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Cyclophosphamide enhances the release of tumor exosomes that elicit a specific immune response *in vivo* in a murine T-cell lymphoma

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ABSTRACT

Exosomes are 60–150 nm small extracellular vesicles (EVs) released by most cells. Tumor-cell-derived exosomes, used as a vaccine, elicit a specific cytotoxic response against tumor cells, usually with a greater immunogenicity than tumor-cell lysates. However, the number of exosomes isolated from culture cells is limited. In recent studies, it was observed that cells respond to different stressor stimuli such as cytotoxic drugs, hypoxia, acidosis, or radiation by increasing the release of EVs.

In this study, using the murine LBC T-cell lymphoma, we found that cyclophosphamide significantly increased EVs yield. These EVs express exosome marker proteins such as TSG-101, CD9, CD81, and CD63. Furthermore, similar humoral and cellular immune responses were induced *in vivo* by EVs isolated from LBC-tumor cells whether they were grown under normal culture conditions (EVs C) or in the presence of cyclophosphamide (EVs CTX). Mice vaccinated either with EVs C or EVs CTX were similarly protected against an intraperitoneal challenge with LBC tumor cells. CD4+ and CD8+ IFN-γ secreting cells were induced in immunized mice and a specific cytotoxic cellular immune response was elicited *in vitro*. These results demonstrate that a Th1 response was induced by immunization with the EVs. Our findings suggest that treatment of tumor cells with cyclophosphamide is a useful method to enhance the secretion of EVs in sensitive cell lines without altering their antitumor properties and thus may be used to produce antigens for future design of cancer vaccines.

1. Introduction

Exosomes are small extracellular vesicles (EVs) that range in size from 60 to 150 nm [1]. They are of endosomal origin and are effective carriers of proteins, RNAs (including miRNAs), DNA and other bioactive molecules [2–4]. Depending on the cell types from which they are derived and the state of differentiation, exosomes play a role in diverse physiological and pathological processes, serving as a novel and more complex form of cell-cell communication [5].

Tumor exosomes (tex) were isolated from malignant effusions and tumor-conditioned medium [6–9]. The biggest difference between tex and other exosomes is the presence of tumor-associated antigens. Comparisons between tumor-cell-lysates and tex often revealed an enrichment in tumor antigens in exosomes such as HER2/neu, mela-A [6], Silv [10], carcinoembryonic antigen [11], or mesothelin [12]. Immunization of mice with exosomes derived from tumor cells or from dendritic cells pulsed with tumor antigens demonstrated that it is possible to induce an antitumor protective immune response using exosomes as a source of antigens [10]. Similarly, in a human *ex vivo* model, exosomes extracted from malignant effusions proved to be an effective source of tumor antigens for the cross-presentation by dendritic cells to CD8 cytotoxic T-cells. Tex not only act as passive forms of antigens but also are superior to other forms of antigens such as complete cell lysates [10] or soluble antigens [13]. The apparent advantage of exosomes could be due to the expression of specific molecules on the surface, such as costimulatory molecules, MHC molecules, or heat shock proteins (HSP), that act as a cofactor for efficient receptor-mediated uptake and also, communicate danger signals that trigger the maturation of the dendritic cell and consequently the activation of the immune system [14,15].

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The use of exosomes as a defined source of easily obtainable and stable tumor antigens to modulate the immune system is an innovative technique for the treatment of cancer. However, only a limited number of exosomes can be obtained from tumor effusions or conditioned medium.

Recent studies, have documented that cells react to different stressors such as: hypoxia [16–18], acidosis [19], radiation [16], cytotoxic drugs [20–24], and oxidative and thermal stress [25–29] modulating the secretion of EVs in quantity and/or their content (quality).

Cyclophosphamide is a cytotoxic agent commonly administered for the treatment of lymphomas, leukemias, neuroblastomas, and retinoblastomas as well as ovarian, breast, endometrial and lung carcinomas due to its cytotoxic effect on tumours. Due to its toxicity, cyclophosphamide is usually used in combination with another chemotherapeutic agent [30]. However, more recent work have also demonstrated that low-dose cyclophosphamide treatment results in the enhancement of effector T-cell function promoting anti-tumour immunity, by selectively depleting regulatory-T cells (Treg) [31] and enhancing effector T-cell function [32]. Interestingly, metronomic administration of cyclophosphamide alters the integrity of the intestinal epithelium, promoting the translocation of defined bacteria in secondary lymphoid organs that elicit tumor specific subpopulation of Th17 and Th1 [33] the increase of intratumoral CD8/Treg ratio, a decrease Treg in the tumor microenvironment and the infiltration of IFN-γ-producing γδ T-cells in cancer lesion [34]. Furthermore, low dose of cyclophosphamide produces apoptosis of endothelial cells in the tumor microvasculature therefore inducing an antiangiogenic effect [35].

In the present study, we investigated the effect of cyclophosphamide on the secretion and modulation of the immune properties of tumor cell-derived exosomes. Using the T-cell lymphoma LBC, we found that cyclophosphamide significantly enhances the secretion of exosomes by tumor cells without altering their immune properties.

2. Materials and methods

2.1. Cell culture and reagents

The syngeneic BALB/c T-lymphoma cell line, LBC (H-2^d) was maintained in RPMI 1640 (Gibco™, Invitrogen, CA, USA) supplemented with 10% heat-inactivated, fetal-bovine serum (FBS) (Internegocios, Mercedes, Buenos Aires, Argentina), 2 mM glutamine, 25 mM HEPES buffer, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM 2-mercaptoethanol, as previously described [36]. Cyclophosphamide (Laboratorio KLM; Buenos Aires, Argentina) used to stress LBC cells was dissolved in RPMI medium as a stock solution.

2.2. Mice

Six-to-ten-week-old female immunocompetent BALB/c mice were purchased from the School of Veterinary Sciences, Universidad de Buenos Aires (Buenos Aires, Argentina). Animals were fed on Cargill pellets and water *ad libitum*. All animal procedures were conducted in accordance with Institutional Animal Care and Use Committee (CICUAL) guidelines of University of Buenos Aires Medical School.

2.3. EVs isolation

EVs from LBC cell culture were isolated from bovine-EVs-depleted medium after 24 h of LBC cell culture at 37 °C and 5% CO_2 atmosphere, under normal culture conditions or in the presence of cyclophosphamide. EVs were isolated as previously described [37]. Briefly, 30 ml per T75 culture flask with 1.0×10^6 LBC cells/ml were cultured for 24 h at 37 °C in RPMI without (normal culture conditions) or with 3 mM cyclophosphamide. The LBC-conditioned medium (exactly the same volume from treatment) was centrifuged at 300*g* for 10 min to separate floating cells. Supernatants were removed and centrifuged successively at 800*g* for 30 min, 10,000*g* for 30 min. Supernatants were filtered through a 0.22 μm porous membrane and centrifuged at 100,000*g* for 80 min. EVs contained in the pellet were washed in phosphate-buffered saline (PBS), and were concentrated in a last step of 80 min. ultracentrifugation at 100,000*g*. The EVs were resuspended in PBS and kept at −80 °C.

2.4. Bradford assay

EVs concentrations were indirectly measured by protein quantification in a Bradford assay [37]. Briefly, 10 μL of exosomes sample were incubated with 190 μL of Bradford reagent (Bio Rad Laboratories, Hercules, CA) at RT. Absorbance was read 5 min after at 595 nm, and protein concentration was extrapolated from a standard concentration curve of Bovine Serum Albumin.

2.5. EVs immunofluorescence staining and flow cytometry

Five μg of EVs were incubated with 4 μm-diameter aldehyde/-sulfate latex beads (Invitrogen, CA, USA), as detailed in [37,38]. Bound EVs were spun down and the unoccupied sites were saturated with 100 mM glycine. Then, the EVs were incubated with anti-mouse CD24-PE, anti-CD8-PE, and isotype-matched controls (e-Bioscience, CA, USA). After incubation with anti-MHC I-Biotin, CD9-Biotin, CD81-Biotin (e-Bioscience, CA, USA), anti-HSP-60-Biotin, anti-HSP-70-Biotin, and anti-HSP-90-Biotin (Santa Cruz Biotechonoly, TX, USA) primary antibodies, and then, EVs-coated beads were incubated with a streptavidin-phycoerythrin conjugated (Invitrogen, CA, USA) secondary antibody. Fluorescence was measured in a BD FACSCalibur flow cytometer (BD Biosciences, CA, USA). The data was analyzed with the *FlowJo* 10 software.

2.6. Electron microscopy

To visualize the EVs preparations by electron microscopy, EVs were fixed with 4% paraformaldehyde and loaded into electron microscopy grids [37]. Samples were then observed in a transmission electron microscope Phillips Tecnai-10. Vesicle size was assessed with ImageJ analysis software.

2.7. Dot blot

Samples containing 1 μg of EVs or 10,000 LBC cells were spotted onto a nitrocellulose membrane (Hybond-ECL nitrocellulose membrane, 0.2-μm transfer membrane; GE Amersham Life Sciences, US). Membranes were blocked and incubated overnight at 4 °C using different dilutions (from 1:200 to 1:6400) of sera from naïve mice or from mice immunized with irradiated-LBC-cells, or EVs CTX or, EVs C (EVs isolated from conditioned medium of LBC treated or not with cyclophosphamide). Blots were washed and incubated with anti-mouse HRP conjugated sera for 1 h. Membranes were revealed with an ECL kit (GE, Amersham Life Sciences, USA) following the manufacturer's instructions. GE Healthcare, Image Quant TM-RT ECL, Version 1.0. software was used to detect specific proteins [37].

2.8. Mice immunization and tumor challenge

Mice were randomly divided into groups and vaccinated i.p. once a week for 2 weeks. For each injection, each group of mice received EVs C or EVs CTX doses at 20 μg/ml/mouse, or as a control, PBS alone. Seven days after the last immunization, animals were challenged with 1.0×10^6 LBC tumor cells i.p, which is a dose that has been determined to cause 100% lethality 21 ± 4 days after injection. Mice survival was monitored daily and the mortality rate and survival were recorded [39]. This immunization protocol and its subsequent challenge was carried out twice.

2.9. Intracellular staining for IFN-γ

For the detection of IFN-γ secreting cells, 80 days after LBC cells inoculation, spleen cells were obtained from 3 mice from each group (mice immunized with either EVs CTX or EVs that have rejected the tumor challenge, and naïve mice). Splenocytes (1.0×10^6/well) were co-cultures in a 96-well plate during 48 h with 10 μg of EVs C, 10 μg EVs CTX or irradiated LBC cells (LBCi, at a ratio 1:50). Splenocytes were incubated with Golgi Stop® (Monensin, BD Biosciences, CA, USA), according to the manufacturer's recommendations, for the final 6 h and centrifuged at 250 *g* for 5 min. Cells were resuspended in staining buffer (PBS supplemented with 0.1% sodium azide and 5% FBS, pH 7.4–7.6) and the intracellular staining for IFN-γ expression was accomplished as previously detailed [40]. Double-color surface staining was first performed with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and phycoerythrin (PE)-conjugated anti-CD8 monoclonal antibodies, and then cells were fixed with 1% paraformaldehyde, permeabilized with a permeabilization buffer (PBS supplemented with 0.1% sodium azide, 1% FBS, and 0.1% saponin) and stained with an Allophycocyanin (APC) labeled–anti–IFN-γ monoclonal antibody (e-Bioscience, USA). Samples were acquired in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). For the flow cytometric analysis, a lymphocyte gate based on forward and side scatter properties (FSC/SSC) was set and 200.000 events were acquired from this gate. A second gate was drawn around the CD4+ or CD8+ lymphocytes in the CD4+ versus CD8+ dot plot. Multiple control experiments were performed to validate the applicability of intracellular staining for cytokines according to previously reported methods [41]. Accordingly, we stained unstimulated splenocytes from naïve and immunized mice and lymphocytes that had been cultured for 6 h, with phorbol-12-myristate-13-acetate (PMA, ICN Biomedicals, CA, USA) at 50 ng/mL and Calcium ionophore ionomycin (ICN Biomedicals, CA, USA) at 1 μM. The evaluation of IFN-γ gamma secreting cells was performed twice. For each experiment, duplicates samples were analyzed separately (from each mouse out of 3 mice per experimental group; EVs CTX; EVs C and naïve mice. N = 6).

2.10. 7-AAD/CFSE cell-mediated cytotoxicity test

The cytotoxic activity behavior of the lymphocytes was quantified by the carboxy-fluorescein succinimidyl ester (CFSE) dilution and 7ADD [42]. Briefly, target LBC-cells were stained with CFSE before seeding, using the CFSE (Invitrogen, CA, USA) at a final concentration of 1 μM for 10 min at room temperature, followed by immediate quenching with culture medium. CFSE stained LBC were adjusted at 1.0×10^6/ml and seeded in a 24-well plate.

Effector cells were harvested, counted, washed, and resuspended to 1×10^6 cells/ml. Effectors and CFSE-labeled target cells

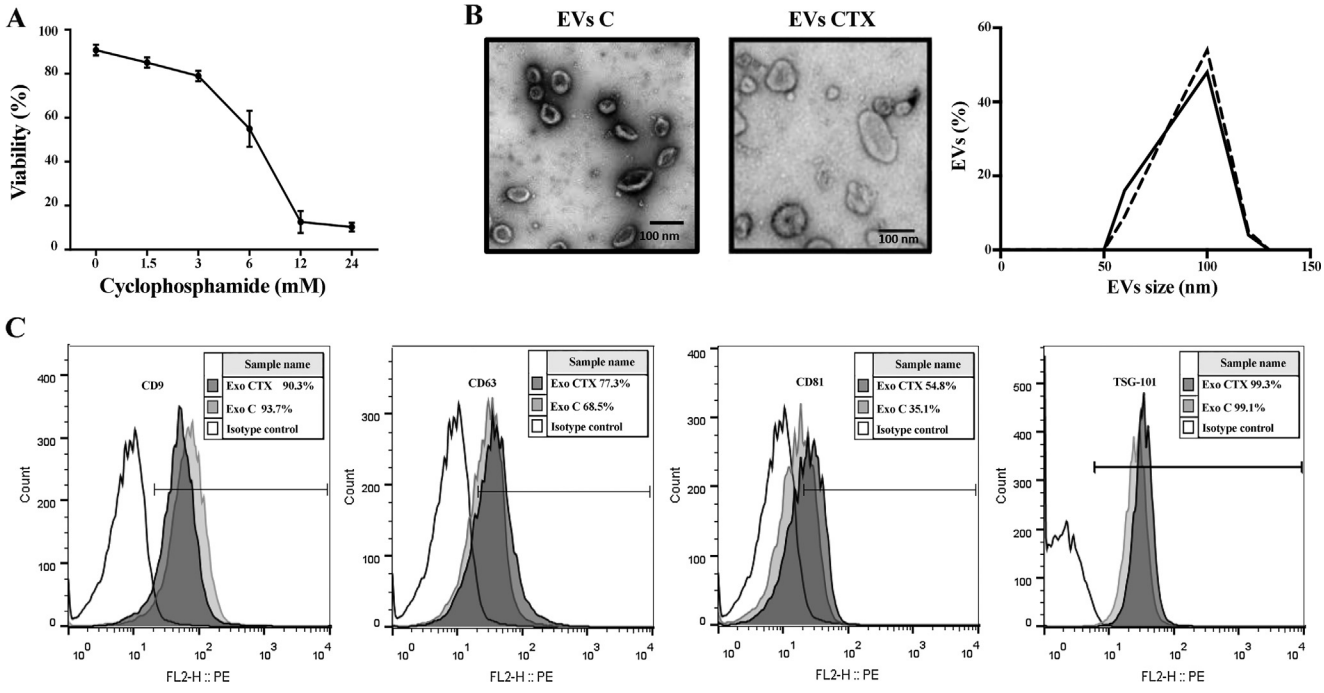

Fig. 1. Dose response curve of LBC cells to cyclophosphamide and characterization of EVs isolated from LBC cells under standard culture conditions or incubated with cyclophosphamide. (A) Dose response curve of LBC cells to cyclophosphamide. LBC cells were exposed to different doses of cyclophosphamide during 24 h. The viability was measured by the Trypan Blue exclusion method. The mean ± SD is expressed on the graph. The graphs correspond to 3 independent experiments. (B) Morphological characterization by Transmission-electron microscopy: Electron microscopy of purified small extracellular vesicles (EVs) isolated from LBC cell conditioned medium under normal culture conditions or incubated with cyclophosphamide, demonstrating the typical shape and size (60–120 nm) of exosomes, and graphic of the size-distribution of the vesicles measured with ImageJ software of at least four micro-photos. Small EVs from unstressed LBC cells (EVs C; straight line). EVs from LBC cells incubated with cyclophosphamide (EVs CTX; dashed line). (C) Flow cytometry detection of surface molecules on EVs. EVs were incubated with aldehyde–sulfate latex beads and stained with monoclonal antibodies or specific isotype-matched control antibodies. Analysis was performed on singlet gate of a forward scatter versus side scatter dot plot. Filled histograms represent exosome–bead complexes stained with specific monoclonal antibodies, the unfilled histogram represents isotype control antibodies.

were mixed at a range of E:T, 20:1; 40:1 and 80:1, with duplicate wells/condition. Cultures were incubated for 4 h at 37 °C under 5% CO_2. The 7-AAD was then added to samples, and cultures were incubated for 30 min in the dark, washed, and resuspended in 900 μL FACS buffer (PBS, 0.5% BSA, 0.006% Sodium Azide). Fluorescence was measured in a BD FACSCalibur flow cytometer (BD Biosciences, CA, USA). The data analysis was performed with the FlowJo 10 software [41]. The gating strategy employed by Cao et al. was used [42].

2.11. Statistical analysis

The survival fractions were calculated using the product-limit Kaplan-Meier method and differences between treatments were evaluated by log-rank statistic adjusting the p value for multiple comparisons. Statistical significance of differences between the experimental and control groups was analyzed using the paired samples Student's *t* test or repeated measures analysis of variance (one-way ANOVA with Tukey's post-test) where appropriate. Differences were considered significant at p values < 0.05 for all comparisons. The statistical analysis was performed using GraphPad Prism software 6 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Cyclophosphamide enhances secretion of exosomes by tumor cells

The response to cellular stress, in general, prevents cell apoptosis up to a certain point. If the intensity of the stress is such that the response is not sufficient to keep the cells healthy, then the cells enter into apoptosis [43]. Cells undergoing apoptosis also release EVs, generally called "apoptotic bodies", with a wide range of sizes (from 50 nm to bigger than 1000 nm) [5,44]. Therefore, it was considered that a sub-lethal dose that maintained a good viability in culture (>80%) would be an appropriate dose to induce cellular stress. To define this dose, LBC cells were exposed for 24 h to increasing concentrations of cyclophosphamide (in the range of maximum tolerated dose determined for other tumor cell lines [45]). The cells were then subjected to stress for 24 h, a period of time to obtain a good yield of EVs but not long enough to induce apoptosis and generate apoptotic bodies that could contaminate the exosome preparations. The graph shown in Fig. 1A indicates the highest dose required to induce cell stress in LBC cells preventing cell death. Three mM was the dose selected to induce stress as is the highest concentration that maintained LBC cell viability greater than 80% for 24 h.

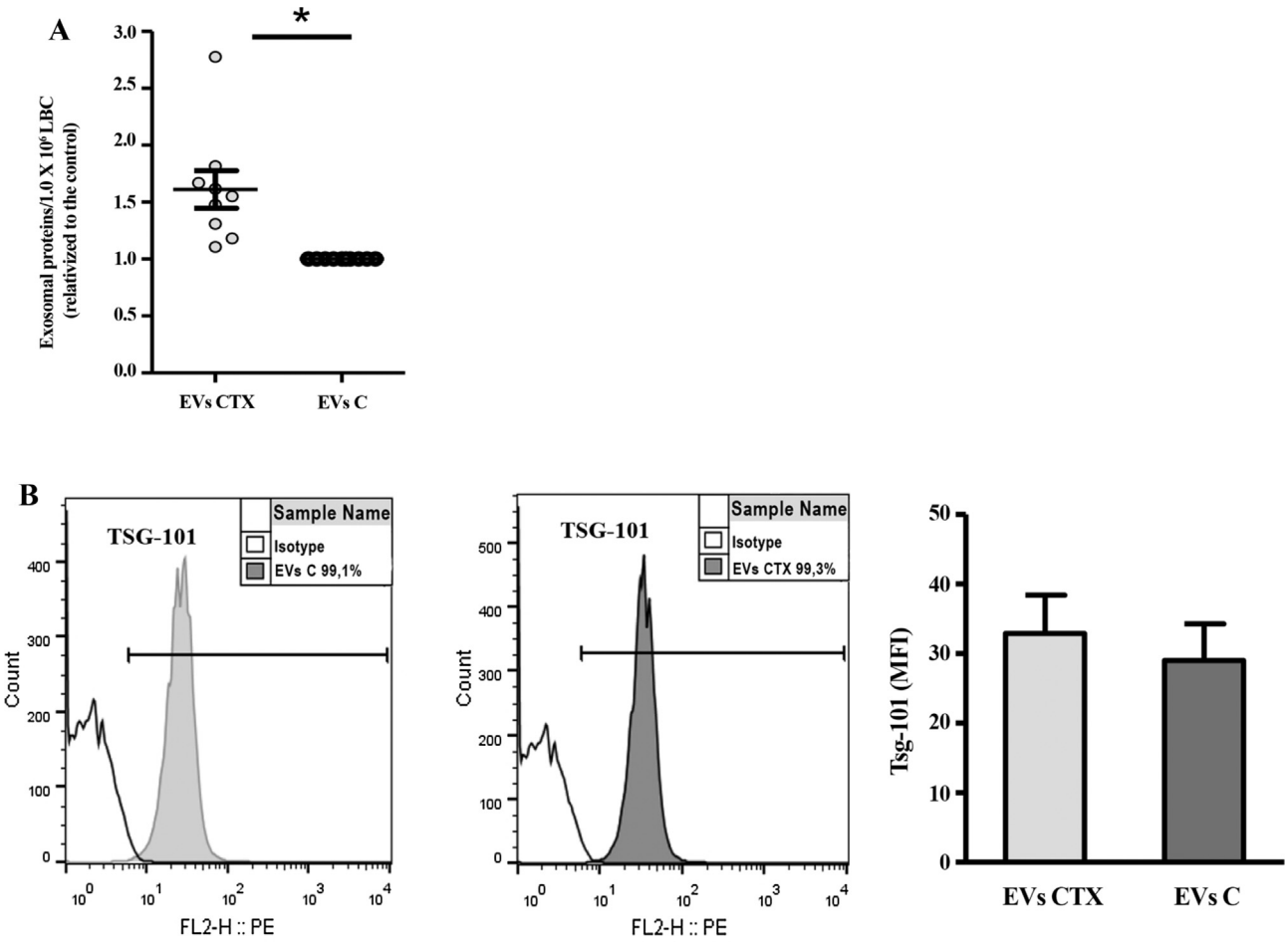

Fig. 2. Quantification of EVs released by LBC cells incubated with cyclophosphamide. EVs were isolated through a series of centrifugations, filtration, and ultracentrifugation from equal volume of cell culture supernatant of the identical number of LBC cells incubated for 24 h under standard culture (Control) and with 3 mM of (cyclophosphamide). (A) The yield of EVs was measured as the amount of total proteins in the exosomal fraction by the Bradford method. Protein content recovered in the pellet of LBC cells incubated or not with cyclophosphamide was expressed as micrograms per 1.0×10^6 secreting cells and results were normalized to control (y axis). The mean + SD of 8 independent experiments is expressed. The statistical comparison was made between control and cyclophosphamide ($^*p = 0.004$). (B) Flow cytometric analysis of EVs CTX and EVs C incubated with anti-TSG-101 antibody. Empty histograms represent the controls without specific antibody, from which the positive region was defined for each marker. Analysis was performed on singlet cell gates of a forward scatter versus side scatter dot plot. One of 3 representative experiments is shown.

Once the appropriate dose of cyclophosphamide was found, EVs secreted in an LBC-conditioned medium treated with or without cyclophosphamide were isolated. Small EVs were recovered from the pellet obtained from the conditioned medium ultracentrifugated at high-speed (exosomal fraction) and characterized by their morphology and exosomes markers as detailed in *Materials and methods*.

A morphological identification of the EVs obtained was made through Transmission-electron microscopy (Fig. 1B). It was possible to observe a heterogeneous population of EVs with a size (ranging from 60 to 100 nm) and a morphology generally described for exosomes. These EVs were positive for several protein markers characteristic of exosomes, such as the tetraspanins CD63, CD9, CD81, and TSG-101 a protein that is part of the ESCRT complex responsible for the formation of exosomes in endosomes and MVB (Fig. 1C). Therefore, we suggest that *bona fide* exosomes were contained in the pellets isolated after ultracentrifugation from tumor-cell culture supernatants before and after cyclophosphamide treatment.

In the next step, we investigated whether cyclophosphamide also affected the quantity of exosomes secreted by LBC cells. To this end, the exosomal yield from an equal volume of cell-conditioned medium produced by an equal amount of LBC cells cultured with or without cyclophosphamide was indirectly quantified through the measurement of total proteins by the Bradford method. As shown in Fig. 2A, LBC cells growing in the presence of 3 mM of cyclophosphamide release 61 ± 16% more EVs than under normal conditions. Since, exosomal fractions may contain a certain proportion of impurities in the form of protein aggregates or other types of EVs. To confirm that the quantified EVs corresponded to exosomes and not to impurities, we determined the expression of the exosome marker TSG-101, using flow cytometry. Four µm latex-beads were coated with the same amount of proteins from the exosomal fraction, labeled with a specific antibody for the exosome marker TSG-101, and measured using flow cytometry. Fig. 2B shows that, more than 99% of the events expressed the exosomal markers TSG-101 with similar mean fluorescence intensities (MFI). It should be noted that each event represents a microsphere in the region of singlet of a dot plot, to which the same micrograms of sample obtained from the exosomal fraction was bound, thereby the MFI of the surface marker gives an idea of he purity of exosomes in the exosomal fraction isolated.

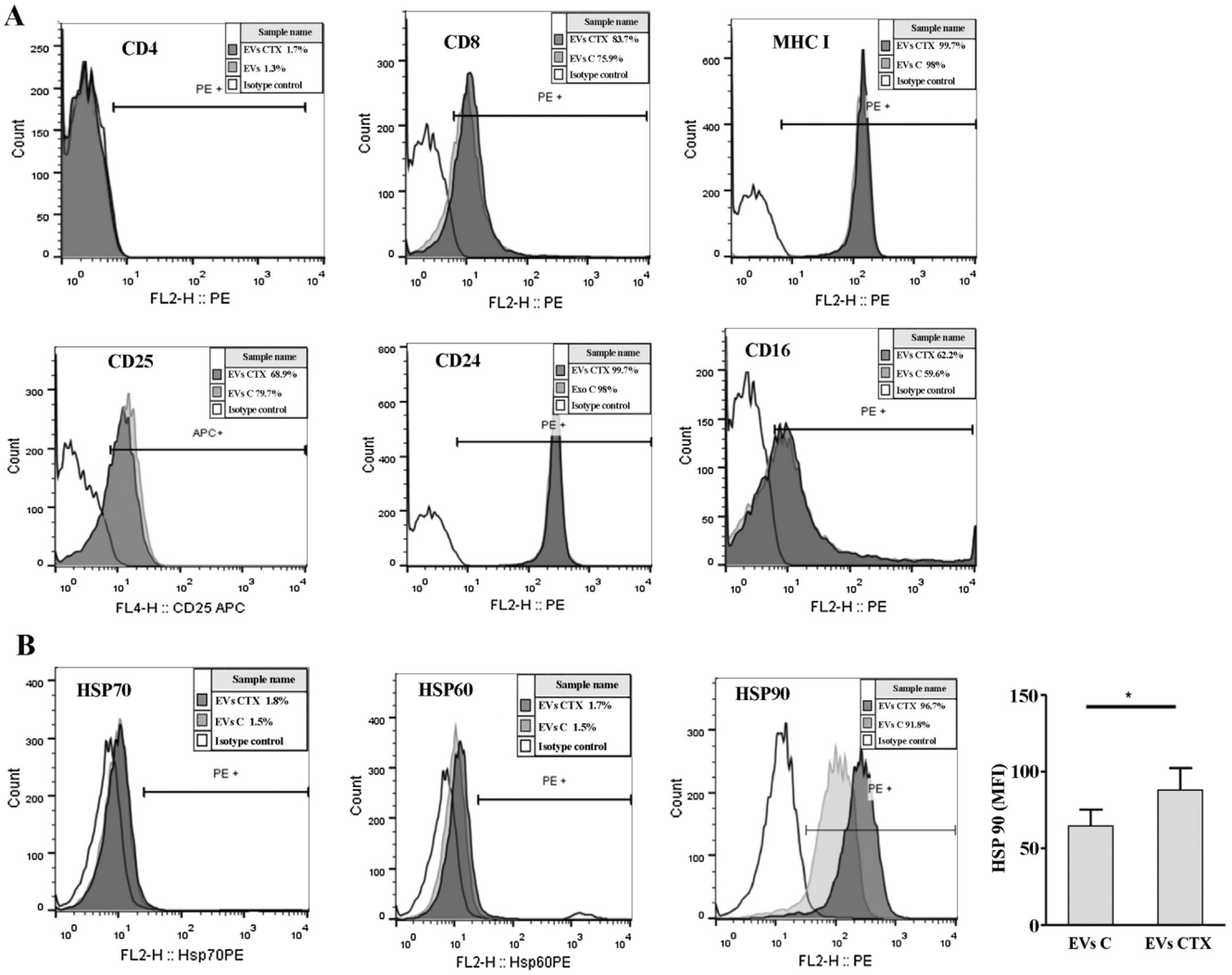

Fig. 3. Characterization of exosomes from LBC cells under standard culture conditions and under cyclophosphamide cell stress. The exosomal fractions isolated from the supernatant of the LBC cells were labeled on membrane with antibodies specific against HSP-60, HSP-70, HSP-90, and markers of the LBC cells as described in *Materials and methods* and analyzed by flow cytometry. A: Histograms of the different membrane markers of exosomes under normal and stress conditions. B: Comparison between the MFI of the markers expressed on EVs C and EVs CTX. The mean + SD of 3 independent experiments is expressed. Asterisks indicate significant differences ($^{*}p < 0.05$); analyzed by the t Wilcoxon rank test for paired samples. The number of positive events is expressed as a percentage.

The internal and external composition of exosomes depends not only on the cell type that they come from, but also on the state in which the cell is found, whether in a replicative or stationary phase, differentiated or undifferentiated, under stress or under normal conditions [46]. For this purpose, heat shock proteins HSP-60, 70 and 90, and proteins expressed on LBC cells, such as CD24, MHC I, CD8, CD25, CD16, and CD4 were also evaluated by flow cytometry (Fig. 3). It was observed that both EVs CTX and EVs C highly express CD24, MHC I and HSP-90 on the surface. Furthermore, they express CD25, CD16 and CD8 to a lesser extent, and do not express HSP-60, HSP-70 and CD4. When comparing the marker expression levels between the exosomes isolated from LBC cells cultured with or without cyclophosphamide, no significant differences were observed for CD24, MHC I and CD8, remarkably there was a significative higher expression of HSP-90 on exosomes released by LBC cells incubated with cyclophosphamide, according to the mean fluorescence intensities (MFI) of the surface proteins ($p < 0.05$).

3.2. Cyclophosphamide does not alter tumor antigen expression on exosomes

Depending on the tumor cell from which they come and the stage in which they are, tumor exosomes may be either immunogenic or immunosuppressive. We have demonstrated that exosomes isolated from ascites of mice bearing LBC tumor are immunostimulant both *in vitro* and *in vivo* [9]. Furthermore, vaccination with these exosomes induced a potent immune response that lead to the rejection of the tumor in 60% of the animals immunized and challenged with the tumor. Once the EVs C and EVs CTX have been characterized in terms of protein expression and knowing that expression of certain proteins expressed on those EVs such as HSP-90 and 70 are involved both in the presentation and in priming an immune response [47–49], we next proposed to evaluate the immunogenicity of exosomes derived from LBC cells cultured with cyclophosphamide and compare it with cells growing under standard culture conditions. We first evaluated if the treatment with cyclophosphamide could have induced a modification in tumor-antigen expression on exosomes from LBC cells. To this end, 2 sera were used: a specific serum for LBC cells and a normal serum as a control.

As shown in Fig. 4, anti-LBC antibodies reacted in a similar way either with LBC cells or EVs C or EVs CTX, whereas no specific signal was obtained with normal serum. These results demonstrated the antigenicity of the EVs C and EVs CTX while an increase in the expression of tumor antigens due to the treatment cannot be determined by this methodology.

3.3. Cyclophosphamide does not alter the immune response induced in vivo by exosomes

Given the variability of functions reported for tumor exosomes and the fact that *in vitro* experiments only reveal a portion of the modulation that could eventually influence the immune system of an organism, we decided to conduct an *in vivo* experiment to evaluate the immunogenicity of EVs C and EVs CTX. To achieve this goal, 3 groups of 10 mice were immunized intraperitoneally with 2 doses of 20 μg of EVs C, or EVs CTX per mouse, or 0.5 ml of PBS in the control group, with a 7-day interval between inoculations (Fig. 5A).

To evaluate the induction of a specific antitumor response, the day before the challenge with the tumor cells, a blood sample was taken from the mice and the presence of specific antibodies for the LBC cells were evaluated, using the dot blot technique. A pool of sera from each treatment was incubated with the intact LBC cells, with EVs C or EVs CTX. A high titer was obtained for sera

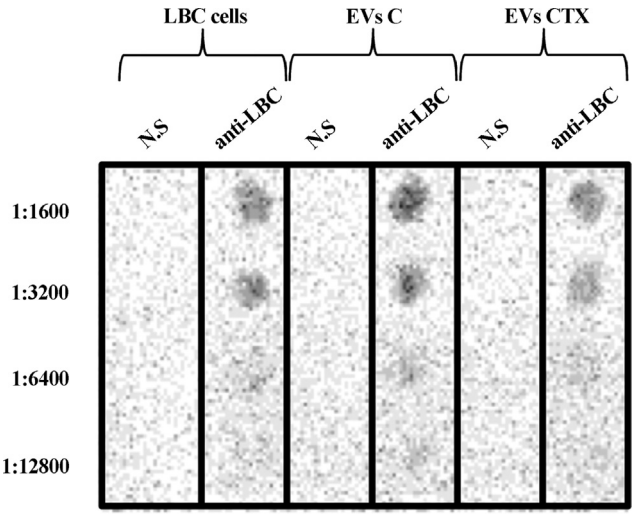

Fig. 4. Tumor antigen expression on exosomes from LBC cells culture with or without cyclophosphamide, evaluated by the dot blot. Lanes 1 and 2) 10,000 LBC cells, lanes 3 and 4) 1 μg of EVs C, lanes 5 and 6) 1 μg of EVs CTX. Samples were incubated with different dilutions of normal serum (N.S.) and serum from mice immunized with LBC cells that developed an effective immune response against the tumor cells (anti-LBC serum) and with anti-mouse IgG antibodies conjugated to HRP as a second antibody. Subsequently, they were revealed by chemiluminescence, as indicated in *Materials and methods*.

from mice immunized either with EVs CTX or EVs C (1:12800). This result demonstrates that immunizations with exosomes generated an immune response and that the humoral response was similar in mice immunized with EVs C and EVs CTX. As a positive control, the sera were incubated with the respective exosomes with which each group of mice was immunized (Fig. 5B). Sera from mice immunized with EVs C and EVs CTX react to the exosomes that induced the immune response with similar titres. This would indicate that antibodies present in the sera of immunized mice recognize in the same way both the exosomes that generated them, and the tumor cell.

In order to investigate if the immune response induced by the EVs C and EVs CTX could protect the animals against a challenge with the tumor, 7 days after the last immunization, each mouse received i.p. 1.0×10^6 LBC cells and the survival time was recorded (Fig. 5A and C). As shown in Fig. 5C, immunization with EVs CTX or EVs C significantly improved the survival of mice as compared to control non-immunized animals ($p < 0.017$). Immunization with EVs CTX protected 90% of mice and 70% of those immunized with EVs C remained tumor-free. While in non-immunized animals 87.5% died around day 21. However, when comparing the survival curves of mice immunized with both EVs CTX or EVs C, no significant differences were observed between the them.

In order to evaluate whether a Th1 response was induced in mice that had been immunized with EVs C or EVs CTX and had rejected the tumor, the effect of exosomes on the generation of IFN-γ secreting cells was determined. Splenocytes were cultured in the presence and absence of 10 μg EVs C or EVs CTX for 2 days and intra-cellular levels of IFN-γ were determined on CD4+ and CD8+ T-cell subsets. Our results showed that, at day 2, the percentage of intracellular IFN-γ was enhanced when splenocytes (in both T-cell subsets) were cultured with the EVs, when compared with unstimulated splenocytes (Fig. 6A). These results demonstrated that EVs C and EVs CTX both significantly enhanced IFN-γ secretion primarily by CD4 T-cells and to a lesser degree by CD8 ($p < 0.05$; Fig. 6B). No difference between EVs C and EVs CTX was observed.

To determine whether a specific cytolytic response was elicited, an *in vitro* cytotoxic assay determined by flow cytometric of CFSE

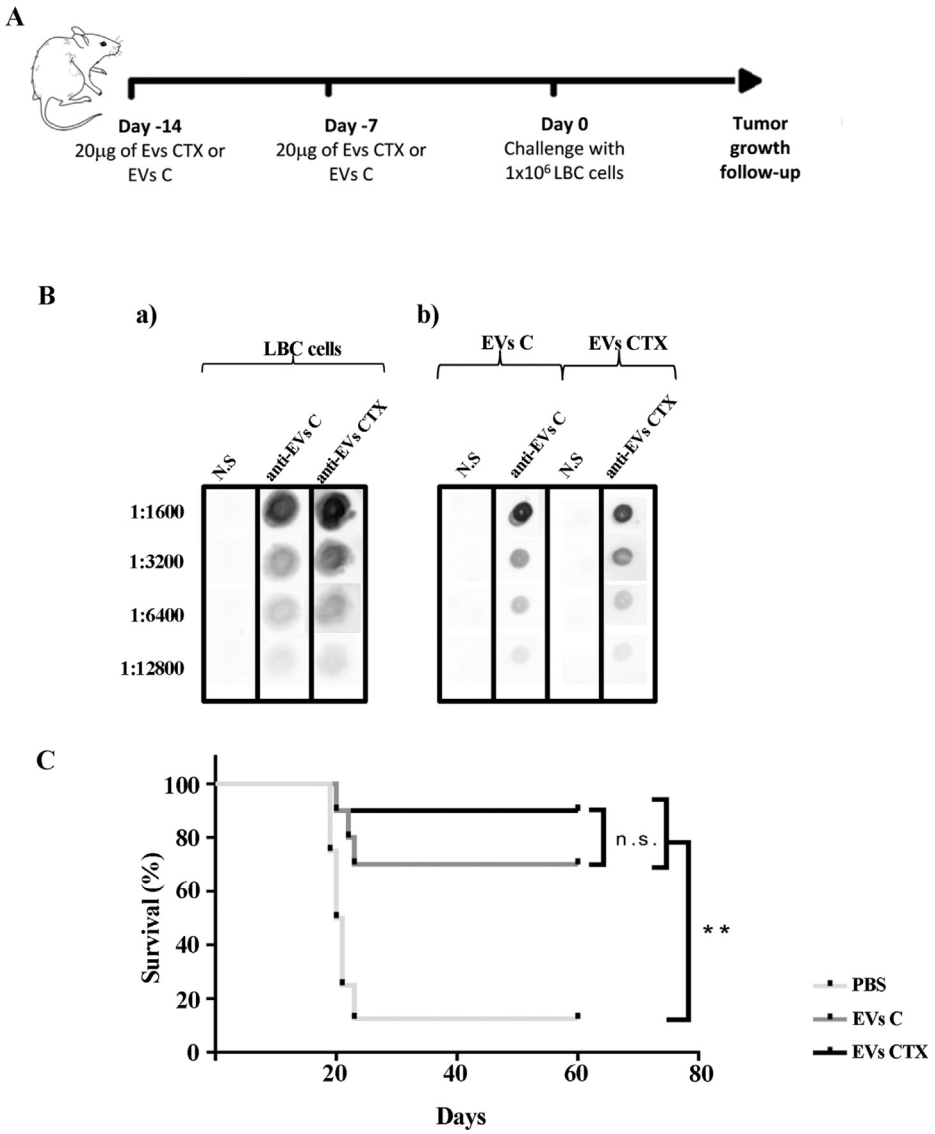

Fig. 5. Immunization protocol with EVs C and EVs CTX. (A) Immunization protocol with EVs C and EVs CTX. (B) Detection of antibodies against LBC in the serum of mice immunized with EVs C or EVs CTX prior to the challenge with the tumor cells, by dot blot. A: 10,000 LBC cells or B: 1 µg of EVs C or EVs CTX were spotted on a nitrocellulose membrane and incubated with different dilutions of a pool of sera from mice inoculated with PBS (N.S.), or sera from mice immunized with EVs C (anti-EVs C) or EVs CTX (anti-EVs CTX). To demonstrate the reaction, anti-mouse IgG antibodies bound to HRP were used and revealed by chemiluminescence as described in *Materials and methods*. (C) Survival of mice immunized with EVs C and EVs CTX and subsequently challenged with LBC lymphoma cells. Three groups of 10 BALB/c mice were immunized once a week for two weeks with PBS, EVs C and EVs CTX. Seven days later, 1.0×10^6 viable LBC cells were inoculated intraperitoneally, and the survival time was recorded. A multiple comparison (3) was made with the Log-rank test and the Bonferroni correction of p. $^{*}p < 0.017$. Two independent experiments were performed; data from one representative experiment were shown. N = 10.

labelled tumor LBC cell was performed as detailed in *Materials and methods* (Fig. 7A). From the three-different effector: target (E:T) ratios evaluated (80:1, 40:1 and 20:1), the 40:1 ratio provided the best responses and is the one depicted in Fig. 7. As shown in Fig. 7B, cell cytotoxicity induced against LBC in BALB/c mice immunized with EVs C (12.35 ± 0.15%) was not higher than that obtained from mice vaccinated with EVs CTX (11.5 ± 0.2%), even though there was a significant increase ($p < 0.01$) with respect to the naïve control (7.89 ± 0.21%).

Our results demonstrate that immunization with either EVs C or EVs CTX elicits a humoral and cellular immune response with the same magnitude as the one elicited with irradiated tumor cells. The generation of specific IFN-γ CD4 and CD8 T-cells and the induction of specific tumor-cell cytotoxicity suggest a Th1 profile which may play an important role in the observed tumor rejection.

4. Discussion

The use of EVs as antitumor vaccines has been the subject of numerous investigations in tumor immunology [50–54], particularly the EVs derived from tumor cells [55–58] or dendritic cells as acellular vaccines [59,60,61]. The use of EVs as tumor antigens for the development of cancer vaccines presents several advantages over the use of irradiated-tumor cells or tumor-cell lysates. For example, EVs are defined acellular antigens which can express specific molecules of the cells from which they originate, with the same properties as tumor cells but with the added advantage of being more effective (as express concentrating tumor antigens). EVs are easier to conserve than irradiated cells that require cryopreservation at −196 °C. They are also more stable, reproducible, and safe. Consequently, they may constitute a better immunologi-

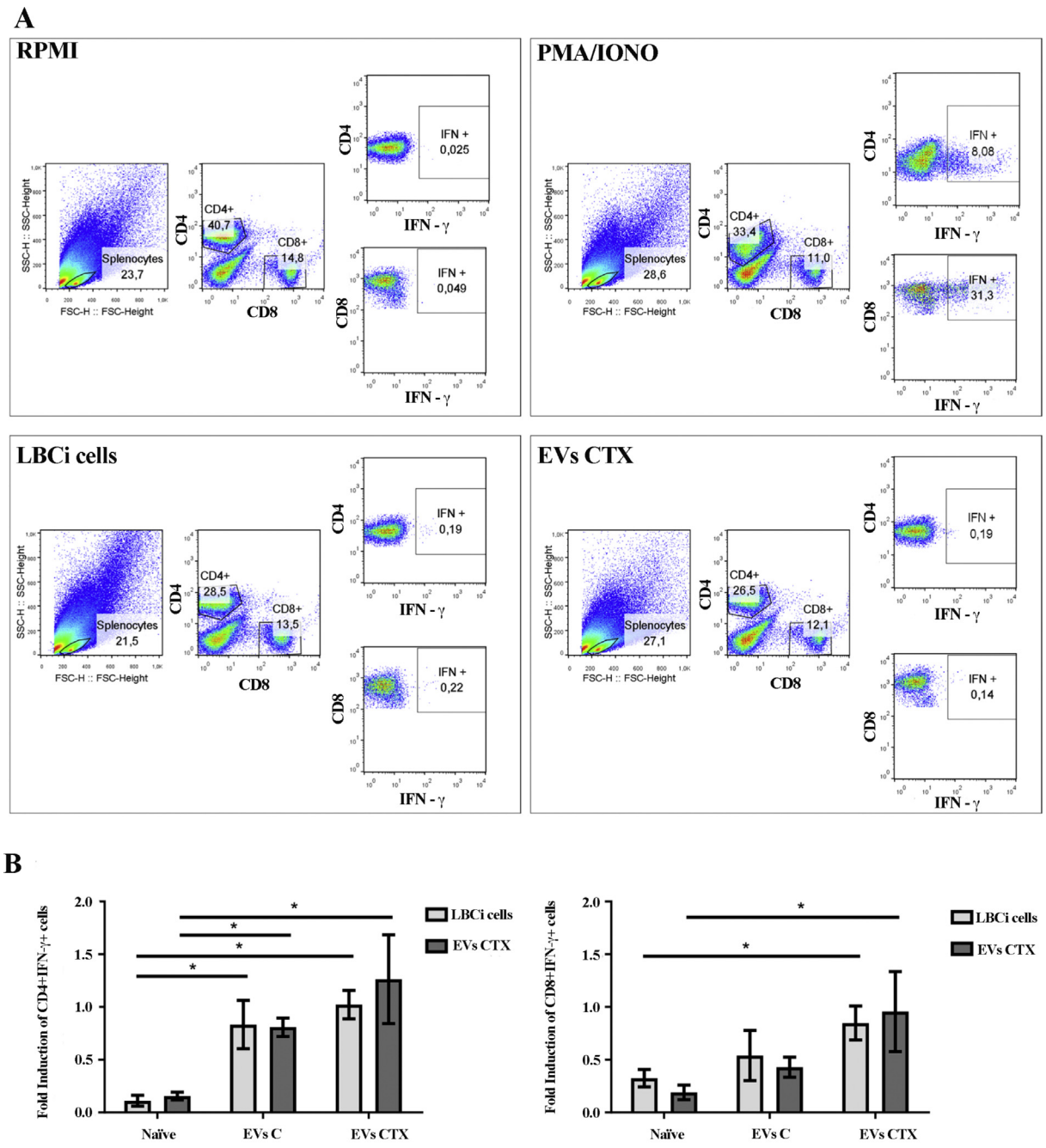

Fig. 6. Immune response induced in mice immunized either with EVs CTX or EVs C. (A) Intracellular staining for IFN-γ in splenocytes from mice immunized with EVs C or EVs CTX. Spleen cells obtained from naïve or mice previously immunized with EVs CTX or EVs C were incubated for 48 h at 37 °C. Double-color surface staining was first performed with conjugated mAbs FITC-anti-CD4 and PE-anti-CD8, and then cells were permeabilized with saponin and stained with APC-anti-IFN-γ. To analyze intracellular cytokine expression by CD4+ and CD8+ lymphocytes, a gate was first drawn around the lymphocytes in a dot plot of forward scatter versus side scatter dot plot and 200.000 events were acquired from this gate. A second gate was drawn around the CD4+ or CD8+ lymphocytes in the CD4 versus CD8 dot plot. Non-stimulated splenocytes were used as negative control (RPMI). Splenocytes cultured 6 h with PMA at 50 ng/ml and ionomycin at 1 μM were used and as positive control. (B) Average fold increase in IFN-γ-producing cells. This index is defined as the ratio between % stimulated IFN-γ-producing cells and % IFN-γ-producing cells of unstimulated splenocytes. The % IFN-γ-producing cells used for the calculation was previously subtracted by the % of unstimulated control. The evaluation of IFN-γ secreting cells was performed in two independent experiments. For each experiment, duplicates samples were analyzed separately (from each mouse out of 3 mice per experimental group. N = 6). Values represent the mean of 6 replicas +SD). Asterisks indicate significant differences ($^{*}p < 0.05$).

cal strategy for the development of an antitumor vaccine than those using irradiated-tumor cells or dendritic cells. Currently, it is possible to obtain EVs derived from tumor cells on a large scale that also have the necessary quality to be administered to patients, following the *Good Laboratory Practices* [58,62,63]. However, one of the great disadvantages that remains to be overcome is the low number of EVs produced by the cell.

The research presented in this paper aimed to study the influence of cyclophosphamide on the quantity and quality of tumor EVs secreted by the cells of a murine T-cell lymphoma growing *in vitro* to be used as an acellular source of tumor antigens. The hypothesis was developed taking into account the recent discovery that certain cells respond by inducing the secretion of EVs and/or the modulation of their content against different stressors. In

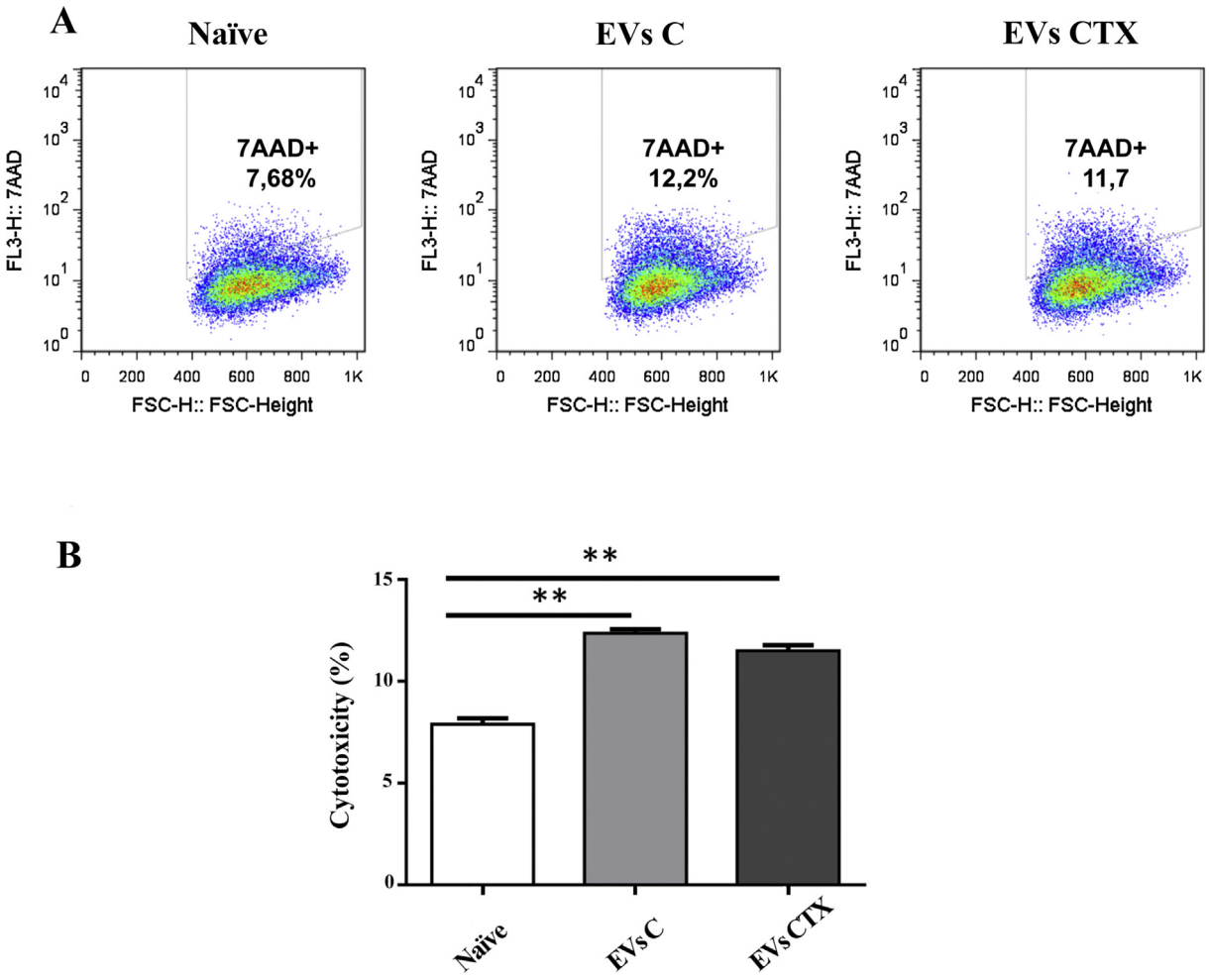

Fig. 7. Antitumor cytotoxic response induced by either EVs C or EVs CTX. Spleen cells were isolated 7 days after the final immunization with EVs C or EVs CTX and cultured as described in *Materials and methods*. LBC-target cells were labeled with CFSE (LBC-CFSE) and co-cultured for 4 h at 37 °C with splenocytes from immunized mice. An E:T ratio of 40:1 is displayed. At the end of the experiment, dead cells were labeled with 7-AAD. The percentage of LBC-CFSE cell death was analyzed by flow cytometry. (A) Density plots of 7-ADD versus forward scatter are displayed. The gating strategy followed was according to Cao et al. [42]. An initial CFSE+ gate was established on LBC-CFSE-stained target cells. Flexible quadrants were determined to optimally establish the 7-AAD quadrants. Percentage of live cells were recorded. (B) % of specific cytolysis. The % of LBC-CFSE was calculated according to Cao et al. [42]. When LBC-CFSE cells were cultured alone, there is little cell death (2%). Values represent the mean of duplicate cultures +SD. Asterisks indicate significant differences ($^{**}p < 0.01$).

particular, chemotherapeutical drugs such as bortezomib, carfilzomib, and melphalan markedly stimulate the secretion of exosomes and alter their composition [23]. Previous investigations has characterized the effect of various anti-cancer drugs (paclitaxel, etoposide, carboplatin, irinotecan hydrochloride) on the release of EVs from a human hepatocarcinoma, the expression of HSP, and the enhanced NK-cell activity *in vitro* [20]. However, at present, there is no study on how cyclophosphamide, one of the drugs most commonly used in chemotherapy, affects the production and molecular composition of EVs derived from tumor cells. Nor is there a record of an *in vivo* study of the immune response generated by these EVs from tumor cells stressed with chemotherapeutic drugs.

The first objective of this work was to determine the appropriate dose of cyclophosphamide to be used in the experiments. To avoid apoptosis and the formation of apoptotic bodies that chronic cellular stress can induce, a 3 mM dose of cyclophosphamide was chosen. This was the highest dose that maintains cell viability over 80% during 24 h. Remarkably, LBC cells were more sensitive than other lymphomas cells to cyclophosphamide. In fact, Wang et al. reported that the percentage of cell death induced by incubating several lymphoma-cell lines with 10 mM cyclophosphamide ranges between 12 and 40%, depending on the cell line [45]. In the present work however, it was observed that LBC cells cultured in similar conditions showed a cell death percentage between 50 and 90%.

Once the appropriate dose of the cyclophosphamide was determined, the EVs were isolated from the conditioned medium of LBC cells by employing the classical method of purification of EVs. This consists of a series of differential centrifugations to eliminate the non-viable cells first, followed by cellular debris, organelles, and large microvesicles. Recently, it was demonstrated that large EVs are restrained [64] on a 0.22 μm membrane filtration. Thus, this filtration step was added to obtain a higher purity in terms of EVs in our samples. Finally, the EVs were recovered by ultracentrifugation at 100,000*g* with a final wash with PBS to eliminate further contaminants, such as proteins.

Exosomes are part of a set of heterogeneous EVs in size, biogenesis, and the molecules that compose them. During the recent years, several microvesicles have been defined including apoptotic bodies, ectosomes, and exosomes all of which are secreted by most cells through different mechanisms and with characteristics that differentiate these microvesicles. Thus, it was necessary to characterize the EVs obtained before performing any functional analysis. The evaluation of the exosomes' purity as well as their size and morphological analysis was determined by electron microscopy. From the microphotographs obtained, the characteristic shape

and size attributed to the exosomes was determined [65]. One of the most convincing ways to define the quality of a sample of EVs is the identification of marker molecules, such as the components of the endosomal sorting complex required for transport (ESCRT): TSG-101 and Alix, or the tetraspanins proteins CD63, CD9, and CD81 [1,66]. The size and morphology evaluated by TEM and the expression of the proteins TSG-101, CD9, CD63, and CD81 suggest that the EVs in the pellet secreted by LBC cells corresponds to exosomes.

On the other hand, proteins expressed in the LBC cells CD24, MHC I, HSP-90, CD25, CD16, and CD8 [36] were also found on the surface of both EVs C and EVs CTX. These molecules are candidates to confer a possible specific function to these EVs. The expression of CD24 in the EVs is important because it is an indicator that these EVs come from the LBC cells since CD24 is expressed specifically in immature T-cell lymphoblasts but not in differentiated T lymphocytes [36]. The expression of CD24 on LBC lymphoma correlates to the early stage of T-cell differentiation when the neoplastic transformation of the tumor cells took place.

The measurement of total proteins presents in the preparations of the EVs gives an indication of the number of EVs secreted by the cells and allows the calculation of the yield of the samples obtained by different treatments. It is important to stress that EVs CTX and EVs C samples were normalized as the amount of EVs in the exosomal pellet secreted by 1.0×10^6 cells present exactly the same volume of conditioned medium. Using an indirect method of quantification, we demonstrated that a large quantity of EVs are secreted, evidenced by the beads that express an equal amount (% of events) and an equal intensity (MFI) of the TSG-101 marker. The amount of total proteins in the exosomal fraction produced by non-stimulated LBC cells (0.20 ± 0.036 μg/1.0×10^6cells) obtained through the method of differential centrifugations, ultracentrifugation, and subsequent washing, is below the average production of EVs calculated for this type of purification (3.018 μg/1.0×10^6cells) by Gudbergsson et al. [67]. Here it was demonstrated that the genotoxic drug cyclophosphamide, when administered in doses that produce cellular stress but not cell death, induces the secretion of EVs in a murine model of T-cell lymphoma *in vitro*, thereby achieving a significant increase in production ($61\% \pm 16$). Our findings are support by those reported by other authors who reported that chemotherapy drugs considerably enhanced the production of EVs by human hepatocarcinomas [20], ovarian carcinomas[21], and myeloma cells[23].

In our previous work, we demonstrated that immunization with exosomes isolated from ascites of tumor-bearing LBC lymphoma could induce an immune response that protected 60% of the immunized mice from a tumor challenge [9]. The next step was to study whether the EVs C and EVs CTX have the same ability to stimulate a specific immune response against the LBC tumor cells. In this study, we were able to establish a very effective antitumor immune response in mice using either EVs CTX or EVs C. The observed protection of 90–70% of the immunized mice against the challenge with live tumor cells was as effective as the one obtained either with irradiated LBC cells or with exosomes isolated from the ascites of LBC-tumor bearing mice[9]. Immunization with either EVs CTX or EVs C prior to tumor challenge led to tumor rejection with both T-cell activation and a high titre of serum antibodies specific for EVs CTX, EVs C, or LBC cells. Our results indicate that either EVs CTX or EVs C can be processed and presented not only to CD4+ but also to CD8+ T-cells, as IFN-γ CD4+ and CD8+ secreting cells as well as tumor-specific cytotoxic response were generated.

A high surface expression of HSP-90 on EVs CTX and EVs C with a slightly significant difference between the exosomes was demonstrated. Exosome surface HSPs could potentiate the transportation of antigens and promotion of the maturation of dendritic cells or directly stimulate NK cells and macrophages [68–70]. Although exosome surface HSPs expression may promote interactions with and/or internalization by antigen-presenting cells, the rest of the exosomal content may determine the outcome of an immune response. Further study on how the unusual expression of HSP-90 may contribute to EVs CTX and EVs C reactivity with antigen-presenting cells or with natural killer cells will be of great value.

Overall this work demonstrated the feasibility of increasing the EVs secretion *in vitro* using cyclophosphamide. In addition, the exosomes obtained by this method retain their antigenicity inducing an immune response *in vivo* comparable to the one elicited with irradiated tumor cells or tumor lysates with the added advantage of being cell-free antigens.

On the other hand, it has already been demonstrated that pancreatic carcinoma cells treated with gemcitabine undergo significant enhancement of exosome secretion. This enhancement provides chemoprotection to subsequent toxicity and chemoresistance to gemcitabine [24]. The evidence of increased exosome secretion by tumor cells incubated with cyclophosphamide could have an implication for this drug's use in the treatment of patients with cancer since neoplastic cells are exposed to the drug in concentrations ranging from mere stressors to lethal for the tumor cells [30].

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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