separately (Figure 1), to determine more precisely the changes in activation elicited by the injection treatments. Representative dot plots of the analyzed
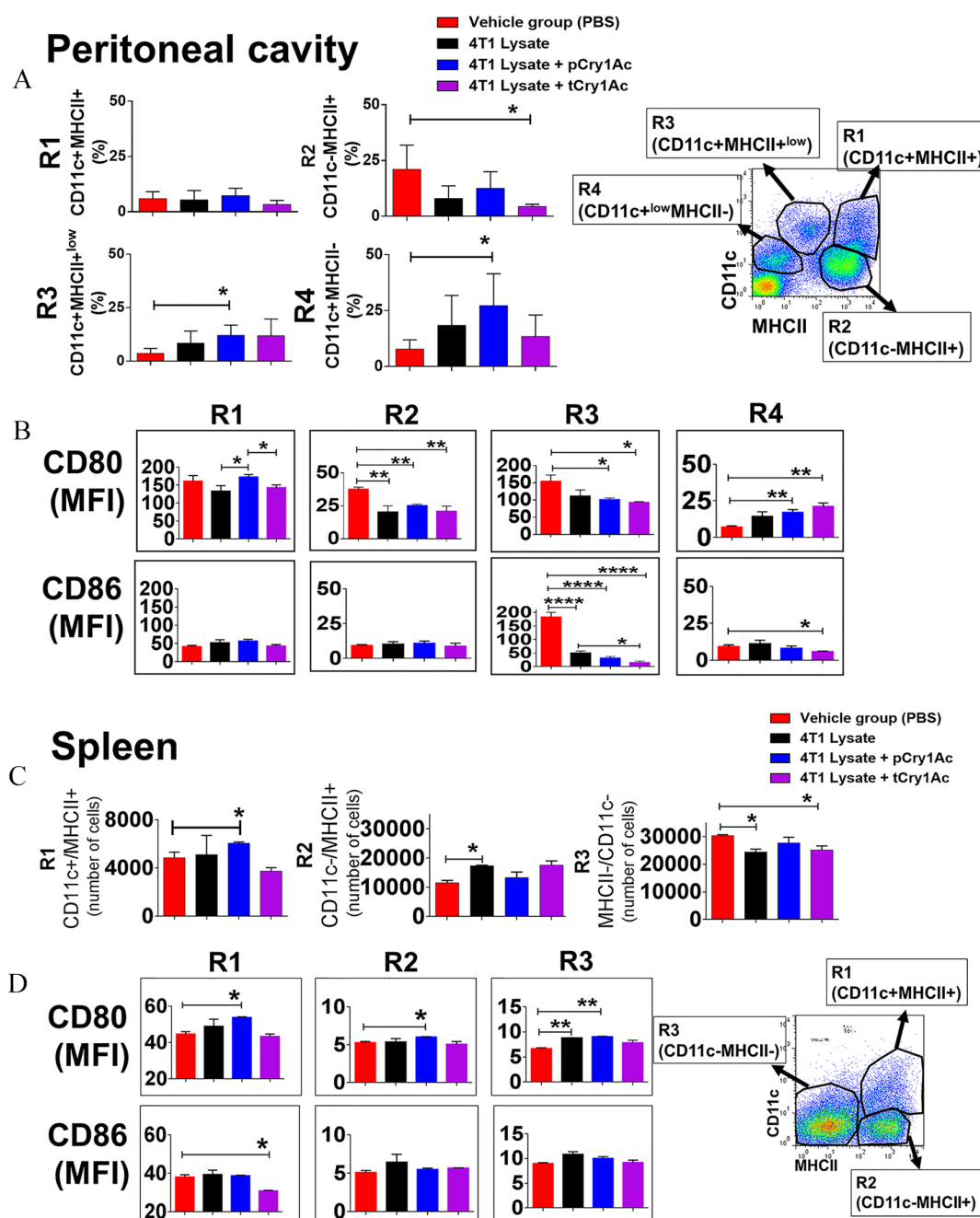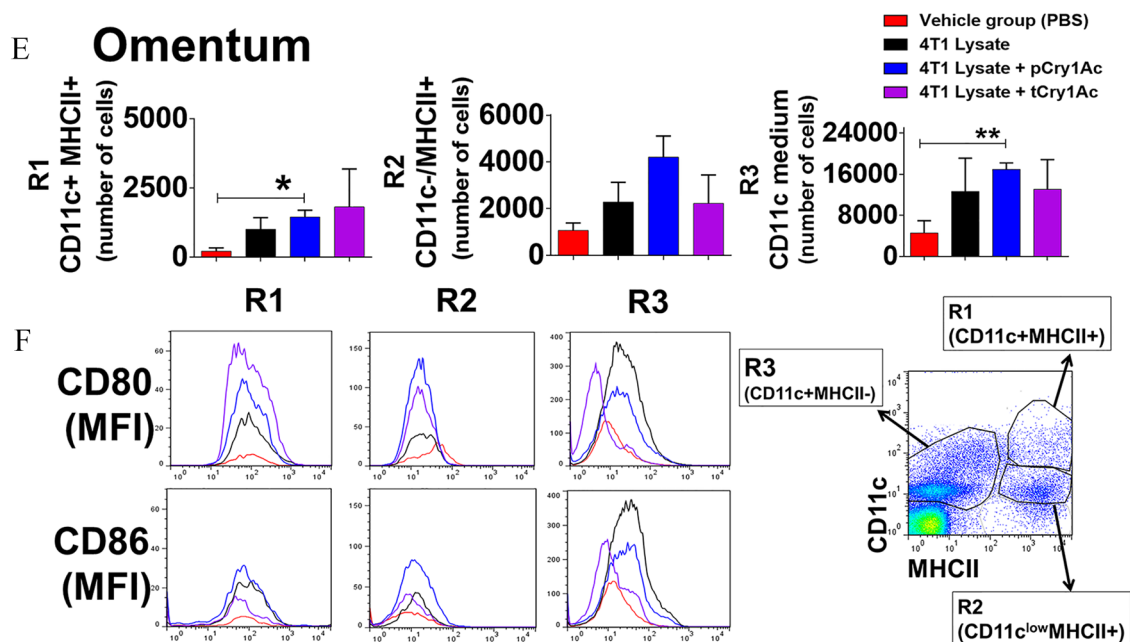

Figure 1. *(Continued)*

Figure 1. Immunization with 4T1 lysate + Cry1Ac protoxin increases the activation of dendritic cells (CD11c+MHCII+) and the amount of CD11c+ in omentum. BALB/c mice were intraperitoneally (i.p.) injected with a single administration of 4T1 lysate alone or with Cry1Ac protoxin (pCry1Ac) or Cry1Ac toxin (tCry1Ac). Lysates and proteins were administered at 50 µg doses each. After 48 hours, mice were killed, and leukocytes were obtained from the peritoneal cavity (A and B), spleen (C and D), and omentum (E and F). Cells were stained for flow cytometry analysis as described in the material and methods section with anti-CD11c, anti-MHCII, anti-CD80, and anti-CD86 antibodies to determine activation of dendritic cells (DC). One hundred thousand events per sample were recorded. Representative dot plots showing the regions displaying differential expression of CD11c and MHCII are shown in Supplementary Figure 2. In the peritoneum, 4 regions were distinguished, while in the spleen and omentum 3 main populations were defined. These regions were analyzed separately to evaluate CD80 and CD86 expression. The values in the bar plots in A, C, and E show the percentage of cells in each region, expressed as mean ± SD. Values shown in the bar plots (B and D) represent the mean fluorescence intensities, expressed as the means ± SD. One-way ANOVA and Dunnett's multiple comparison test and *t* test were used. Representative data of 2 independent experiments are shown. *P < .05, **P < .01, ****P < .0001 versus the indicated group.

regions for each treatment in the distinct tissues analyzed are shown in Supplementary Figure 1.

In mice immunized with the Cry1Ac protoxin as an adjuvant, the amount of DC (CD11c+MHCII+) did not increase in the peritoneal cavity. However, we observed an increase in the proportion of cells expressing CD11c+/MHCII+low(R3) and CD11c+lowMHCII-(R4), in which CD80 was low and upregulated, respectively, compared with the vehicle control group PBS (Figure 1A and B). Whereas, mice that received the Cry1Ac toxin as an adjuvant showed upregulation of CD80 in CD11c+lowMHCII- (R4) cells.

Notably, the mice that received the 4T1 lysate + Cry1Ac protoxin showed an increase in the expression of CD80 in DC CD11c+MHCII+(R1) in the spleen compared with the control group (Figure 1D). In contrast, mice that were immunized with 4T1 lysate + Cry1Ac toxin showed a decrease in CD86 expression (Figure 1D).

These results indicate that mice immunized with the Cry1Ac protoxin showed an increase in the number of CD11c+MHCII+low and CD11c+lowMHCII- (with upregulation of CD80) locally in the peritoneal cavity and an increase in the activation of DC at the systemic level in the spleen.

Intraperitoneal immunization with the Cry1Ac protoxin increases dendritic cells (CD11c+/MHCII+) of the omentum

To determine whether the activation of DC occurred in major magnitude in other nearby sites following i.p. injection with tumor lysate plus Cry1Ac proteins, the population of DC in the omentum and MLN was analyzed. These sites were chosen because it has been described that following i.p. injection with diverse inflammatory stimuli, such as lipopolysaccharide (LPS), thioglycolate, or even with Cry1Ac protoxin, monocytes are recruited but the main resident population of macrophages appear to migrate mainly to the omentum.[22,38] It was observed that mice that received the Cry1Ac protoxin showed an increase in the number of DC in the omentum compared with the control group PBS and with the mice that received only 4T1 lysate. In mice immunized with 4T1 lysate + Cry1Ac toxin, we did not find any significant differences (Figure 1E). No differences were found in the number and activation of DC recorder in the MLN by any experimental treatment (Supplementary Figure 1D).

Altogether, these results showing protoxin-induced DC activation, in the peritoneal cavity, spleen, and omentum, led us to propose the use of the Cry1Ac protoxin as an adjuvant that could activate the prime-T cell antitumor response.

Cry1Ac protoxin treatment increases the proliferation of TCD4+ and TCD8+ in spleen and MLN

The induction of cellular responses of TCD4+ and TCD8+ lymphocytes is required to induce adequate antitumor immune responses, as these T lymphocytes improve cytokine production and eliminate tumor cells, respectively. To determine whether the Cry1Ac protoxin or Cry1Ac toxin used as adjuvants coadministered with 4T1 lysate were able to induce an increase in the cellular response after immunization, we evaluated local and systemic proliferation of TCD4+ and TCD8+ lymphocytes in the spleen and MLN, which were re-stimulated in vitro with a pool of peptides corresponding to tumor epitopes described in the 4T1 tumor line.[33]

It was observed that mice immunized with 4T1 lysate + Cry1Ac protoxin induced a marked increase in the proliferation of TCD4+ lymphocytes (45.1%) in the spleen in comparison with mice that were immunized only with 4T1 lysate (29.5%) and control group PBS (9.8%) treatment. The high proliferative rate recorded was probably due to the in vivo stimulus as spleen cells were collected from mice receiving the second immunization 2 days before the killing. Consequently, no further increase in proliferation was observed after re-stimulation with the pool peptides corresponding to tumor epitopes described in 4T1 cells, possibly because the specific T cells were already proliferating (Figure 2A). Notably, proliferation of TCD8+ lymphocytes in the spleen was higher in mice that received the 4T1 lysate + Cry1Ac protoxin treatment (55%) in comparison with mice immunized only with the 4T1 lysate (41.9%) and those that received the vehicle (PBS) (16.7%) (Figure 2A).

In addition to evaluating the cellular response in the spleen, we evaluated proliferation of TCD4+ and TCD8+ lymphocytes in mesenteric lymph nodes (MNL), as these lymphoid nodules are located near the immunization site (i.p. immunization). It was observed that mice immunized with both the 4T1 lysate + Cry1Ac protoxin or Cry1Ac toxin had increased proliferation of TCD8+ lymphocytes (18.7% and 11%, respectively) in comparison with immunization of 4T1 lysate alone (3.4%) (Figure 2B). Therefore, these results show that mice immunized with the Cry1Ac protoxin or Cry1Ac toxin as adjuvants increase the local (MLN) and systemic (spleen) proliferation of TCD4+ and TCD8+ lymphocytes.

Cry1Ac protoxin has a significant antitumor adjuvant effect in vivo

To evaluate whether the Cry1Ac protoxin and Cry1Ac toxin had an adjuvant antitumor effect in vivo, we used the TNBC model induced by injection of 4T1 cells and measured the size of tumors in mice that received prophylactic immunization of the 4T1 lysate + Cry1Ac protoxin or Cry1Ac toxin with 2 different immunization schemes. The first scheme, a short prophylactic scheme in which immunizations were administered every 3 days, was based on the reported literature in which the antitumor response of some adjuvants[39,40] was evaluated (Figure 3A to C). The second scheme, a long prophylactic scheme in which immunizations were administered every 7 days, was evaluated to improve the adaptive response (Figure 3D to F).

In the short prophylactic immunization scheme, we observed that mice that received the 4T1 lysate + Cry1Ac protoxin or Cry1Ac toxin showed 60% tumor-free mice (6 out of 10) and the mice that developed tumors were smaller compared with the mice that only received the 4T1 lysate, which had 20% tumor-free mice (2 out of 10) (Figure 3A and B). Interestingly, with the long prophylactic immunization scheme, mice that received the 4T1 lysate + Cry1Ac protoxin were completely protected from the development of tumors (Figure 3D to F).

Mice that received the 4T1 lysate + Cry1Ac toxin had 20% tumor-free mice and the size of the tumors was similar to mice that only received the 4T1 lysate (Figure 3D to F). It is important to mention that the mice that received only the 4T1 lysate showed lower tumor growth compared with the short immunization scheme, indicating that the extended immunization allowed the development of adaptive immune responses. Intraperitoneal prophylactic immunization of the Cry1Ac protoxin or Cry1Ac toxin alone (without coadministration of the 4T1 lysate) had no effect on tumor growth, confirming the adjuvant effect of both proteins (Figure 3G).

In addition, the potential in vivo therapeutic antitumor effect of the Cry1Ac protoxin was also evaluated by injection of 3 peritumoral doses (50 μg/100 μL, once a week for 3 weeks); however, we did not observe any decrease in the growth of tumors (Figure 3H). The null therapeutic effect observed could be explained by the characteristics of the 4T1 breast cancer model, which is very aggressive, since it represents an advanced stage, in which the TME and the antitumor response at a systemic level are diminished.

We also tested whether these proteins had any cytotoxic effect on 4T1 cells as this antitumor activity has been reported[41] in other tumor cell lines. The results of MTT assay show slight or null cytotoxic effects of Cry1Ac protoxin and Cry1Ac toxin, respectively, on 4T1 cells. While by MTT viability assay and Annexin V assay, no significant effects on apoptosis were induced by Cry1Ac toxin nor by Cry1Ac protoxin (Supplementary Figure 2). In these assays, relative high doses of Cry1Ac proteins from 50 to 500 μg/mL were tested. Although with the protoxin at the higher doses a reduction of mitochondrial succinate dehydrogenase activity of 4T1 cells by the MTT assay was observed, this activity was unaffected by the toxin even at the highest doses tested. Notwithstanding, we consider that no direct cytotoxic effects of the protoxin on 4T1 cells, might participate on the antitumor adjuvant effect observed, since these antitumor effects were only observed by the prophylactic coadministration of Cry1Ac protoxin, that was long before the inoculation of 4T1 cells.
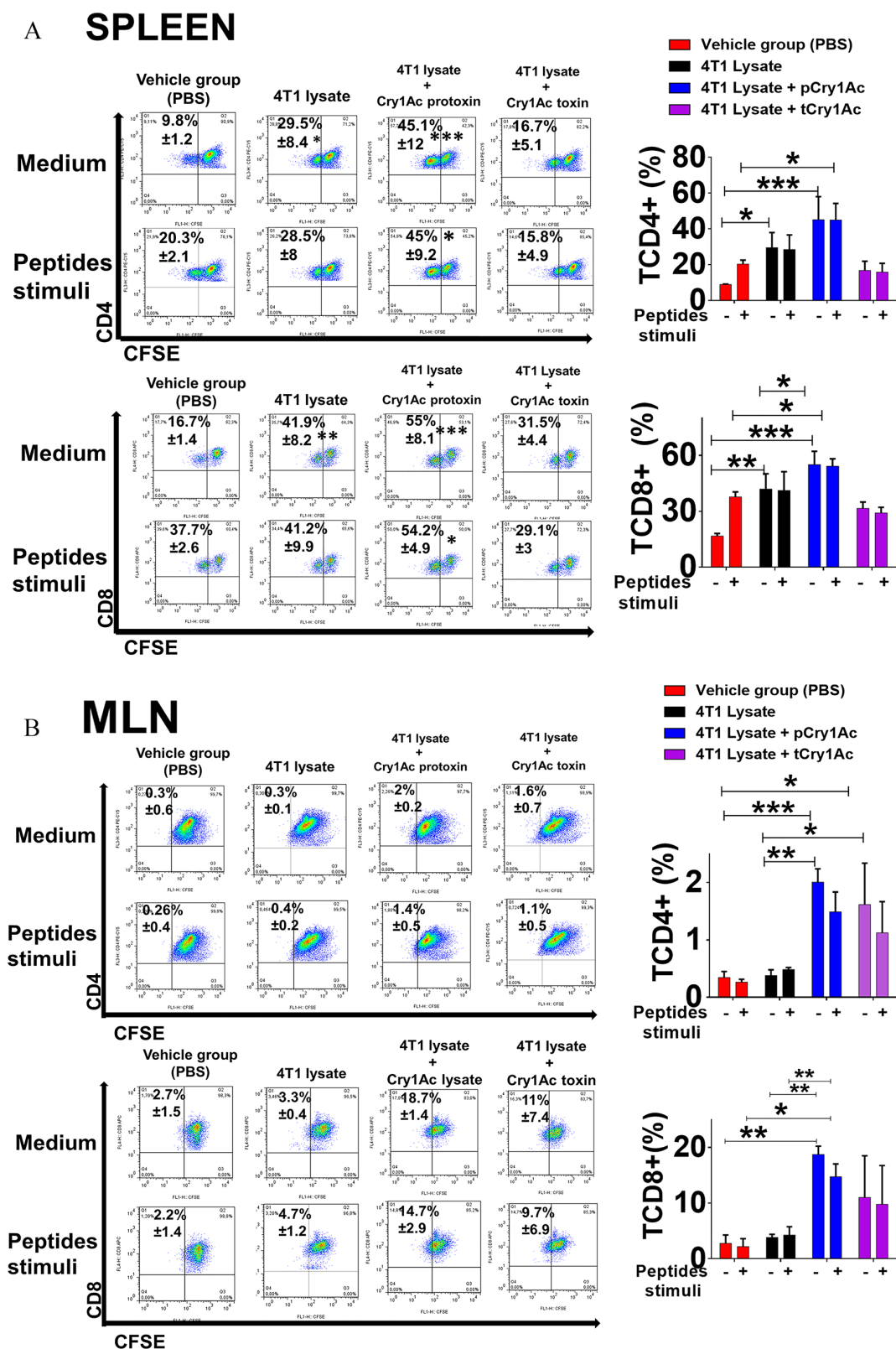
Figure 2. Immunization with the 4T1 lysate + Cry1Ac protoxin induces an increase in the proliferation of TCD4+ and TCD8+ lymphocytes in the spleen and MLN. BALB/c mice were immunized with 4T1 lysate alone or with Cry1Ac protoxin or Cry1Ac toxin (at 50 μg doses each) by intraperitoneal (i.p.) route; immunizations were applied once a week for 2 weeks. Mice were killed 2 days after the second immunization and leukocytes were obtained from the (A) spleen and (B) mesenteric lymph node (MLN) and stained with CFSE. To analyze proliferation, lymphocytes were cultivated in 96-well plates and stimulated with a pool of peptides (10 μg/1 mL) (as described in the materials and methods), 72 hours after the lymphocytes were obtained and stained with anti-CD3, anti-CD4, and anti-CD8 antibodies and analyzed by flow cytometry. One hundred thousand events per sample were recorded. n = 3 per group. *$P < .05$, **$P < .01$, ***$P < .001$ versus the indicated group. Representative dot plots are shown. Values shown in the right bar graphs indicate the means ± SD, expressed as percentages.

Prophylactic treatment of the 4T1 lysate + Cry1Ac protoxin as an adjuvant prevents the decrease of TCD3+ and TCD4+ lymphocytes in the spleen

A well-described characteristic of the 4T1 breast tumor model is the marked reduction of T lymphocytes.[42] Therefore, to know how the populations of T lymphocytes were found in the mice protected from the development of tumors, we evaluated

changes in population of TCD3+, TCD4+, and TCD8+ lymphocytes in the spleens of mice that received the long (Figure 4) or short (Supplementary Figure 3) prophylactic immunization schemes.

With the long scheme, the mice that received the Cry1Ac protoxin as an adjuvant did not present a decrease in the proportion of TCD3+ lymphocytes (38%) in the spleen, in
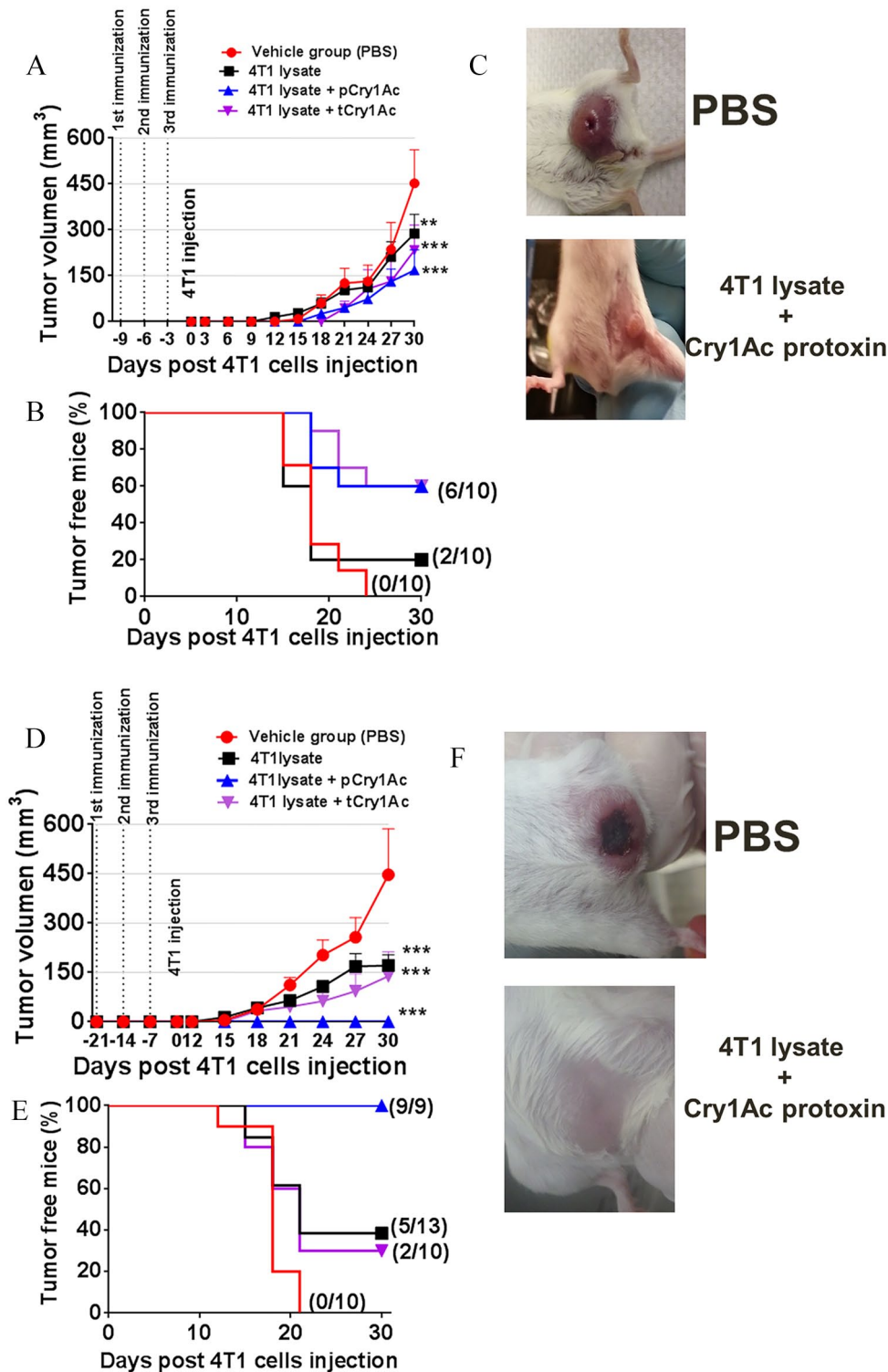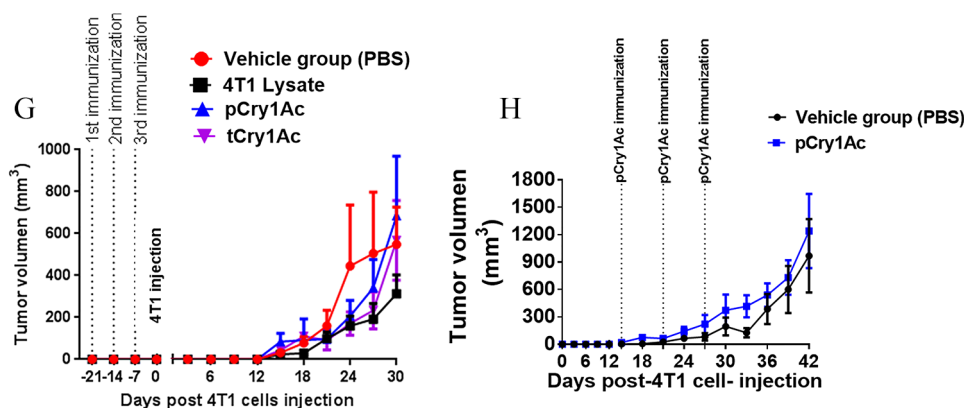

Figure 3. *(Continued)*

Figure 3. Cry1Ac protoxin has adjuvant antitumor effects in 2 different prophylactic immunization schemes. In both (short and long) schemes, BALB/c mice received 3 administrations of the vehicle (PBS) or the 4T1 lysate alone or coadministered with the Cry1Ac protoxin or Cry1Ac toxin as adjuvants, proteins were administered at 50 μg doses each as specified in materials and methods. In the short scheme, each dose was applied every third day (A and B); while in the long scheme each dose was applied every seventh day (C and D) for 3 weeks. Subsequently, breast cancer was induced by the inoculation of 3×10^3 4T1 cells by the subcutaneous (s.c.) route; the growth of the tumors was monitored every third day. Growth of tumors (A) and percentage of tumor-free mice (B) of the short immunization scheme, each point represents the mean tumor volume (mm³) ± SEM. ANOVA and Dunnett's multiple comparison test. **P < .01, ***P < .001. * indicates significant differences with respect to the PBS group. Tumor photos of mice that received immunizations in the short scheme, control group (PBS) and 4T1 lysate + Cry1Ac protoxin 30 days after inducing tumors (C). Growth of tumors (D) and percentage of tumor-free mice (E) of the long immunization scheme, each point represents the mean tumor volume (mm³) ± SEM. (F) Tumor photos of mice that received immunizations in the long scheme, vehicle group (PBS), and 4T1 lysate + Cry1Ac protoxin 30 days after inducing tumors PBS, 4T1 lysate, and 4T1 lysate + Cry1Ac protoxin (n = 10). (G) Growth of tumors in mice prophylactically administered with Cry1Ac protoxin or Cry1Ac toxin alone (with long scheme) or with vehicle or 4T1 lysate. (H) Growth of tumors in mice therapeutically administered with Cry1Ac protoxin 14 days after tumor induction. Cry1Ac protoxin was applied once a week for 3 weeks. Tumor growth curves include only values from tumor-bearing mice. ANOVA and Dunnett's multiple comparison test. ***P < .001. * indicates significant differences with respect to the PBS group.

comparison with the mice that developed tumors, control group PBS (16.3%), 4T1 lysate (37.1%), and 4T1 lysate + Cry1Ac toxin (19%) (Figure 4A). When the total number of TCD3+, TCD4+, and TCD8+ lymphocyte subpopulations were analyzed, we found statistically significant differences in the mice that received the immunization with the Cry1Ac protoxin as an adjuvant, as they did not show a decrease in the number of TCD3+ and TCD4+ lymphocytes in comparison with mice that received the control group PBS, while mice that received the Cry1Ac toxin as an adjuvant showed a decrease in both the percentage and number of TCD3+, TCD4+, and TCD8+ lymphocytes (Figure 4B).

While the analysis of T cell populations, in mice prophylactically immunized with the short scheme (performed in the groups immunized with 4T1 lysate alone or with Cry1Ac proteins or just receiving the vehicle; that were then inoculated with 4T1 cells and killed 30 days after), showed that mice coadministered with 4T1 lysate + Cry1Ac protoxin or + Cry1Ac toxin presented a minor reduction in the percentage of TCD8 lymphocytes and significantly higher numbers of T lymphocytes than the vehicle group (Supplementary Figure 3A and B respectively).

We also examined the lymphocyte proliferative responses and the cytokine production in lymphocyte culture supernatants in the same mice prophylactically immunized with the short scheme and killed at day 30. However, similar lymphocyte proliferative responses were elicited among the distinct treatments (data not shown), while the cytokine levels

(TNF-α and IFN-γ) tended to be higher in mice with tumors (in any of the treatments) while mice immunized with Cry1A protoxin (which did not develop tumors) had a lower production of these cytokines similar to the one presented in healthy mice (Supplementary Figure 3C and D). Therefore, further characterization of cytokine profile rest to be done, to determine, at early time points after immunization, whether or not the antitumor adjuvant of Cry1Ac is related with its ability to increase the levels of TNF-α and IFN-γ or of other cytokines. The experimental conditions used in present study may not have been optimal for assessing changes in cytokines due to the adjuvants; possibly cytokine evaluation should have been performed at earlier time points after tumor induction, or perhaps should have been assessed prior to tumor induction; or it may be necessary to include some type of stimulation in the assay, such as tumor peptides, or anti CD3/anti CD28 or phorbol 12-myristate 13-acetate (PMA).

We also analyzed a population of cells known as myeloid-derived suppressor cells (MDSC), which play a critical role in maintaining the immunosuppressive tumor environment. These cells are a heterogeneous population of myeloid progenitor cells, comprising immature granulocytes, macrophages, and DC.[43] In mice, MDSCs express both the myeloid lineage differentiation antigens, Gr1 and CD11b.[44] In the 4T1 TNBC model, MDSCs increase in the peripheral blood, spleen, lymphoid nodes, and improve the growth of tumors and metastasis, and favors the evasion of recognition by the immune system.[45] Remarkably, we observed that mice that received the 4T1
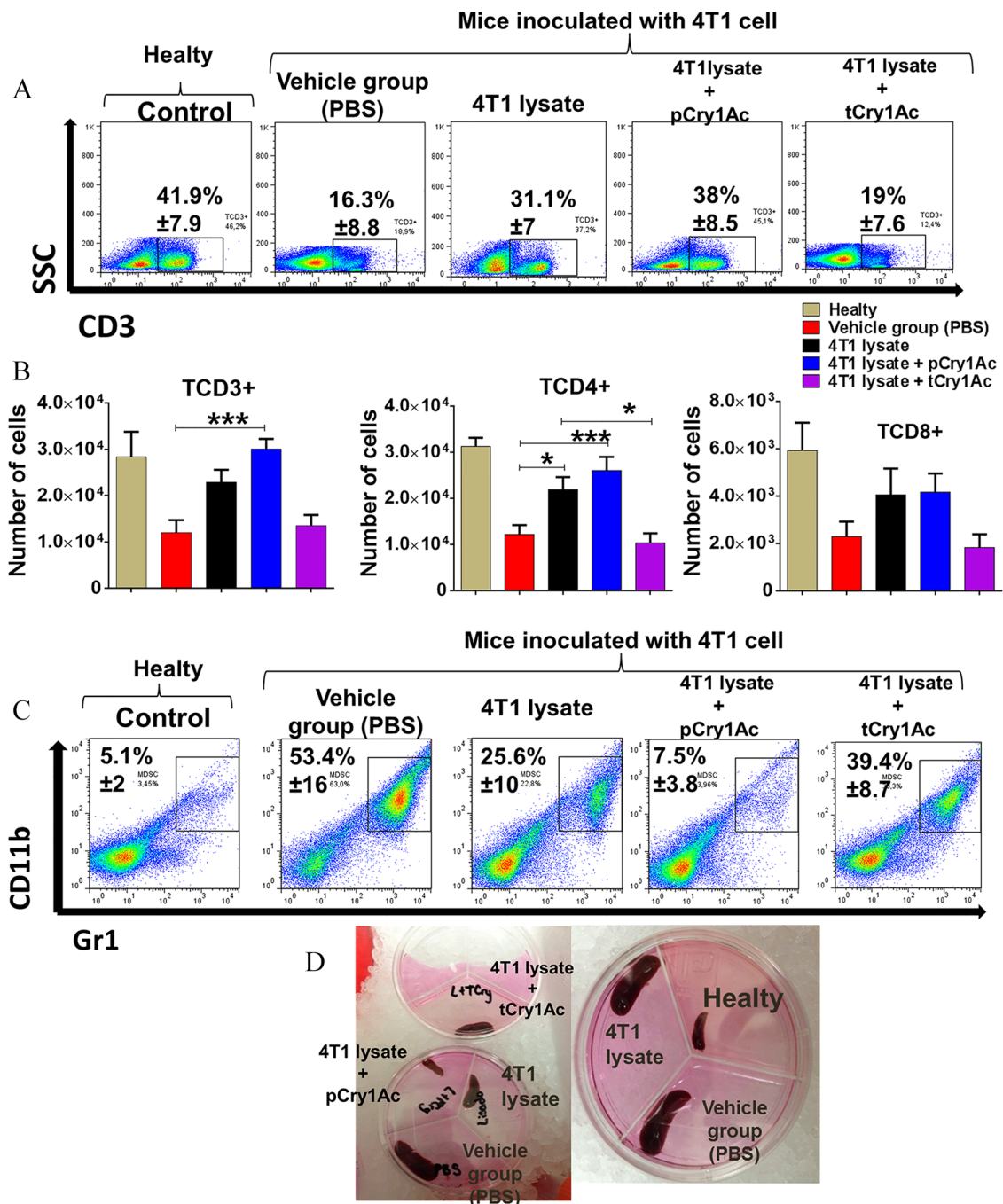
Figure 4. Prophylactic immunization with the long scheme, with 4T1 lysate + Cry1Ac protoxin prevents tumor-induced decline of TCD3+, TCD4+, and TCD8+ lymphocytes and increased MDSC in the spleen. Spleen lymphocyte populations were obtained from healthy mice, mice receiving the vehicle or were immunized with the long prophylactic scheme (1 immunization every week for 3 weeks) with 4T1 lysate alone or coadministered with Cry1Ac protoxin or toxin (at 50 µg doses each). Tumors were induced by inoculation of 3×10^3 4T1 cells and killed at day 30. (A) Representative dot plots of the percentage of TCD3+ lymphocytes in the spleen of mice that received different treatments. (B) Total number of TCD3+, TCD4+, and TCD8+ lymphocytes obtained from the spleen ± SEM. Total cell counts were estimated by analysis of equal cell counts, 100 000 events per sample were acquired. (C) Representative dot plot of the percentage of myeloid-derived suppressor cells (MDSC) in the spleen. (D) Size of spleen obtained from mice that received different treatments. TCD3+ cells were gated from lymphocyte region defined on basis of FSH and SSH parameters. CD4 and CD8 were calculated from CD3 + gated cells. MDSC region was defined as F4/80+CD11b+ cells. Bar graphs show mean percentages or number ± SEM of gated CD3+ or MDSC populations. ANOVA and Dunnett's multiple comparison test. *P < .05, ***P < .001. n = 7–10.

lysate + Cry1Ac protoxin treatment did not have an increase in MDSC (7.5%) compared with mice that developed tumors, control group PBS (53.4%), 4T1 lysate (25.6%), and 4T1 lysate + Cry1Ac toxin (39.4%) (Figure 4C). The increase in the number of MDSCs is related to an increase in the size of the spleens, which was observed on the day of killing (Figure 4D).

These results suggest that the protective and adjuvant antitumor effect of the protoxin Cry1Ac is related to
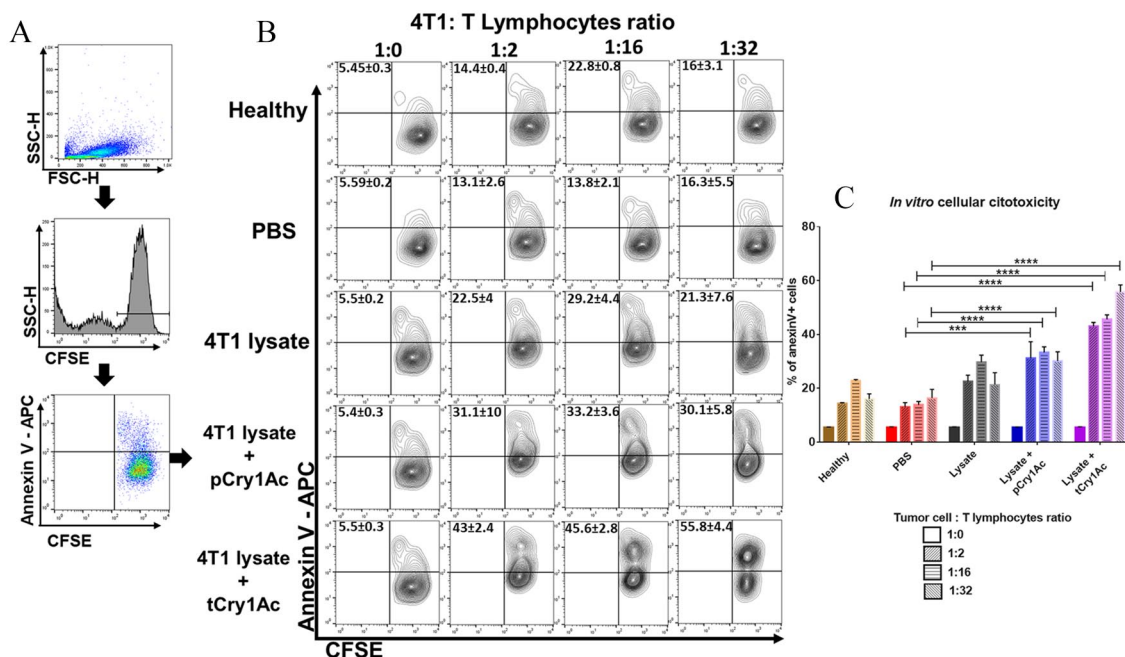
Figure 5. Cry1Ac protoxin and Cry1Ac toxin administrated with 4T1 lysates induce spleen T cell-mediated cytotoxicity in vitro. For the analysis of cell cytotoxicity mediated by T cells, the mice were immunized with 4T1 lysate alone or coadministered with Cry1Ac protoxin or toxin (at 50 μg doses each). Mice received 2 immunizations or vehicle administrations 1 per week and were killed on day 21. The spleens of healthy mice or from immunized mice were collected and T lymphocytes were isolated by negative selection with magnetic beads and were cocultured with 4T1-CFSE cells for 4 hours. Cells were recovered, stained with Annexin V-APC, and analyzed by flow cytometry with a FACSCalibur cytometer, in which 10 000 events per sample were recorder. (A) Flow cytometry gating strategy, the figure shows the regions used to isolated 4T1- CFSE+ cells for the analysis shown in B. (B) Representative dot plots of 4T1- CFSE+ cells positives to Annexin V with different ratios of T cells, mean of the CFSE+-Annexin V+ quadrants are shown. (C) Percentage of cytotoxicity of 4T1 cells; the data in bar graphs are represented as mean ± SEM. N=3. *** and **** denote significant differences, where the *P* value was ⩽ .01 or .001, respectively.

the ability to improve cellular immune responses, with the capability to prevent the decrease of TCD4+ and TCD3+ lymphocytes and to avoid the increase of MDSCs at a systemic level.

Immunization with the 4T1 lysate+ Cry1Ac proteins increases specific cytotoxic T-cell responses

Furthermore, the in vitro evaluation of the cytotoxic response of T lymphocytes on CFSE labeled 4T1 cells confirmed the increased specific cytotoxic capacity of T lymphocytes isolated from spleen of prophylactically immunized mice with the 4T1 lysate coadministered with the Cry1Ac protoxin or Cry1Ac toxin in comparison with T cells from unimmunized mice or immunized with 4T1 lysate alone. Surprisingly, Cry1Ac toxin showed a better capacity to activate T cells to kill 4T1 cells in vitro, than Cry1Ac protoxin, which promotes a better antitumor response. The increased apoptosis was evaluated by the expression of Annexin V in CFSE-stained 4T1 cells incubated with distinct ratios of T cells from mice immunized in comparison with lymphocytes from unimmunized mice (Figure 5).

Improved antibody-mediated cytotoxicity of 4T1 tumor cells might be an additional immune mechanism participating in the enhanced antitumor response elicited by the adjuvant Cry1Ac protoxin, but this will require further investigation.

The coadministration of Cry1Ac protoxin activate immune cells in tumor microenvironment

To evaluate whether the distinct immunization treatments had effects on the immunological cells in the tumor environment, we evaluated populations which may be associated with improvements in the capability to induce tumor immunity. To this aim, a 2 immunization protocol with 4T1 lysates alone or with Cry1Ac protoxin toxin was performed, then we induced tumors, and after 3 weeks, were analyzed in the tumors the following immunological parameters: the number of T lymphocytes and its activation, the amount of regulatory T cells, the number of macrophages F4/80 and its activation, and the number of MDSC (Figure 6). Unimmunized mice presented a low amount of T cells, while mice immunized with Cry1Ac protoxin presented the highest amount of T cells, followed by the mice immunized with lysate alone and finally mice immunized with the Cry1Ac toxin presented a low amount similar to the found in immunized mice. However, the amount of CD4T cells was higher in mice immunized with lysate alone followed by the group coadministered with Cry1Ac protoxin and unimmunized mice and mice coadministered with Cry1Ac toxin presented the lowest amounts of CD4T cells. While no statistically significant changes were recorded for CD8 T cells. Interestingly, mice immunized with the Cry1Ac protoxin presented the lowest amount of regulatory T cells CD4
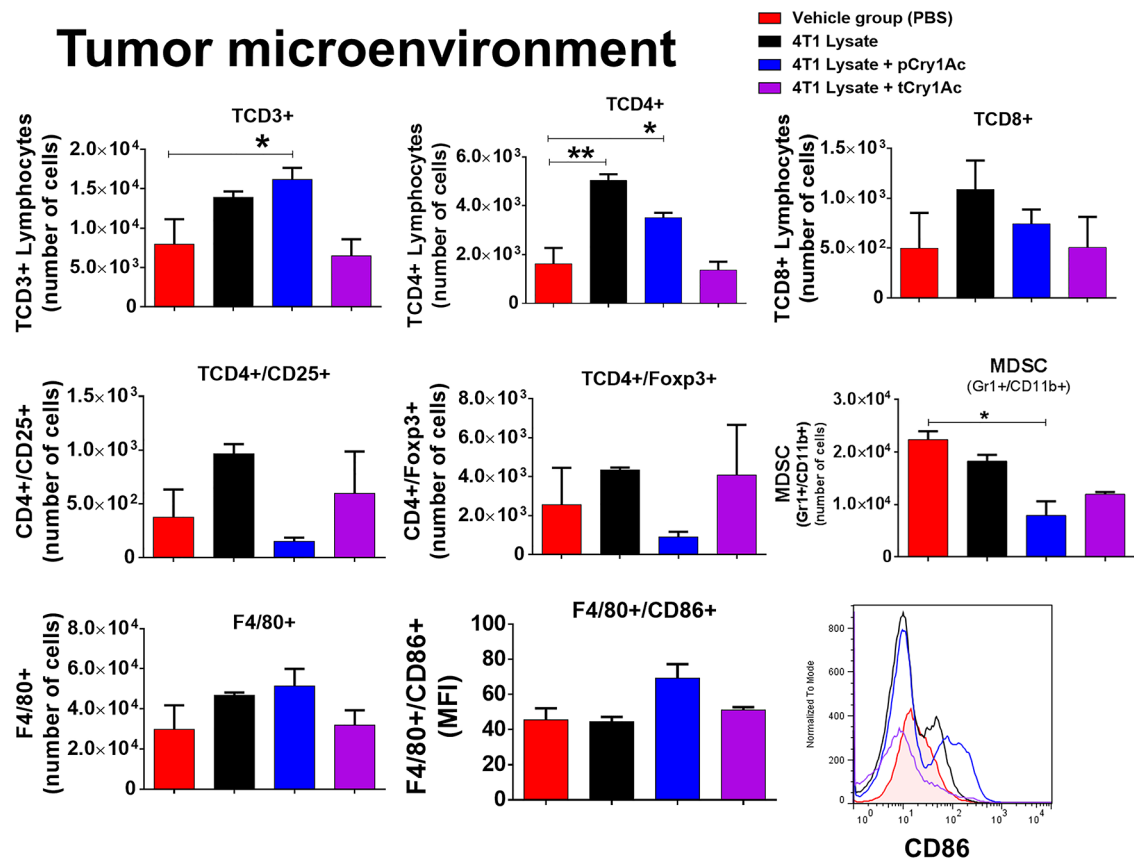
Figure 6. Immunization with 4T1 lysate + Cry1Ac activate immune cells in tumor environment. The effect of immunization in the immune populations in tumor environment was evaluated by flow cytometry. In this experiment was performed a two i.p. immunization scheme, applied once a week with 4T1 lysate alone or coadministered with Cry1Ac toxin or protoxin, 7 days after mice were inoculated with 1×10^4 4T1 cells and mice were killed to collect tumors 3 weeks after induction. The populations analyzed were T lymphocytes TCD3+, TCD4+, TCD8+, or T regulatory lymphocytes TCD4 + CD25 +, TCD4 + FoxP3 +, the population of MDSC cells CD11b + Gr1 + cells and the population of macrophages F4/80 +. We estimated the amount of all these cell populations and the amount of F4/80 + upregulating CD86. Values shown are means ± SD expressed as number of cells or the mean fluorescence intensities of CD86 in the representative histograms of gated macrophages F4/80 +. In the figure, representative data of 2 independent experiments are shown. Total cell counts of each analyzed population were estimated by analysis of equal cell counts, 100 000 events per sample were acquired.

FoxP3+ and CD25+ which had been associated with immunosuppression in tumor environment.[46] While these populations of regulatory T cells were higher in the rest of the groups (unimmunized or immunized with lysate alone or with Cry1Ac toxin), but without significant differences among groups, although in mice immunized with lysate alone tended to be higher. The number of MDSC which also had been related with immunosuppressive effects within the tumors was reduced in mice immunized with Cry1Ac protoxin and also in mice immunized with the toxin but in lesser magnitude. In contrast, mice administered with the vehicle or immunized with the 4T1 lysate alone presented a marked increase of MDSC populations within the tumors. Mice immunized with Cry1Ac protoxin presented higher number macrophages with upregulation of CD86 in relation with the rest of treatments.

Cry1Ac protoxin and doxorubicin therapy reduces tumor growth and decreases the severity of metastases. Given that the potential application of Cry1Ac protoxin in humans, in a prophylactic approach would be limited, due to the heterogeneity of tumors between patients and the lack of specific antigens for breast cancer. We decided to evaluate a therapeutic approach to determine whether the adjuvant antitumor effect of Cry1Ac protoxin could improve therapy with doxorubicin, an anthracycline used in patients with TNBC. BALB / c mice 6 to 8 week old were injected with 2×10^4 4T1 cells, when the tumors were palpable; the mice were separated into 3 groups: no treatment (PBS vehicle), doxorubicin and doxorubicin + Cry1Ac protoxin. Mice that received doxorubicin + Cry1Ac protoxin combination therapy showed less tumor growth and tumor weight (Figure 7A and B) and decreased incidence of lung metastases, compared with mice that only received doxorubicin or the vehicle. Therefore, the adjuvant effect of Cry1Ac protoxin in combination therapy with doxorubicin offers therapeutic benefit in TNBC (Figure 7C).

No significant benefit was observed in mice that only received doxorubicin therapy; indeed this group showed the highest number of lung metastases. The absence of antitumor effect may be related to the high number of 4T1 cells used in this experiment to induce tumors, along with the aggressiveness of the model.
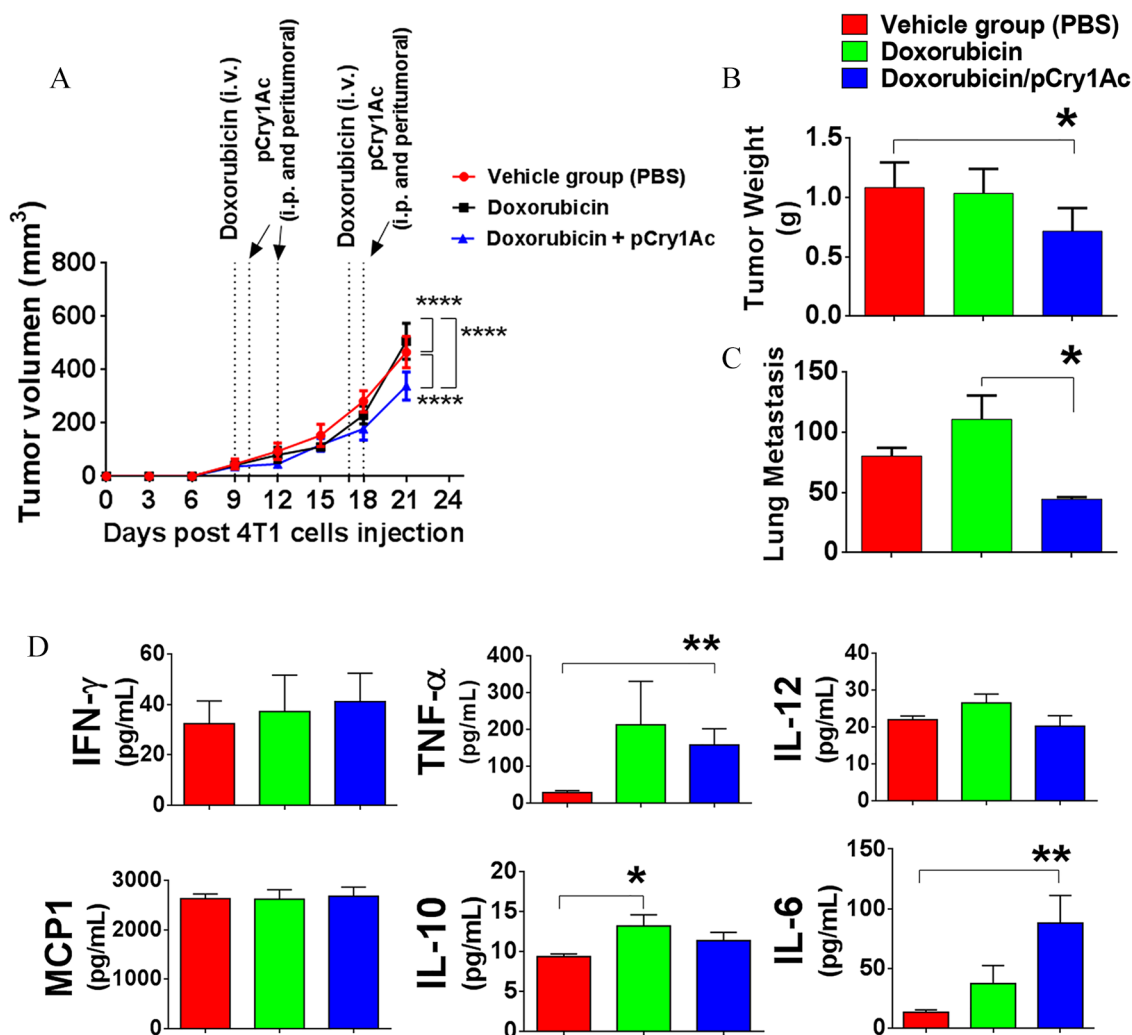
Figure 7. Cry1Ac protoxin and doxorubicin therapy reduces tumor growth and decreases the severity of metastases. BALB/c mice 6 to 8 weeks of age were injected with 2×10^4 4T1 cells to induce tumor growth, when the tumors were palpable, the mice were separated into 3 groups: no treatment (PBS vehicle group), doxorubicin, and doxorubicin + Cry1Ac protoxin. Doxorubicin was administered intravenously (i.v.) at a concentration of 5 mg /kg in 100 μL on days 9 and 17 after tumor induction. Cry1Ac protoxin was administered after doxorubicin therapy on days 10, 12, and 18 after tumor induction by intratumoral route at a concentration of 50 μg in 50 μL and intraperitoneally at a concentration of 50 μg in 100 μL. The growth of the tumors was monitored on the third day. Mice were killed on day 24 after tumor induction. Tumors were obtained and processed from the mice with the different treatments as described in materials and methods to evaluate the production of cytokines in the tumor. Volume was normalized by tumor mass as described in methods. Mice lungs were obtained for staining with India ink and assessing metastasis. (A) Growth of tumors in the therapy protocol. Each point represents the mean tumor volume (mm³) ± SEM. Two-way ANOVA and Dunnett's multiple comparison test. ***P < .0001. n = 5. (B) Weight of tumors. (C) Metastasis in the lung. (D) Cytokines in mice tumor (TNF-α, IFN-γ, IL-12, IL-6, IL-10, and MCP1). ANOVA and Dunnett's multiple comparison test. *P < .05, **P < .01. n = 5.

The TME has a very important role in the therapy and elimination of tumor cells; therefore, we decided to evaluate the cytokine production in the tumor of the mice that received the combined Doxorubicin + Cry1Ac protoxin therapy. Mice that received doxorubicin + Cry1Ac protoxin combination therapy had greater production of IL-6 and TNF-α in the tumor, compared with mice that received no treatment and those that received only doxorubicin (Figure 7D).

Discussion

The major findings in the present study is, first, the evidence showing the significant antitumor adjuvant effect conferred by

the prophylactic coadministration of the Cry1Ac protoxin with 4T1 lysate in a TNBC model. This effect is related to its capacity to improve tumor immunity and is much higher than the one conferred by the Cry1Ac toxin. Moreover, using a therapeutic approach with doxorubicin protoxin Cry1Ac also conferred antitumor benefit.

These findings are relevant because, although there are various strategies to enhance tumor immunity, such as TLR agonists, anti-CD40 antibodies, cytokines, like IL-2 and IFN-α, or growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), that have been investigated,[47] in general, these have shown limitation in capacity, safety, costs,

or stability.[48,49] A desired condition for an adjuvant capable of enhancing antitumor immunity is the capacity to activate APCs. In agreement with this requirement, we found that both proteins Cry1Ac protoxin and Cry1Ac toxin were able to activate DC following i.p. coadministration with 4T1 lysate. We chose the i.p. route because the administration of adjuvants by the i.p. route has been reported to induce DC activation and a strong TCD8+ lymphocytes response in the spleen.[50]

The differential capability between Cry1Ac toxin and Cry1Ac protoxin to induce the activation and recruitment of DC may explain the higher adjuvant effect conferred by the Cry1Ac protoxin compared with the Cry1Ac toxin. Since in the spleen, MLN, and peritoneal cavity, the recruitment and activation of DC upon i.p. injection occurred particularly with Cry1Ac protoxin, unlike with Cry1Ac toxin. It is important to mention that in steady conditions, DC represents 1% of the resident cells in the rat peritoneum cavity,[51] while in mice, macrophages can represent up to 50% of cells and the majority correspond to the called large peritoneal macrophage population (LPM).[38] In the present study, 4 populations with differential expression of MHCII and CD11c were distinguished in the peritoneal cavity. The populations that increased in number with Cry1Ac protoxin plus 4T1 lysate were CD11c+ MHCII+ low and CD11c+ low MHCII- cells with the upregulation of CD80 in the latter, which might correspond to immature DC. However, they also could be macrophages, as some macrophages in serous cavities also express CD11c.[52,53]

Although we presumed that the activation of DC could be better appreciated in the MLN, considered a key determinant of the flow of the peritoneal cavity,[54] changes upon Cry1Ac protoxin injection were not detected in this tissue but were detected in the omentum. Likewise, following i.p. injection with diverse inflammatory stimuli, such as LPS, thioglycolate, or even with the Cry1Ac protoxin, mainly monocytes are recruited to the peritoneal cavity but the main resident population of macrophages appeared to migrate mainly to the omentum.[22,38] Leukocytes found in the murine omentum are concentrated in milky spots, composed of both myeloid and lymphoid cells; these spots increase in size and number upon peritoneal inflammatory stimuli. While the low number of DC found in the omentum of unstimulated mice led to propose that the omentum is an inflammatory-induced lymphoid structure.[55,56] In accordance with these notions, we observed that the size and total number of leukocytes recovered from omentum were increased in Cry1Ac protoxin stimulated mice. It has been reported that the omentum contains DC that are able to cross-present exogenous antigens that promote rapid early activation of T lymphocytes. In addition, there is a constant flow of T and B lymphocytes from the peritoneal cavity to the omentum.[32] Similarly, we observed an increase in the number of DC in the omentum but only in mice that received the Cry1Ac protoxin plus 4T1 lysate; it is possible that these DC might have cross presented tumor antigens and

contributed to the enhanced tumor immunity observed in this group. Interestingly, in the spleen, we also observed that only mice that received the Cry1Ac protoxin enhanced CD80 expression in DC, compared with the control group PBS. These results indicate that the Cry1Ac protoxin, unlike the Cry1Ac toxin, increases the activation of DC and other APCs, locally (in the peritoneal cavity and omentum) and systemically (spleen), and this effect might be related to the better antitumor response.

Some evidence indicates that to achieve effective antitumor immunity, not only a local immune response (within the tumor) is required but a systemic response (at blood, secondary lymphoid organs, and bone marrow) of TCD4+ and TCD8+ lymphocytes[57,58] also contributes. Notably, we observed that only use of the Cry1Ac protoxin as an adjuvant with the 4T1 lysate increased the specific response of TCD4+ and TCD8+ lymphocytes, obtained from both systemic and local tissues (spleen and MLN, respectively). These results showed that the Cry1Ac protoxin promotes the increment and activation of DC accompanied with enhanced proliferative responses of T lymphocytes, which led us to presume that it could confer an antitumor adjuvant effect, as it was evidenced. Certainly, the Cry1Ac protoxin, unlike the Cry1Ac toxin, conferred a significant adjuvant effect, preventing tumor induction when the effect was tested in the murine breast cancer model induced by 4T1 cells. These results are outstanding because this 4T1 model, done in the immunocompetent BALB/c mice, resembles stage IV human TNBC, which provokes most deaths due to breast cancer because of its aggressiveness and lack of therapeutic targets available.[2] In this 4T1-induced tumor model, similarly as occurs in women, the TNBC tumor grows in situ and generates metastasis to the lung, liver, bone, and brain.[59,60] Due to its aggressiveness, there are no known vaccine strategies able to prevent tumor development in TNBC as we accomplished in the present work in TNBC model, using the prophylactic scheme. Two of the main factors known to contribute in the efficacy of cancer vaccines are the type of TAAs and the adjuvants employed.[61,62] In the present study, we chose tumor cell lysates as a source of TAAs because the 4T1 tumor model is considered non-immunogenic, and in therapeutic approaches, this strategy has proved to be effective to induce a strong antitumor response and prevent tumor escape, in comparison with the use of some peptide vaccines. This greater effect had been attributed to the large amount of TAAs. The higher antitumor effect achieved with the longer prophylactic immunization schedules in comparison with the short scheme may be attributed to the effective induction of adaptive immunity with the former. This antitumor effect was significantly enhanced by the coadministration of Cry1Ac proteins but the Cry1Ac toxin showed partial antitumor adjuvant effects in both immunization schedules, whereas the antitumor adjuvant effect of protoxin that was partial in the short scheme was enhanced so that it prevented tumor development. These findings clearly supporting the antitumor adjuvant effect of the Cry1Ac protoxin

are relevant because it is not easy to achieve complete tumor immunity in this aggressive TNBC model. Indeed, when the effect of a DNA vaccine target to interleukin-13 receptor α2 chain as a tumor antigen was evaluated in the same TNBC 4T1 mouse model, the vaccine did not completely protect against tumor development, but it was able to reduce tumor growth compared with the control group. In that study, the DNA vaccine was coadministered with IFA (incomplete Freund's adjuvant) and CpG as adjuvants in prophylactic and therapeutic schemes.[63] Likewise, when the prophylactically effect of a heterogeneous vaccine (multiepitope DNA and peptides-based cancer vaccine) coadministered with IFA as an adjuvant was evaluated in the TNBC 4T1 mouse model, a reduction in tumor growth was observed, but mice were not fully protected against tumor development.[64] The greater effect achieved by us with Cry1Ac protoxin as an adjuvant could be due the antigen source, as the 4T1 lysate contained a higher amount of TAAs than the antigen sources used in the mentioned studies.

A hallmark in the TNBC model with the 4T1 cell is the systemic decrease of lymphocytes in mice that develop tumors.[42] A proposed mechanism to explain this decrease is thymic atrophy, which causes a spread decline of TCD4+ and TCD8+ lymphocytes in both peripheral organs, such as the spleen.[65,66] Remarkably, we observed that mice immunized with the 4T1 lysate + Cry1Ac protoxin prevented the decrease in the percentage and number of TCD3+ and TCD4+ lymphocytes in the spleen, compared with the control group PBS. These results suggest that immunization with the Cry1Ac protoxin with tumor antigens improves tumor immunity, allowing the maintenance of normal lymphocytes numbers and function. Likewise, the induction of specific cytotoxic T cell responses able to induce apoptosis of 4T1 cells was particularly achieved following coadministration of the Cry1Ac toxin and Cry1Ac protoxin. The analysis of immune cells within tumor environment reinforces the potential benefit of the use of Cry1Ac as an antitumoral adjuvant, as particularly following immunization with 4T1 lysate plus Cry1Ac protoxin were found changes in the populations which has been associated with improved tumor immunity such as reduction in the number of immune suppressive cells such as MDSC and regulatory T cells and increased numbers of macrophages upregulating CD86.

The marked effect in the reduction of the immunosuppressive population of MDSC, observed following coadministration of the Cry1Ac protoxin with 4T1 lysate, is a relevant effect that deserves further investigation, as the elimination of MDSC is considered an efficient strategy to be able to induce efficient antitumor immune responses.[67,68] It is important to mention that despite both proteins having a similar capacity to activate macrophages,[22,23,29] only the Cry1Ac protoxin showed the potential capability to improve tumor immunity, preventing tumor development. Despite the Cry1Ac toxin used as an adjuvant showed a partial antitumor adjuvant effect, the induction of specific cytotoxic T cell responses was an effect elicited with both proteins,

which was even better elicited with the Cry1Ac toxin than with the protoxin. These differences may be due to the differential effects on the activation of APCs elicited by each protein.[37]

The outcomes indicate that the Cry1Ac protoxin confers a better antitumor adjuvant effect than the toxin in TNBC 4T1 mouse model, since mice that were immunized prophylactically with the 4T1 lysate were fully protected against the development of tumors. These protective effects correlated with the maintenance of normal numbers of lymphocytes, avoidance of MDSC increments, a better capacity to activate DC and induce T lymphocyte proliferative responses and an immunological improvement in tumor environment, with lower amount immunosuppressive populations. The distinct capacity between Cry1Ac protoxin and Cry1Ac toxin to improve tumor immunity might be also explained by the distinct capacity to induce local and systemic immune activation, with the Cry1Ac protoxin able to induce local (at peritoneal cavity, omentum, and MLN) and systemic (in spleen) activation of DC, accompanied with enhanced T cell responses, unlike the Cry1Ac toxin, which only induced local activation of T cell responses (in the MLN).

A limitation of present results is to having not determined the extent of the prominent antitumor effect conferred by coadministration with the protoxin Cry1Ac; this critical point should be examined in further immunological memory studies by repeating the injection with 4T1 cells at distinct time points in immunoprotected mice, which resisted the first inoculation challenge with 3×10^3 4T1 cells. The antitumor immunoprotection capacity also could be evaluated injecting a higher amount of 4T1 cells, but due to the aggressiveness of this model protection is unlikely to occur under these conditions, as we have observed that injection with 1×10^4 4T1 cells surpassed the 100% immunoprotection capacity conferred by coadministration with Cry1Ac protoxin. Thus to strength the potential utility of this protein to improve tumor immunity, further studies will be required to validate whether the significant antitumor adjuvant effect observed in present study in the TNBC mouse model induced with 4T1 cells are also achieved using alternative less aggressive mouse models of TNBC such as EMT6 model or to test the effect in a distinct type of cancer model.

The potential application of Cry1Ac protoxin in a prophylactic approach in humans would be limited, as it might present several difficulties, such as the heterogeneity of tumors between patients, or the lack of specific antigens for breast cancer. Therefore, we decided to evaluate in a therapeutic approach whether the adjuvant antitumor effect of Cry1Ac protoxin could enhance therapy with doxorubicin, an anthracycline used in the treatment of patients with TNBC. The results indicate Cry1Ac might confer benefit for cancer immunotherapy, since mice receiving doxorubicin + Cry1Ac protoxin combined therapy showed less tumor growth and decreased the incidence of lung metastases. A possible explanation for these effects

could be due to the adjuvant effect of Cry1Ac, which allowed a better presentation of tumor antigens, both in the TME and possibly at the systemic level; since doxorubicin therapy has been reported to be capable of inducing the release of antigens in the TME.[69] However, more studies are needed to describe the exact mechanism by which Cry1Ac protoxin enhances the antitumor effect of doxorubicin therapy.

The presence of cytokines in the TME plays an important role in mediating an antitumor response. The mice that received the combination therapy of doxorubicin + Cry1Ac protoxin had a higher production of IL-6 and TNF-α in the tumors. Although TNF-α and particularly IL-6 are widely described as cytokines involved in promoting tumor growth and metastasis,[70-73] it has also been described that the presence of IL-6 can provoke an "acute" inflammatory response that favors the elimination of tumor cells.[74] IL-6 might contribute to antitumor immunity by mobilizing T cell responses, by providing survival, differentiation, recruitment and proliferative signals to many leukocyte populations.[75] It has been reported that the presence of TNF-α in tumors can have a vasomodulator effect that improves the concentration of different drugs in the tumor tissue, favoring the elimination of tumors.[76] Assessing cytokine production in the tumor at different stages could help us to better describe the adjuvant effect of Cry1Ac protoxin in combination therapy with doxorubicin.

Mice that received only doxorubicin therapy showed even a greater amount of metastasis than mice that received no therapy; a possible explanation for this effect could be due to pro-metastatic effects elicited by doxorubicin through the release of extracellular vesicles that enhance pre-metastatic niches in the lung.[77,78] Together, these results suggest that combination therapy of doxorubicin with Cry1Ac protoxin may improve the decrease in tumor growth and decrease lung meta, further supporting the potential benefit Cry1Ac may confer for cancer immunotherapy.

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Author Contributions

Conception or design of the work: LMF and RRSG; methodological standardization: RRSG, DIA, and AJJC; experimental design: RRSG, LMF, DIA, and AJJC; experimentation and data collection: RRSG, DIA, and AJJC; data analysis and interpretation: RRSG, LMF, and AJJC; drafting the article: RRSG; critical revision of the article: LMF and RRSG; final approval of the version to be published: LMF, DIA, RRSG, and AJJC.

Ethical Approval

The authors state that all procedures performed in present study which involved experimentation with animals were approved by a research ethics committee at the institution UNAM FES Iztacala. The authors declare that all experimental procedures were performed in accordance with the ethical standards of the institutional and national research ethical committee following the international, national, and institutional guidelines for the care and use of animals.

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Supplemental material

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