



# Systemic administration of imiquimod as an adjuvant improves immunogenicity of a tumor-lysate vaccine inducing the rejection of a highly aggressive T-cell lymphoma

Paula Di Sciullo<sup>a,\*</sup>, Florencia Menay<sup>a</sup>, Federico Cocozza<sup>a</sup>, María José Gravisaco<sup>b</sup>,  
Claudia I. Waldner<sup>a</sup>, Claudia Mongini<sup>a,c</sup>

<sup>a</sup> Centro de Estudios Farmacológicos y Botánicos-Consejo Nacional de Investigaciones Científicas y Técnicas (CEFYBO-CONICET), Facultad de Medicina, Universidad de Buenos Aires, 2155 Paraguay, Ciudad Autónoma de Buenos Aires C1121ABG, Argentina

<sup>b</sup> Instituto de Biotecnología, Instituto Nacional de Tecnología Agropecuaria (INTA), Nicolas Repetto and de los Reseros, Buenos Aires 1686, Argentina

<sup>c</sup> Instituto de Virología, Instituto Nacional de Tecnología Agropecuaria (INTA), Nicolas Repetto and de los Reseros, Buenos Aires 1686, Argentina

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## ABSTRACT

T-cell lymphomas include diverse malignancies. They are rare, some have low survival rates and they lack curative therapies. The aim of this work was to assess whether employing the TLR7 agonist imiquimod and the T-cell costimulatory molecule CD40 or the combination of both as adjuvants of a cell lysate vaccine could enhance the antitumor immune response using a murine T-cell lymphoma model.

Immunization with LBC-lysate and imiquimod protected almost all vaccinated animals. A specific humoral and a Th1-type cellular immunity were induced in mice that rejected the lymphoma, characterized by an elevated number of CD4 + T-cells and secretion of IFN- $\gamma$ , locally and systemically. In contrast, CD40 alone or in combination with imiquimod did not improve the protective response obtained with LBC-lysate and imiquimod. Systemic administration of imiquimod proved to have high potential to serve as a vaccine adjuvant for the treatment of T-cell lymphomas and was effective in this immunotherapy model.

## 1. Introduction

T-cell lymphomas are a group of diverse histologic subtypes of non-Hodgkin neoplasms that represent 12 to 15% of all lymphoid tumors. They include very heterogeneous diseases such as T-lymphoblastic lymphoma/leukemia, angioimmunoblastic T-cell lymphoma, follicular T-cell lymphoma, anaplastic large-cell lymphoma, adult T-cell leukemia/lymphoma, extranodal lymphomas, and cutaneous T-cell lymphomas to name a few [1]. They can occur in children or adults, they can also localize systemically or in skin and can be fast or slow-growing depending on the type. These various factors will determine their aggressiveness. The aggressive types, even though they are infrequent, have a 5-year progression-free survival rate of only 31%–36%, which varies according to the presence of prognostic factors and respond poorly to chemotherapy. Currently, with standard treatment CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) or

similar schemes, remission is achieved in only half of patients. Some alternative therapies involve approaches of intensive chemotherapy or combined chemotherapy with autologous stem cell transplantation. However, they failed to improve response rates or showed limited benefits [2]. One reason for the poor outcome for these patients is the lack of new treatment strategies to improve survival, so immunotherapy using tumor vaccines appears to be a promising therapy.

Immunotherapies based on tumor cell lysates have proved to be effective vaccines in humans [3,4]. Immunological adjuvancy is a necessary tool in the field of tumor vaccines to enhance immunomodulatory pathways leading to functional response. To initiate an antitumor immune response and to generate effector and memory T-cells, APC<sup>2</sup> must undergo activation and maturation through recognition of danger signals. In this sense, direct stimulation of APC mediated by adjuvants would modulate the type of immune response elicited. One strategy to achieve this stimulation is through the interaction of

\* Corresponding author.

E-mail addresses: [pauladisciullo@yahoo.com.ar](mailto:pauladisciullo@yahoo.com.ar) (P.D. Sciullo), [cmongini@yahoo.com](mailto:cmongini@yahoo.com) (C. Mongini).

<sup>1</sup> Present address: Instituto Multidisciplinario de Investigaciones Biológicas San Luis (IMIBIO-SL-CONICET), Universidad de San Luis, 917 Chacabuco, San Luis 5700, Argentina.

<sup>2</sup> APC: antigen presenting cells.

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antigens with pattern-recognition receptors such as TLR<sup>3</sup> present in the APC.

To date, many specific agonists for TLR have been identified. However, whether these ligands induce an effective therapeutic anti-tumor response remains to be determined. A member of the imidazoquinoline family called imiquimod is a TLR7 agonist. Imiquimod is a nucleoside analogue that mimics the immune response to viral single-stranded RNA. TLR7 stimulation increases the production and secretion of proinflammatory cytokines, chemokines and adhesion molecules through activation of the transcription factor NF- $\kappa$ B [5]. Imiquimod induces, recruitment and transformation of plasmacytoid DC,<sup>4</sup> into cytotoxic plasmacytoid DC [6], stimulating the expression of costimulatory molecules. It also promotes a Th1-cellular response and the suppression of the Th2-pathway, enhances the activation of CD8+ T cytotoxic cells and macrophages, improves NK<sup>5</sup> cell activity, and induces proliferation and differentiation of B-cells [7]. As a topical formulation, imiquimod has been demonstrated to have an antiviral effect and it has been approved by US Food and Drug Administration (FDA) for the treatment of external genital and perianal warts, superficial basal cell carcinoma, and actinic keratosis [7]. Furthermore, anti-angiogenic [8] and proapoptotic effects on malignant keratinocytes, melanoma cells [9] [10], as well as T-lymphoma cells, both murine and human [11] have been demonstrated for this compound.

CD40 is one of the most important TCR<sup>6</sup> costimulatory molecules, which binds to CD154 (CD40L) found on activated T-cells, B-cells, NK, DC, monocytes, and granulocytes among others cells. The direct binding of CD40 to CD40L can regulate DC functions and T-cell activation, and both T-cell and DC signaling. CD40 triggering can also induce innate immune cells activation leading to antitumor mechanisms. An effective antitumor response must also be capable of stimulating the expression of adhesion and costimulatory molecules. T-cell activation requires two different signals from APC. The first one involves antigen presentation to the TCR in the context of MHC and the second signal needs the engagement of each costimulatory molecule with its ligand to avoid anergy. In previous works we have demonstrated that LBC tumor cells transfected with CD40 (LBC.CD40)<sup>7</sup> induced a significant increase in specific cytotoxic T-cell activity, IFN- $\gamma$  secretion, and significant tumor rejection after LBC.CD40 inoculation [12]. CD40 has also been used as a molecular adjuvant to enhance weak immunogenic vaccines, as is the case of DNA vaccines. CD40-CD40L interaction has been shown to enhance cellular and humoral immune responses to vaccination against HIV, hepatitis C, H5N1 influenza, and foot-and-mouth disease virus [13–16].

The aim of this study was to determine whether the use of imiquimod and CD40 (two adjuvants directed against two different immunological targets) could enhance the antitumor immune response elicited by a cell lysate vaccine in order to achieve tumor rejection in a murine model of T-cell lymphoma.

## 2. Material and methods

### 2.1. Cell line

The syngeneic BALB/c T-cell lymphoma cell line, LBC (H-2<sup>d</sup>) was maintained in RPMI 1640 (Gibco™, Invitrogen, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, CA, USA), 2 mM glutamine, 25 mM HEPES buffer, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.05 mM 2-mercaptoethanol, as previously described [17]. LBC.CD40 cells were established from LBC cells after transfection

with pcD-SR $\alpha$ -CD40 as previously described [12]. CD40 expression on LBC.CD40 was evaluated by flow cytometry before using them in the experiments.

### 2.2. Mice

Six to ten-weeks 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 procedures involving animals followed guidelines of Institutional Animal Care and Use Committee (CICUAL) of University of Buenos Aires Medical School.

### 2.3. Freeze-thaw lysate

LBC and LBC.CD40 cells were harvested and washed twice with PBS. Cells were then suspended in PBS ( $2 \times 10^6$  cells/ml) and lysed by means of 3 to 4 cycles of freezing at  $-70^\circ\text{C}$  and thawing at  $37^\circ\text{C}$ , vortexing after each round. Total cell death was confirmed by trypan blue dye exclusion. Protein concentration was determined by the Bradford method. LBC-lys<sup>8</sup> and LBC.CD40-lys<sup>9</sup> were then aliquoted and stored at  $-70^\circ\text{C}$  until used.

### 2.4. Reagents

Imiquimod (3M Pharmaceuticals R-837) was dissolved in sterile acidic deionized distilled water (pH 2.5) at a concentration of 1 mg/ml [18], sterilized by 0.22  $\mu$ m membrane filtration and diluted at working concentration in PBS. Corresponding control was identically prepared with the same volume of acidic deionized distilled water (pH 2.5) and PBS, but without imiquimod. The final pH value of imiquimod solution and control solutions used to inject the mice was always adjusted between 6.5 and 7.

### 2.5. Immunization and tumor challenge

Mice were randomly divided into six groups and vaccinated *i.p.*<sup>10</sup> once a week for 2 weeks. For each injection, each group of mice received a final treatment of one of the following: 0.5 ml of 100  $\mu$ g of LBC-lys proteins; 100  $\mu$ g of LBC-lys proteins mixed with 100  $\mu$ g of imiquimod; 100  $\mu$ g of LBC.CD40-lys proteins; 100  $\mu$ g of LBC.CD40-lys proteins mixed with 100  $\mu$ g of imiquimod; 100  $\mu$ g of imiquimod, or PBS alone as control. Seven days after the last immunization, animals were challenged with  $1 \times 10^6$  LBC tumor cells *i.p.*, a dose that has been determined to cause 90–100% lethality at  $21 \pm 4$  days after injection. Mice survival was monitored daily and the rate of mortality and survival were recorded. It has previously been determined in our laboratory that 100  $\mu$ g of imiquimod is the optimum dose to be used as adjuvant without any side effect in immunized mice.

To evaluate whether the mice had developed a memory response *in vivo*, the animals previously vaccinated that had rejected the first tumor challenge were re-challenged 80 days after the first tumor injection with  $1 \times 10^6$  LBC tumor cells *i.p.* The survival time of the animals was recorded daily. Survival curves from these treated mice were compared with survival curves from naïve mice of the same age inoculated *i.p.* with  $1 \times 10^6$  LBC cells at the same time.

### 2.6. In vitro specific T-cell amplification and T-cell cytotoxicity assay

Splenocytes ( $5 \times 10^6$ /ml) from naïve and immunized mice that rejected LBC lymphoma were harvested and stimulated *in vitro* with

<sup>3</sup> TLR: Toll-Like-Receptors.

<sup>4</sup> DC: dendritic cells.

<sup>5</sup> NK: natural killer cell.

<sup>6</sup> TCR: T-cell receptor.

<sup>7</sup> LBC.CD40: LBC tumor cells transfected with CD40.

<sup>8</sup> LBC-lys: LBC cell lysate.

<sup>9</sup> LBC.CD40-lys: LBC.CD40 cell lysate.

<sup>10</sup> *i.p.*: intraperitoneal.

$1 \times 10^5$ /ml irradiated LBC cells (3000 cGy) for 7 to 10 days at 37 °C in 5% CO<sub>2</sub>. Recombinant IL-2 (33 U/ml) (eBioscience, CA, USA) was added on days 2 and 5 of culture. Activated splenocytes were washed, counted and used as effector cells to analyze cytotoxic-induced DNA fragmentation measured by the Jam test [19]. Briefly, LBC cells were labeled with 5  $\mu$ Ci/ml of [<sup>3</sup>H] thymidine (NEN™, MA, USA) during 4 h at 37 °C, washed 3 times and used as target cells. Then,  $5 \times 10^3$  [<sup>3</sup>H] thymidine-LBC cells were cultured with effector cells for 4 h at different effector: target ratios (90:1, 60:1 and 30:1) in a round-bottom 96-well plate at 37 °C in 5% CO<sub>2</sub>. Cells were harvested on a PHD™ Sample Harvester (Brandel, MD, USA) and radioactivity was measured in a liquid scintillation beta counter (Tri-Carb 2800TR, Perkin Elmer). Induced and spontaneous releases were determined by incubating LBC target cells with effector cells or with culture medium alone, respectively. The percentage of specific lysis was calculated as [(spontaneous release – experimental release)/spontaneous release]  $\times$  100.

## 2.7. Immunofluorescence cell staining and flow cytometric analysis

Cells isolated from spleen or the peritoneal cavity of mice that had rejected the tumor, or splenocytes stimulated *in vitro* (as described above) were stained with different monoclonal antibodies and analyzed by flow cytometry. Cells from naïve mice were used as control. Briefly,  $5 \times 10^5$  cells were stained with specific FITC-conjugated anti-CD4 (L3T4) (clone GK1.5), FITC-conjugated anti-mouse/human CD45R (B220) (clone RA3-6B2), or PE-conjugated anti-mouse CD8b (Ly-3) (clone eBioH35-17.2) (eBioscience, CA, USA) in FACS buffer (PBS, 5% fetal bovine serum and 0.1% NaN<sub>3</sub>) for 30 min on ice. Isotype-matched murine IgG was used as control. Cells were then washed twice in a FACS buffer and fixed with 1% paraformaldehyde for 20 min. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA).

## 2.8. Measurement of cytokines by ELISA

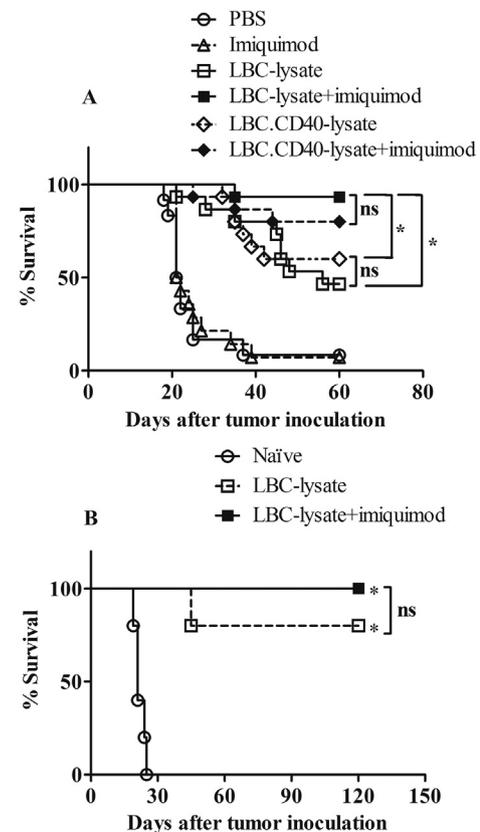
Splenocytes ( $5 \times 10^6$ /ml) or cells from the peritoneal cavity ( $2.5 \times 10^6$ /ml) obtained from naïve mice and from vaccinated mice that had rejected the tumor were stimulated in culture with  $1 \times 10^5$ /ml irradiated LBC cells (3000 cGy) for 48 h and supernatants were then collected. Concentrations of IFN- $\gamma$  and IL-4 were quantified using specific ELISA kits (eBioscience, CA, USA).

## 2.9. Dot blot

Sera were collected one day before the sacrifice, from naïve and immunized mice that had rejected the tumor and the presence of specific anti-LBC antibodies was evaluated by dot blot using LBC cells as antigens. Briefly,  $1 \times 10^4$  LBC cells/2  $\mu$ l PBS were loaded in duplicate onto strips of a nitrocellulose membrane (Hybond™-ECL™ Amersham Bioscience) and allowed to adsorb completely (about 3–5 min). Membrane strips were placed in 35 mm culture dishes and blocked overnight at 4 °C in blocking buffer (10% non-fat dry milk in TTBS solution: 100 mM Tris-HCl pH 7.5, 0.9% NaCl and 0.1% Tween 20). After washing with TTBS 3–4 times, strips were incubated overnight at 4 °C with serial dilutions of each serum. Membranes were then washed and incubated with a goat anti-mouse IgG-HRP antiserum (1/2000) (eBioscience, CA, USA) for 1 h at room temperature and revealed using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA). The immuno-recognition signals were acquired using ImageQuant ECL (GE Healthcare, Piscataway, USA).

## 2.10. Statistical analysis

Survival fractions were calculated using the product-limit Kaplan-Meier method and differences between treatments were evaluated by log-rank statistics. Data obtained by flow cytometry were analyzed



**Fig. 1.** Imiquimod enhanced the protective antitumor response elicited by the LBC-lysate vaccine. A) Mice were i.p. injected twice with either LBC-lysate with or without 100  $\mu$ g of imiquimod, LBC.CD40-lysate with or without 100  $\mu$ g of imiquimod, or PBS alone or with 100  $\mu$ g of imiquimod as control groups. Seven days after the last immunization mice received an i.p. tumor challenge with  $1 \times 10^6$  viable LBC cells and subsequently followed up for survival. The graph depicts a standard Kaplan-Meier survival curve analyzed by means of log-rank test. \*,  $p < .05$ . Ns, non-significant, ( $n = 12$ –15). B) Mice previously immunized with LBC-lysate, alone or with 100  $\mu$ g of imiquimod that had rejected first tumor challenge were i.p. injected with  $1 \times 10^6$  tumor LBC cells. The control group was made up of naïve animals of the same age that were i.p. inoculated with  $1 \times 10^6$  tumor LBC cells at the time of challenge. Survival was monitored and data were analyzed by log-rank test. \*,  $p < .05$  with respect to naïve control group. Ns, non-significant, ( $n = 5$ ). Data from two independent experiments are shown.

using CellQuest (Becton Dickinson, CA, USA) or Win.MDI (Windows Multiple Document Interface) Flow Cytometry Applications. For statistical comparisons, a one-way ANOVA with Tukey's post-test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Values of  $P < .05$  were considered significant.

## 3. Results

### 3.1. Tumor cell lysate vaccination using imiquimod as an adjuvant enhanced the protection from tumor growth

To evaluate the efficacy of tumor lysate vaccination using either imiquimod or CD40 as adjuvants, BALB/c mice were injected i.p. with LBC-lys, LBC.CD40-lys, LBC-lys + imiquimod or the combination LBC.CD40-lys + imiquimod twice every 7 days. The third week, the mice were challenged i.p. with  $1 \times 10^6$  viable LBC tumor cells, and survival was recorded daily. As shown in Fig. 1 A, all vaccines prolonged survival as compared to control non-immunized animals and to mice receiving only imiquimod ( $p < .001$ ). Imiquimod used as an

adjuvant in LBC-lys vaccine, markedly improved survival when compared with mice immunized with LBC-lys without adjuvant ( $p < .05$ ) and with mice that received LBC.CD40-lys ( $p < .05$ ). However, a combination of both adjuvants, imiquimod and CD40, did not improve survival compared to the use of imiquimod as the only adjuvant and compared to those animals who received only CD40 as adjuvant. While LBC-lys vaccine protected only  $46.7 \pm 13\%$  of mice and only  $60 \pm 13\%$  of the immunized with LBC.CD40-lys survived,  $93.3 \pm 6\%$  of animals immunized with LBC-lys + imiquimod remained free of any observable tumor at the end of the study (60 days after tumor cell injection). Surprisingly, the presence of CD40 did not improve the effect of imiquimod. No difference was observed when combining LBC.CD40-lys + imiquimod since  $80 \pm 13\%$  of mice were protected from tumor challenge. We studied protein expression of CD40 molecule in lysates of LBC cells transfected with pcD-SR $\alpha$ -CD40 plasmid. Western blotting indicated a protein of the expected mass (~45 kDa) as shown by SDS-PAGE analyses under reducing conditions (data not shown), confirming that CD40 protein expression was present in the lysate vaccine. However, no clinical effect was observed with CD40 used as an adjuvant of the immunization. All mice that have survived the tumor challenge were still alive after one year from the vaccination.

To further investigate if LBC-lys + imiquimod could induce a long-term antitumor immunity against a second parent tumor challenge, mice that had survived over 80 days after tumor cell injection and naïve mice that were used as control were inoculated with LBC cells *i.p.* Almost all mice that had been vaccinated and had rejected the first tumor challenge survived the lethal tumor re-challenge over 120 days, whereas 100% of mice in the control group died (Fig. 1 B).

### 3.2. Tumor cell lysate vaccination using imiquimod as an adjuvant induced a Th1-type as well as humoral immune responses against LBC cells

In order to investigate the immunomodulatory effect of imiquimod on LBC-lys vaccine in mice that had rejected the tumor, 47 days after the last booster we analyzed the immune response induced locally (in the peritoneum) and systemically (in the spleen). We first evaluated changes in lymphocyte subpopulations by flow cytometry. The addition of imiquimod to LBC-lys significantly induced an increase in the absolute number of CD4+ T-cells ( $p < .05$ ) in the peritoneum in comparison with the group immunized with LBC-lys (Fig. 2 A). Mice inoculated with LBC-lys + imiquimod developed splenomegaly with an increase in the absolute number of CD4+ T-cells ( $p < .05$ ) in spleen (Fig. 2 B). Moreover, the percentage of CD4+ T-cells primed *in vivo* was markedly amplified after co-culture *in vitro* splenocytes from immunized mice with irradiated LBC cells during 7 days, (Fig. 2 C). A significant difference was observed in specific CD4+ T-cells obtained from mice that received LBC-lys + imiquimod that were expanded *in vitro* ( $p < .05$ ) ( $33.5 \pm 3.5\%$ ) compared to those animals inoculated with LBC-lys ( $21.5 \pm 2.1\%$ ), to unimmunized mice (PBS group) ( $19.5 \pm 2.1\%$ ) and to mice that received imiquimod alone ( $21 \pm 2.8\%$ ). On the contrary, the percentage and absolute number of CD8+ T-cells, as well as B220+ cells (B-cells), did not change significantly in the peritoneum or in the spleen.

To further elucidate whether the vaccine elicited a Th1 or Th2-profile, the levels of IFN- $\gamma$  and IL-4 were determined. The levels of IFN- $\gamma$  in the supernatant of splenocytes obtained from mice immunized with LBC-lys + imiquimod that had rejected the tumor were higher than those obtained from mice immunized with LBC-lys, and from those obtained from naïve mice ( $p < .001$ , Fig. 3 A). Similar results were found when the local response was analyzed. The levels of IFN- $\gamma$  found in the supernatant of peritoneal cells obtained from mice inoculated with LBC-lys + imiquimod were higher than those found in either the supernatant of peritoneal cells obtained from mice immunized with LBC-lys alone; from naïve mice, or from unimmunized animals that had rejected tumor challenge ( $p < .001$ , Fig. 3 B). On the other hand, the vaccination did not elicit a higher production of IL-4 in any of the

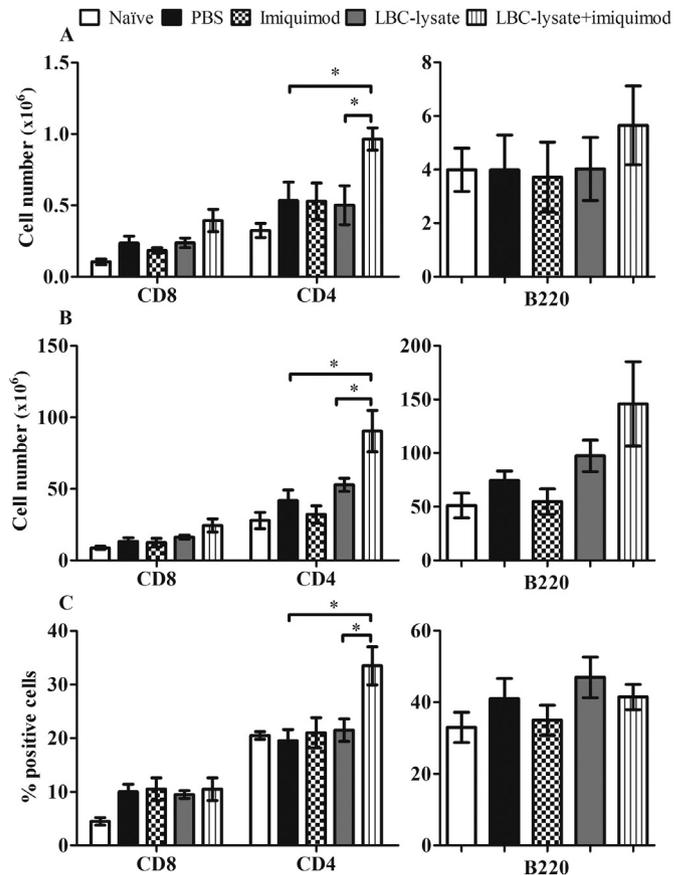


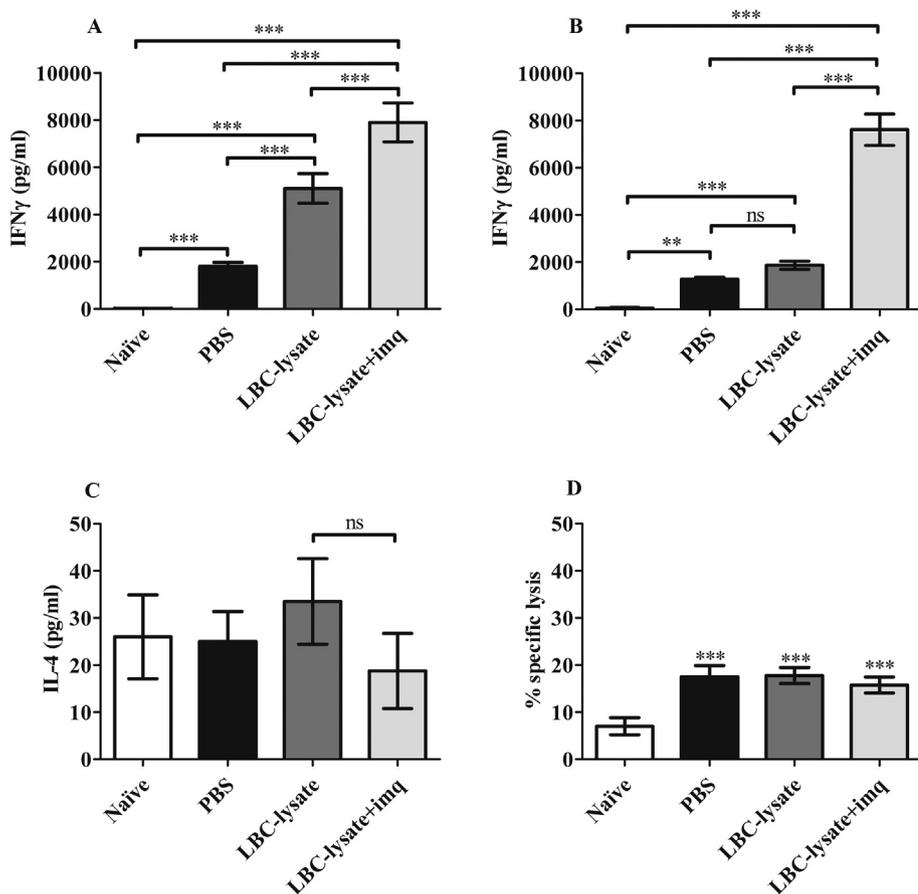
Fig. 2. Analysis of CD4+ and CD8+ T-cells and B220+ B-cells elicited by the LBC-lysate + imiquimod vaccine. Freshly isolated peritoneal cells (A) and freshly isolated spleen cells (B); or spleen cells *in vitro* re-stimulated for 7 days with irradiated LBC cells (C) were obtained from mice that had rejected LBC tumor and that received PBS, imiquimod alone or LBC-lysate with or without imiquimod (imq). Frequencies and cell number of the different populations were evaluated by flow cytometry. Graphs show positive cells present in the lymphocyte gate. \*,  $p < .05$  (One way ANOVA and Tukey's post-test,  $n = 3$ ). Bars represent mean  $\pm$  standard deviation. Data presented are from two independent experiments.

groups (Fig. 3 C). These data suggest that the vaccines induced a Th1-type pattern of cell-mediated immune response in the immunized mice that could have promoted the antitumor activity.

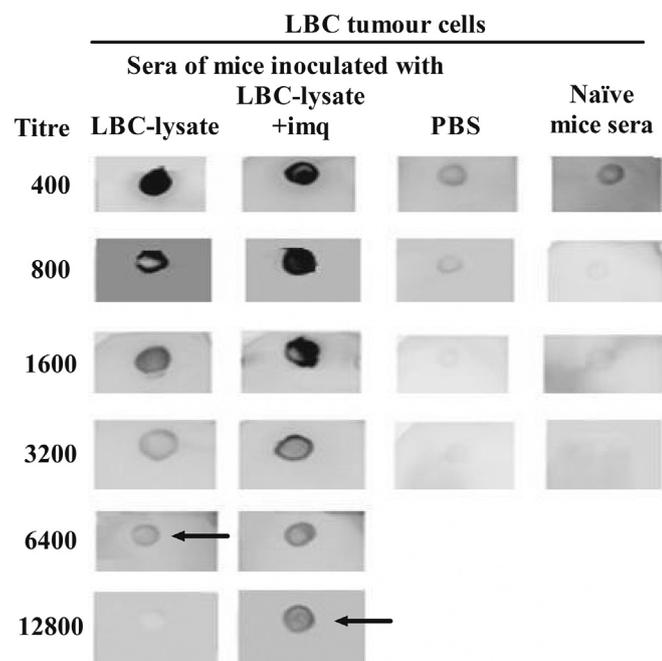
To determine whether vaccines elicited a specific cytolytic response, an *in vitro* cytotoxic assay was performed. From the three-different effector: target ratios evaluated (90:1, 60:1 and 30:1), the 90:1 ratio was the one that provided the best responses and is the one depicted in Fig. 3D. Surprisingly, the cell cytotoxicity induced against LBC in BALB/c mice inoculated with LBC-lys + imiquimod ( $15.7 \pm 1.7\%$ ) was not higher than that obtained from mice vaccinated with the LBC-lys alone ( $17.7 \pm 1.7\%$ ), even though there was a significant increase with respect to the naïve control ( $7 \pm 1.8\%$ ) ( $p < .001$ ). These results are in accordance with the fact that the number on CD8+ T-cells did not differ among the groups.

Thus, it can be concluded that the use of imiquimod as an adjuvant in LBC-lys vaccine increased the cellularity in spleen and peritoneum. This phenomenon could be attributed to a CD4+ T-cell expansion and to the high frequency of specific CD4+ T-cells in spleen.

Finally, to assess the humoral response, antibodies against LBC tumor cells were detected by dot blot in immunized mice that had rejected LBC lymphoma. The addition of imiquimod to LBC-lys vaccine generated a slightly higher humoral response (12,800 titer), than in animals immunized with the LBC-lys vaccine alone (6400 titer) (Fig. 4).



**Fig. 3.** Th1/Th2-cytokine profile and specific cytotoxic response generated by combination of the LBC-lysate with imiquimod. Splenocytes (A, C) and peritoneal cells (B) isolated from mice injected with PBS or immunized with LBC-lysate with or without imiquimod (imq) that had rejected tumor challenge were re-stimulated *in vitro* for 48 h with irradiated LBC cells and the secretion of IFN- $\gamma$  and IL-4 was determined in cell culture supernatants by ELISA. \*\*,  $p < .01$ . \*\*\*,  $p < .001$ . Ns, non-significant (One way ANOVA and Tukey's post-test,  $n = 4$ ). D) Specific cytotoxic activity of splenocytes from mice that received PBS or LBC-lysate with or without imiquimod that survived tumor injection, tested by the Jam test. The optimum effector/target ratio of 90:1 has previously been determined. \*\*\*,  $p < .001$  respect to normal control group (One way ANOVA and Tukey's post-test,  $n = 4$ ). Data represent mean  $\pm$  standard deviation and are from two independent experiments.



**Fig. 4.** The addition of imiquimod to the LBC-lysate vaccine induced a humoral immune response. Sera from mice that received PBS or LBC-lysate, alone or with imiquimod (imq) and had rejected tumor challenge were collected and incubated with  $1 \times 10^4$  LBC cells. Sera from naïve mice were used as control. The antibody titer was determined by Dot Blot as the inverse of the last dilution that was positive (indicated by arrows). Data presented are from one experiment that is representative of two independent experiments performed.

#### 4. Discussion

In this work, we demonstrated that imiquimod alone significantly enhanced the immunogenicity of the LBC cell lysate vaccine against the T-cell lymphoma, since it allowed nearly a 40% increase in the mice survival rate, achieving almost a complete tumor rejection for the overall group. Our results are in agreement with previous studies in which imiquimod proved to be efficient as adjuvant, exhibiting anti-tumor effects in a melanoma model of therapeutic and prophylactic vaccines [20], boosting immune response of an anti-melanoma peptide-vaccine [21] or reducing the incidence of a mammary tumor in comparison with the vaccination alone [22]. On the other hand, costimulatory molecules used as adjuvants have proved effective to eradicate cervical and lung tumors, even more effectively than some TLR agonists, as Sharma et al., demonstrated previously for a soluble form of 4-1BBL with a peptide vaccine [23]. However, in our model of lysate vaccine, the use of CD40 molecule as part of the lysate of LBC transfected cells, did not prolong the life of the animals more than the vaccine alone did. Furthermore, its combination with imiquimod did not synergize the effect of either of the two. Although the reasons for this are not clear, we hypothesized that the protein was functional in fact but the amount expressed of CD40 might have been insufficient to boost the immune response elicited by the vaccine.

The choice of a vaccine to treat a T-cell lymphoma was based on the fact that it could induce an adaptive immune response to tumor antigens that are easily available to immune system and the possibility to overcome the immunosuppressive tumor microenvironment due to the immunomodulation of the adjuvants. Tumor-cell lysate vaccines have advantages over other vaccines, such as those based on purified or recombinant antigens which allow DC to elicit an effective polyclonal immune response in patients with cancer providing a high number of immunogenic CD4+ and CD8+ T tumor epitopes. The need for this

immunotherapy lies in that some T-cell lymphomas can be quite aggressive, such as the one presented here, and have poor survival outcome. In this sense, imiquimod was chosen as adjuvant due to its capacity to act as a TLR7 agonist, activating transcription factors (like NF- $\kappa$ B) that induce expression of proinflammatory cytokines and chemokines. Thus, the antitumor effect of imiquimod would be mediated by improving the viability and the activation of DC, which modulates the immune response by the secretion of IFN- $\alpha$ , TNF- $\alpha$ , IL-2, IL-8, or IL-12, among others, that in turn stimulate T lymphocytes, NK cells and promote Th1-type immunity [7]. Furthermore, we chose the TCR-costimulatory molecule CD40 as adjuvant to stimulate activated specific helper T-cells, protecting them from apoptosis and consequently, increasing the positive signal on APC. Interaction between helper T-cells and APC is essential to enhance antigen presentation and to increase expression of costimulatory molecules on APC, in order to activate CD8+ T-cells and generate cytotoxic memory T-cells [24] thereby inducing an effective antitumor immune response. In our previous studies, the presence of CD40 lengthened survival of LBC tumor-bearing mice [12] and induced tumor rejection in the majority of the immunized mice with an irradiated whole tumor cells vaccine transfected with CD40 as adjuvant (manuscript in preparation). However, the use of a lysate of the tumor cells transfected with CD40 was not as effective to stimulate an antitumor immune response as the intact tumor cells transfected with CD40 and irradiated.

In this study, the effect of imiquimod was stronger than that of the molecular adjuvant CD40. For this reason, the systemic and local immune response was assessed only in mice that received the LBC-lys plus imiquimod vaccine. The immunological profile elicited by this vaccine in our model was essentially of the Th1-type, as we detected IFN- $\gamma$  but not IL-4 in the supernatant obtained from splenocytes and peritoneal cells derived from mice immunized and co-cultured with irradiated LBC cells. An accumulation of CD4+ T-cells in the peritoneum and specific CD4+ T-cells in the spleen of these animals was also observed, indicative of a local and systemic activation of the immune system. Notably, this vaccine induced a long-lasting immune response keeping the mice free of tumor over one year. A contribution of the humoral arm in the antineoplastic response could not be ruled out, as we demonstrated the induction of significantly high titers of serum anti-LBC antibodies. It is also noteworthy that in our *in vivo* experiments, no signs of clinical toxicity were observed in mice inoculated with imiquimod alone, those vaccinated with LBC-lys, or those that received a combination of LBC.CD40-lys and imiquimod. This fact reinforces the idea that imiquimod proved to be safe, administered by systemic route. A vaccine that included imiquimod that induced Th1 polarized response featuring an increase in IFN- $\gamma$  has been reported elsewhere previously [25]. In this case, the response has also been found to be accompanied by high titers of antibodies [22]. Unexpectedly, in the present study, the LBC-lys + imiquimod vaccination did not increase the CD8+ T-cell numbers and their cytotoxic activity *in vitro*. These results are consistent with a previous report where imiquimod, combined with a vaccine for metastatic melanoma increased CD4+ T-cell secretion of IFN- $\gamma$ , but did not increase CD8+ T-cell response [26]. Furthermore, the essential requirement of CD4+ T-cells and IFN- $\gamma$  production, rather than the CD8+ T-cells in rejection of tumors that do not express MHC II molecules, as the LBC lymphoma, has been described before [27]. The induction of a CD4+ T-cell response of the Th1-type, characterized mainly by the secretion of IFN- $\gamma$ , is important to achieve an optimal antitumor response. IFN- $\gamma$  is known to have a cytostatic/cytotoxic effect on tumor cells [28] to induce tumor dormancy [29], and to up-regulate MHC molecules as well as to alter the antigen-processing machinery [30]. IFN- $\gamma$  can also have immunomodulatory properties through the recruitment of innate immune cells, the activation of macrophages and the induction of chemokines secretion which recruit specific effector cells to the site of inflammation. IFN- $\gamma$  also regulates the activation, differentiation, and homeostasis of T-cells; it promotes Th1-profile [31], and induces the secretion of angiogenic chemokines by DC [27].

Together, these biological properties of IFN- $\gamma$  contribute to tumor rejection. The CD4+ T-cells also provide help to generate and maintain the specific CD8+ T-cell antitumor response [32] [33] and a tumor-specific memory CD8 T-cell population [24].

Considering these results, we hypothesize that imiquimod induces the activation of plasmacytoid and myeloid DC loaded with LBC antigens or even stimulates M1-macrophages through TLR7. These cells would promote a proinflammatory microenvironment through the secretion of chemokines and cytokines such as IL-12, IL-6, IL-8, TNF- $\alpha$ , IFN- $\gamma$ , IFN- $\alpha/\beta$ , G-CSF, or GM-CSF, among others. This fact would favor a CD4+ T differentiation towards a Th1-profile; T  $\gamma/\delta$ , NK, and CD8+ T-cells activation and proliferation and differentiation of B-cells. These changes appear to trigger an effective immune response capable of eliminating LBC tumor cells. In this model, we confirmed only an increment of CD4+ T-cells associated with a local IFN- $\gamma$  secretion in the peritoneum. However, it can be hypothesized that these mechanisms are also activated systemically, since the same results were obtained with splenocytes and in addition a humoral immune response was elicited as we found serum anti-LBC antibodies. Likewise, imiquimod might be exerting its effects through other mechanisms, such as the interaction with the adenosine receptor, which is known to be up regulated during inflammatory processes [34].

Our results demonstrate that imiquimod improved the anti-neoplastic effect of a lysate tumor vaccine as an adjuvant. These results also suggest that imiquimod acts as an APC stimulator. Nowadays, the usefulness of imiquimod in the treatment of skin tumors is being widely investigated; however, case reports about its efficacy in remission of clinical lesions in the treatment of lymphomas is scanty [35–40]. Besides, the effect of imiquimod as an adjuvant for tumor vaccines, is already being tested in phase I clinical trials with patients with glioma [41,42] and melanoma [26], but only as a topical formulation. While most studies report the topical administration of imiquimod, we demonstrated that it was also effective as a systemic application to treat lymphoma and consequently, it is not restricted to skin malignancies. The use of imiquimod as an adjuvant in tumor vaccines may also contribute with additional benefits. Previous works carried out in our laboratory have demonstrated that this compound has an anti-proliferative effect *in vitro* on LBC cells through a dose-dependent induction of apoptosis, at concentrations between 1 and 100  $\mu$ g/ml (Di Sciuillo and Mongini personal communication) as it has been reported to have an oncolytic effect on other tumor cells [9–11]. Furthermore, imiquimod was also found to stimulate the proliferation of splenocytes at low doses, as was reported by others [43,44]. The results presented herein could have clinical implications for the treatment of T-cell lymphomas since no reports were found using this type of tumor lysate vaccine. However, further studies evaluating other immunological pathways involved in the response, including regulatory T-cells or myeloid-derived suppressor cells, are required. The lack of targeting negative regulatory receptors on T-cells (PD-1, TIM-3, LAGE-3) and/or immune inhibitory cell populations in the tumor microenvironment could be a limitation of the tumor lysate vaccine with imiquimod. In this regard, the addition of another immunotherapy that modulates these immune checkpoint mechanisms could be necessary in order to overcome immune dysfunctions frequently present in cancer patients.

Imiquimod demonstrated to be a safe drug in this model when administered by the systemic route. Although it has been approved by the FDA only for topical use, it has been found to be effective when administered orally [45], but toxic at high doses [46]. In this sense, the administration of imiquimod as an adjuvant in low doses alone, or in combination with other therapeutic agents that allow the use of lower doses, could renew its applications.

## 5. Conclusions

To sum up, we investigated the antitumor effect of a tumor vaccine that included imiquimod and CD40 molecule as adjuvants and

evaluated their effectiveness in a murine T-cell lymphoma model. We have found that imiquimod administered alone significantly enhanced immune response to a tumor lysate vaccine and produced an elevated number of CD4<sup>+</sup> T-cells and an IFN- $\gamma$  Th1-type response along with specific antibodies.

Slow-growing T-cell lymphomas are generally indolent, but in later stages, patients can develop serious complications and there is no cure. Fast-growing T-cell lymphomas have a poor prognosis. For this reason, the discovery of new immune therapies not only can reduce doses of standard therapy but also it can kill remaining tumor cells. Some of them, like monoclonal antibodies, have achieved moderate efficacy [47]. Thus, the search for more effective treatments is needed. In this regard, we conclude that imiquimod could represent a promising adjuvant to be employed in antitumor vaccines for lymphomas, allowing the design of feasible immunization strategies, like tumor cell lysate vaccines, which usually are safe, inexpensive and easy to produce at large scale. These strategies could be carried out in combination with the standard therapy, not only to achieve an initial complete response, but also to prevent relapses through the induction of immunological memory.

#### Declaration of interest

All authors have declared there are no financial conflicts of interest in regard to this work.

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#### Authorship contributions

P.D. contributed with the study design, performing of experiments, analysis and interpretation of data, statistical analysis, and manuscript preparation. C.M. was the main investigator and contributed with study design, analysis and interpretation of data, statistical analysis, manuscript preparation, and edition. F.M. and F.C. performed some experiments. M.J.G. contributed with analysis and interpretation of flow cytometry data. C.I.W. contributed with the revision of manuscript. All authors read and approved the final manuscript.

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