Novel Adjuvant Based on the Pore-Forming Protein Sticholysin II Encapsulated into Liposomes Effectively Enhances the Antigen-Specific CTL-Mediated Immune Response

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Novel Adjuvant Based on the Pore-Forming Protein Sticholysin II Encapsulated into Liposomes Effectively Enhances the Antigen-Specific CTL-Mediated Immune Response

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Vaccine strategies to enhance CD8+ CTL responses remain a current challenge because they should overcome the plasmatic and endosomal membranes for favoring exogenous Ag access to the cytosol of APCs. As a way to avoid this hurdle, sticholysin (St) II, a pore-forming protein from the Caribbean Sea anemone Stichodactyla helianthus, was encapsulated with OVA into liposomes (Lp/OVA/StII) to assess their efficacy to induce a CTL response. OVA-specific CD8+ T cells transferred to mice immunized with Lp/OVA/StII experienced a greater expansion than when the recipients were injected with the vesicles without St, mostly exhibiting a memory phenotype. Consequently, Lp/OVA/StII induced a more potent effector function, as shown by CTLs, in vivo assays. Furthermore, treatment of E.G7-OVA tumor-bearing mice with Lp/OVA/StII significantly reduced tumor growth being more noticeable in the preventive assay. The contribution of CD4+ and CD8+ T cells to CTL and antitumor activity, respectively, was elucidated. Interestingly, the irreversibly inactive variant of the StI mutant StI W111C, encapsulated with OVA into Lp, elicited a similar OVA-specific CTL response to that observed with Lp/OVA/StII or vesicles encapsulating recombinant StI or the reversibly inactive StI W111C dimer. These findings suggest the relative independence between StII pore-forming activity and its immunomodulatory properties. In addition, StII induced in vitro maturation of dendritic cells might be supporting these properties. These results are the first evidence, to our knowledge, that StII, a pore-forming protein from a marine eukaryotic organism, encapsulated into Lp functions as an adjuvant to induce a robust specific CTL response. The Journal of Immunology, 2017, 198: 2772–2784.

A challenge in the field of vaccinology is the enhancement of cellular immune responses mediated by Ag-specific CD8+ CTLs. Activation of naive CD8+ T lymphocytes and polarization toward effective CTLs requires presentation of MHC class I–peptide complexes by APCs (signal 1), together with costimulation (signal 2) and the presence of cytokines (signal 3), such as IL-12 (1) and type I IFNs (2). Dendritic cells (DCs) are APCs that activate naive T cells (3) and process exogenous Ags for presentation to CD8+ T cells. This event, termed cross-presentation, is crucial for the generation of CTLs in response to tumors or intracellular pathogens (4). Pathogen-associated molecular patterns and other signals, such as inflammatory cytokines, induce the maturation of DCs, triggering their ability to activate T cell responses (3). Induction of tumor-specific CD8+ T cells is an appealing therapeutic strategy because the generated CTLs mediate Ag-specific killing of the targeted tumor via cell–cell contacts, as well as provide the host with long-lasting memory responses that may prevent cancer recurrence (5).

Liposomal vesicles can promote Ag delivery to the cytosol of APCs and subsequently enhance a CTL response, but not effectively.
Different strategies based on liposomal vesicles were designed with the intention of streamlining these functions: examples include acidic pH-sensitive liposomes (Lp) (6, 7), cationic Lp (8, 9), and the inclusion of immunomodulators, such as TLR ligands (10, 11). Liposomal preparations including membranotropic peptides and bacteria pore-forming proteins (PFPs) also were investigated as novel cytosol-delivery systems for cross-priming cytotoxic CD8⁺ T cells (12, 13). Particularly, encapsulation of listeriolysin O (LLO), a cholesterol (Cho)-dependent PFP from the Gram-positive intracellular pathogen Listeria monocytogenes, with Ags in pH-sensitive Lp significantly enhanced functional and protective CTL responses (14–16).

Sticholysins (Sts) I and II are PFPs from the Caribbean Sea anemone Stichodactyla helianthus belonging to a protein family known as actinoporins (17). These cysteine-less and basic proteins of 20 kDa, exhibiting high structural similarity, are able to permeabilize cellular and membrane structures by forming oligomeric pores of 2 nm diameter (18–20). Their permeabilizing activity is significantly enhanced by the presence of sphingomyelin (SM) (21, 22). The pore-forming activity of Sts makes these proteins attractive tools for cytosolic Ag delivery and enhancement of CTL immune responses.

In this study, we assessed the ability of Sts encapsulated into Lp with OVA to enhance an Ag-specific CTL response and their functionality in a murine tumor model. The liposomal formulation containing StI was able to induce a powerful OVA-specific CTL immune response, independent of CD4⁺ T lymphocytes, under the experimental conditions assessed. Furthermore, a strong antitumor response in a preventive or therapeutic scenario was observed in animals immunized with these Lp and challenged with OVA-expressing E.G7-OVA tumor cells. This response was partially abrogated by depleting CD8⁺ T cells. Interestingly, by manipulating the structure of StI, its pore-forming activity and ability to stimulate CTL immune responses were decoupled. Moreover, StII induced the maturation of DCs in vitro, which may support its immunomodulatory properties.

**Materials and Methods**

**Proteins, reagents, and tumor cells**

StI (Swiss Protein Data Bank P07845) was purified from the sea anemone *S. helianthus* and characterized as described by Lania et al. (18). Recombinant StI (rStI) and its mutant StI W111C were obtained according to the procedures described by Pazos et al. (23) and Penton et al. (24), respectively. The protein concentration was determined using absorption coefficients of 1.87, 2.13, and 1.65 mg/ml/cm at 280 nm for StI, rStI, and StI W111C, respectively (18, 24), and their cytolytic activities were monitored by hemolysis assay. These proteins were stored at −20°C until use. The reagent bis(maleimido)hexane (BMH) was purchased from Pierce (Rockford, IL). Chicken OVA grade V, polyinosinic-polycytidylic acid (polyI : polyC), and CFSE were obtained from PeproTech (Rocky Hill, NJ). CFSE was acquired from Invitrogen (Paisley, U.K.). Proteins, reagents, and tumor cells were mixed with their corresponding labeled proteins that were entrapped in Lp/OV A/Sts, and Lp/OV A/Sts formulations were prepared. After centrifugation, the encapsulation percentage was calculated from radioactivity measurements (cpm) of total supernatant (TSN) and the final pellet (SP). The encapsulation efficiency (EE%) was calculated as EE% = [FP cpm/TSN] × 100. Liposomal formulations were kept at 4°C dissolved in PBS, and small aliquots were taken at different times to measure the total radioactivity (T) and the supernatant radioactivity after centrifugation (SN). Retention percentage was calculated as (T cpm – SN cpm) × 100/T cpm. Liposomal preparations were used immediately after reconstitution in all experiments.

To determine the presence of OVA or StI at the liposomal surface, Lp/OV A, Lp/StII, and Lp/OV A/StII preparations (0.962 μmol lipids/ml) were entrapped with mouse anti-OVA (20 μg/ml) or rabbit anti-StI (10 μg/ml) polyclonal Ab. After a 1-h incubation at 37°C with the respective antisera, samples were washed three times with PBS and mixed with secondary anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) or rabbit anti-IgG (Jackson ImmunoResearch, West Grove, PA) horseradish peroxidase-coupled IgG (1:100). Western blot analysis was performed as described (25).

**Entrainment of OVA and Sts in Lp: characterization of vesicles**

The procedure rendering dehydration–rehydration vesicles (DRVs) (27) was used to obtain multimamellar Lp encapsulating OVA with one of the following variants of Sts: StI, rStI, or StI W111C, or rStI W111C (hereafter, the slash notation used with Lp indicates encapsulating). In brief, small unilamellar vesicles composed of DPPC (16 μmol) and an equimolar amount of Cho were prepared using ultrasonication as described in Materials and Methods. The liposomal formulations were kept at 4°C dissolved in PBS, and small aliquots were taken at different times to measure the total radioactivity (T) and the supernatant radioactivity after centrifugation (SN). Retention percentage was calculated as (T cpm – SN cpm) × 100/T cpm. Liposomal preparations were used immediately after reconstitution in all experiments.

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**Hemolytic activity assays**

The hemolytic activity of Sts was assessed, as previously described (29), using a microplate reader (Multiskan EX; Labsystems, Helsinki, Finland). Bovine RBCs were resuspended in TBS (150 mM NaCl, 10 mM Tris-HCl [pH 7.4]), and the concentration of the cell suspension was adjusted with TBS to an apparent absorbance of 0.1 at 650 nm. Proteins were serially diluted 2-fold in saline TBS using flat-bottom 96-well microplates, and

**Production of irreversible inactivated dimeric variant from the mutant StI W111C**

An irreversibly inactive dimer of the mutant StI W111C was obtained by cross-linking the monomeric variant of StI W111C with the homo-bifunctional reagent BMH. First, StI W111C was reduced by incubation with 0.1 M M-2-ME for 30 min at 25°C, followed by filtration in a PD-10 column (GE Healthcare, Uppsala, Sweden) equilibrated with PBS containing 5 mM EDTA to eliminate 2-ME. BMH dissolved in DMSO (Sigma-Aldrich) was immediately added to StI W111C at a 2:1 protein/BMH molar ratio, and the mixtures were incubated for 2 h at 4°C with constant stirring. StI W111C was purified using a Superdex 75 HR 10/30 column (Amersham Biosciences, Uppsala, Sweden) by fast protein liquid chromatography (Pharmacia LKB, Uppsala, Sweden) with PBS at a linear flow of 0.382 cm/min after previous incubation with 0.1 M M-2-ME (reducing agent). SDS-PAGE (26), under reducing conditions, was used to verify production of the irreversible dimer and its purity. StI W111C spontaneously forms a reversibly inactive dimer of the mutant StI W111C stabilized by a disulfide bridge (24) (memStI W111C).
human RBCs were added to each well (200 µl final volume). Hemolytic activity determination of preStI W111C or postStI W111C variants was carried out in the presence or absence of 0.1 M 2-ME.

Permeabilization assays
The permeabilizing activity of StII was assessed by analyzing CF leakage from large unilamellar vesicles (LUVs) comprising DPPC:Cho or PC:SM (1:1 molar ratio) in TBS, as described by Tejeda et al. (21). Phospholipid concentration was determined as previously described (30), and Cho concentration was determined using a commercial kit (cat. no. 1.14830.0001; Merck, Darmstadt, Germany). A FLUOSTAR OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany) was used to measure fluorescence (λex = 490 nm and λem = 520 nm). Each well of the microplate (SPL-Life Sciences, Seoul, South Korea) was filled with 200 µl of TBS plus 0.2 µM LUV. StII was added at final concentrations of 2.5–1400 nM. The fraction of vesicles with at least one pore (f) was calculated as: f = (Ff − F0)/(Fmax − F0), where F0 and Ff represent the fluorescence values before and after adding FFP, respectively, and Fmax is the fluorescence measured in the presence of 0.1% Triton X-100 (Merck).

Mice and immunization schedule
Female C57BL/6 mice (H-2b) were obtained from the National Center for Laboratory Animals Production (Havana, Cuba). CD45.2+ OT-1 (CD8+ T cell TCR-transgenic mice expressing the TCR recognizing SIINFEKL in H-2Kb) and CD45.1+ Ly5-congenic mice on the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were kept in specific pathogen-free conditions at the Center of Molecular Immunology (CIM) animal facilities and were used at 6–8 wk of age. All procedures were conducted in accordance with the protocols approved by the CIM Institutional Committee for the Care and Use of Laboratory Animals (01/07/2008).

Immunizations were performed s.c. or i.m., in the inferior right limb, by delivering 0.2 or 0.1 ml of liposomal preparation, respectively, per mouse. Two injections of Lp/OVA or Lp/OVA/Sts were given at a 12-d interval. Mice were immunized with three doses of the Lp formulations at 1-wk intervals.

Flow cytometry analysis
Cells were stained with specific Abs using conventional protocols. Lymphocyte characterization was performed with the following specific anti-mouse mAbs: CD8/allophycocyanin, CD8/PE, CD4/FITC, CD3/PE/C575, CD62L/FITC, CD45.2/PE, and CD44/PE/C575 (all from eBioScience, San Diego, CA). Cells were acquired using FACScan (BD Biosciences, San Jose, CA) and Gallios (Beckman Coulter, Miami, FL) flow cytometers and analyzed with FlowJo 7.6.1 (TreeStar) software. In BMDC maturation assays, cells were stained with anti-mouse CD11c/PE, CD86/allophycocyanin, CD40/FITC, CD80/FITC, CD80/PE (all from eBioScience), anti-mouse CD11c/allophycocyanin, and CD40/PE Texas Red (both from BD Biosciences). A LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher, Waltham, MA) was used to exclude dead cells. Stained cells were detected using a FACSQuant (BD Biosciences) flow cytometer and analyzed with FlowJo 7.6.1 software. Doublets were excluded from total acquired cells by the analysis of forward scatter (FSC)-area versus FSC-height parameters.

Ag-specific CTL assay in vivo
Total splenocytes of naive C57BL/6 mice were pulsed or not (internal control) with 1 µM SIINFEKL for 60 min at 37°C and 5% CO2. Peptide-pulsed target cells were washed extensively to remove free peptide. Subsequently, target cells were labeled with the fluorescent dye CFSE (CFSE<sup>light</sup>, 5 µM) for 5 min at 37°C and washed twice. Unpulsed cells were simultaneously labeled with the fluorescent dye CFSE (<sup>dark</sup>, 0.33 µM). Cells were mixed at a 1:1 ratio, and 60 × 10<sup>4</sup> cells were injected i.v. into immunized mice on day 20. Sixteen hours later, mice were sacrificed, the inguinal LN closest to the site of immunization was removed, and the total events responsive to the peptide were determined by flow cytometry. The percentage lysis for each well was calculated as: percentage lysis = 100 − (CFSE<sup>light</sup>−h<sub>lum</sub> × 100)/(CFSE<sup>dark</sup><sub>untreated</sub>−h<sub>lum</sub>). Experimental groups (three mice per group) received s.c. PBS, OVA/PIC, Lp/OVA, or Lp/OVA/Sts, as indicated above. In some experiments, depletion of CD4<sup>+</sup> T cells was carried out by i.p. injection of a specific mAb (anti-CD4, 1 mg) for 4 d, starting 1 d before the first immunization. An immunization control group received PBS instead of anti-CD4 mAb. Depletion was checked by flow cytometry.

Tumor challenge assays
Mice (6–10 per group) were immunized s.c. or i.m. with PBS (control), Lp/OVA, or Lp/OVA/StI, as described above. In some experiments, a group of mice received half a dose of OVA, as well as half of the corresponding total lipids and StII. On day 7 postboost, animals were challenged by s.c. injection of 3 3 × 10<sup>6</sup> E.G7-OVA cells in 200 µl of DMEM-F12 into the right flank. Tumor growth was monitored every 2–3 d by measuring the two largest perpendicular diameters with a caliper, and the tumor volume (v) was calculated (v = π/6 × length × width<sup>2</sup>). Mice with one diameter ≥ 2 cm were euthanized following the guidelines of the CIM Institutional Committee for the Care and Use of Laboratory Animals (01/07/2008), and survival was scored as the percentage of surviving animals. The percentage of mice without tumor was also recorded. An antitumor assay was also carried out using a specific mAb to deplete the CD8<sup>+</sup> T cell population (anti-CD8). In this case, 1 mg of this mAb was injected i.p. into immunized mice 1 d before tumor implantation. This dose was shown to deplete >95% of CD8<sup>+</sup> T cells (33). An immunization control group received PBS instead of anti-CD8 mAb. The immunotherapeutic effect of vaccination was also assessed by challenging mice with tumor at day 0 and 3 d after they were immunized with three doses of the Lp formulations at 1-wk intervals.

Analysis of OVA- and StII-specific Ab titers
OVA- and StII-specific IgG titers in serum were measured by ELISA using OVA or StII (10 µg/ml)-coated plates (Greiner Bio-One, Frickenhausen, Germany). Mice sera diluted 1:100 were added to wells in triplicate and further serial dilutions were performed. Specific Abs were detected with alkaline phosphatase–conjugated goat anti-mouse total IgG (Sigma-Aldrich) or biotinylated goat anti-mouse IgG1 or IgG2c Abs (AbD Serotec, Oxford, UK), followed by alkaline phosphatase–conjugated streptavidin (Sigma-Aldrich) in the two last determinations. The absorbance was read at 405 nm using p-nitrophenyl phosphate (Sigma-Aldrich) as substrate.

Statistical analysis
The normal distribution of data was analyzed by the Kolmogorov–Smirnov test, followed by the Bartlett or Levene test to check variance homogeneity.
neity. The means from two independent samples were compared using the unpaired t test (two-tailed) or Mann–Whitney U test. Statistically significant differences between means from three or more unmatched groups were determined using one-way ANOVA or the Kruskal–Wallis test. The Tukey, Dunn test, or Dunn test was used for comparison of multiple means. Survival and tumor-free mice percentages were analyzed using the Kaplan–Meier estimation, followed by the log-rank test. Statistical analyses were conducted using GraphPad Prism 5, version 5.01 (GraphPad) software, with the exception of the Dunnett T3, Kaplan–Meier, and log-rank tests, which were performed using the statistical software package SPSS version 16.0. p < 0.05 was considered statistically significant.

Results

Lp comprising DPPC:Cho are suitable for encapsulating the PFP StII

Sts readily bind to model membrane systems, such as lipidic monolayers, micelles, and lipid vesicles (21, 22, 34–36). These PFPs exhibit high irreversible binding affinity for SM-containing membranes, and the presence of this lipid significantly enhances their pore-forming ability (21, 22). Considering the lipid requirement for the permeabilizing activity of Sts, we evaluated the ability of StII, the most active of Sts (29), to generate pores in DPPC:Cho vesicles by measuring CF efflux from LUVs upon PFP addition (Fig. 1). As expected, the fraction of the PC:SM vesicles permeabilized at the steady-state (fmax, fluorescence achieved at 30 min) increased as a function of StII concentration, exhibiting activity in the nanomolar concentration range (22, 29). In contrast, the permeabilization of DPPC:Cho vesicles by StII was nearly negligible, even at protein/lipid ratios considerable superior to those used in the DRVs formulation (see Materials and Methods) (Fig. 1). Taken together, these results show that DPPC:Cho in a 1:1 molar ratio is an acceptable lipid composition to achieve the encapsulation of Sts together with OVA as a model Ag.

The DPPC:Cho DRVs exhibited a relative high efficacy of encapsulation for StII (52 ± 8%), retaining >70% of this protein after 28 d of storage in suspension at 4°C (Supplemental Fig. 1). In the formulations containing or not containing StII, OVA was encapsulated with similar efficacy (48 ± 7% and 57 ± 10%, respectively) and was similar to that observed for StII (p > 0.05). The retention capacity of DPPC:Cho Lp for OVA (>40%) also was independent of the presence of StII, even after a long incubation period (28 d) at 4°C (Supplemental Fig. 1). The similarity in the OVA encapsulation and retention in Lp in the presence or absence of StII indicates that this protein does not affect Ag entrapment in these vesicles. Although StII was retained inside Lp more efficiently than OVA over the storage time, it is interesting to note that both proteins had a similarly high retention percentage at the first week of incubation (>80%, Supplemental Fig. 1). The higher retention for StII could be related to the ability of this PFP, although very limited, to bind but not destabilize lipid membranes containing PC and Cho (22, 37).

We also evaluated the presence of OVA and StII on the liposomal surface by flow cytometry using specific polyclonal Abs against each protein. Dot plot graphs representative of each liposomal sample illustrate the proportion of vesicles carrying at least one molecule of StII and OVA at the liposomal surface (Fig. 2A). Several liposomal formulations were analyzed, and the percentage of the vesicles positive to labeling for each protein on the surface are shown in Fig. 2B and 2C. The fraction of Lp/OVA vesicles carrying OVA on their surface was similar to that of Lp/OVA/StII vesicles (p = 0.471), and comparable behavior was observed for Lp/StII and Lp/OVA/StII vesicles (p = 0.459) with respect to StII.

FIGURE 1. StII does not show permeabilizing activity on liposomal vesicles composed of DPPC:Cho (1:1). LUVs comprising DPPC:Cho or PC:SM (1:1 molar ratio) encapsulating CF were exposed at different StII concentrations (2.5–1400 nM). LUVs were diluted in 10 mM Tris-HCl, 140 mM NaCl (pH 7.4) to reach a final lipid concentration of 0.2 μM. Vesicles of PC:SM were used as positive control. Excitation wavelength: 490 nm. Emission wavelength: 520 nm. The experiments were carried out at room temperature. The fraction of permeabilized vesicles (f) at a fixed time was estimated as: f = (Ff – F0)/(Fmax – F0), considering that release of fluorescent probe is simultaneous to pore formation, where F0 and Ff are fluorescence values before and after adding StII, respectively, and Fmax is the fluorescence measured in the presence of 0.1% Triton X-100. Data are from a single experiment representative of two independent experiments.

FIGURE 2. Presence of OVA and StII on the surface of DPPC:Cho Lp. Freshly prepared DRVs of DPPC:Cho (1:1) empty (Lp), encapsulating OVA (Lp/OVA), and encapsulating StII with or without OVA (Lp/OVA/ StII, Lp/StII) were incubated with polyclonal Abs from mice and rabbit specific to OVA or StII, respectively. Subsequently, vesicles were treated with an FITC-conjugated Ab anti-mouse IgG or an FITC-conjugated anti-rabbit IgG and examined by flow cytometry. (A) A representative side scatter-area (SSC-H) versus FITC–anti-IgG dot plot graph of each formulation is shown. Asterisks indicate the protein assessed in each case. Mean (± SD) vesicle percentage from different liposomal preparations (n = 6) exposing OVA (B) or StII (C) at the surface. No significant differences (ns) were detected according to the two-tailed unpaired t test (p > 0.05). Data shown are from a single experiment representative of two experiments yielding comparable results.
Furthermore, we measured the size and polydispersity of DRVs carrying OVA, with or without StII, and observed relatively high sizes for both particles (1.6 ± 0.07 and 2.2 ± 0.04 μm for Lp/OVA and Lp/OVA/StII, respectively), in agreement with the previously reported size for this type of Lp (diameters ranging from 0.1 to 2 μm) (38, 39). The entrapment of OVA alone or together with StII into the Lp increased the particle sizes in comparison with empty Lp (data not shown). Additionally, Lp/OVA and Lp/OVA/StII preparations also showed significant differences in particle size (unpaired t test, p = 0.0016), which could result from the necessary changes in vesicle volume to better accommodate the entrapped solutes. In accordance with the observed sizes, vesicles also exhibited relatively high polydispersity indexes, albeit those with larger size showed smaller indexes (Lp/OVA/StII 0.24 ± 0.03 and Lp/OVA 0.4 ± 0.02; p = 0.0056, unpaired t test). The high and low isoelectric point of StII (pI > 9.0) (18) and OVA (pI = 4.5) (40), respectively, could favor the electrostatic interaction among these proteins inside Lp and contribute to the compactness and, hence, homogeneity of the vesicles. Lanio et al. (35) explained the interaction of Sts with anionic surfactants by their high isoelectric points and a preferential electrostatic binding at the working pH (7.4). A similar event could be occurring in this case.

**DPPC:Cho Lp containing StII expand OVA-specific CD8+ T cells with memory phenotype and enhance OVA-specific CTL responses in vivo independent of CD4+ T cell help**

To evaluate the ability of the Lp/OVA/StII formulation to activate CD8+ T lymphocytes in vivo, we first studied the effect of Lp/OVA/StII versus Lp/OVA on the population of OVA-specific CD8+ T cells in the draining LN. For this purpose, we adoptively transferred CD8+ T lymphocytes from OT-1 mice (CD45.2+) into Ly5 mice (CD45.1+). Two days later, Ly5 mice (n = 3) were immunized s.c. twice (12-d interval) with Lp/OVA/StII (50 μg OVA and 6.25 μg StII), Lp/OVA (50 μg OVA), or PBS (as negative control). Seven days after the second immunization, the inguinal LN closest to the inoculation site of each mouse was removed and homogenized, and CD8+ CD45.2+ cells were analyzed by flow cytometry. (A) Each contour graph, with the respective cell percentages in each quadrant referred to the lymphocyte gate, corresponds to an individual animal representative of each group. (B) Percentage (mean ± SEM) of CD8+ CD45.2+ cells for each treatment. (C) Contour graphs showing the percentage of CD44high CD62L− cells (CD8+ memory cells) in relation to the lymphocyte gate of an individual mouse representative of each immunized group. Graphs are from the CD8+ CD45.2+ gate. (D) Percentage (mean ± SEM) of CD44high CD62L− cells for each group. Data in (B) and (D) are from a meta-analysis of two independent assays. *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed unpaired t test.

The results indicate that OVA and StII are exposed in an equivalent proportion of vesicles (~25%) without influencing each other. These results are in agreement with the efficiency of encapsulation and retention for both proteins (Supplemental Fig. 1) and suggest that StII and OVA do not seem to compete with each other during vesicle preparation.

**FIGURE 3.** Immunization with StII coencapsulated into the DPPC:Cho Lp with OVA induces significant expansion of OVA-specific CD8+ T lymphocytes. Splenocytes (25 × 106 equivalent to 5.5 × 106 OVA-specific CD8+ T cells) from OT-1 mice (CD45.2+) were transferred i.v. into Ly5 mice (CD45.1+). Two days later, Ly5 mice (n = 3) were immunized s.c. twice (12-d interval) with Lp/OVA/StII (50 μg OVA and 6.25 μg StII), Lp/OVA (50 μg OVA), or PBS (as negative control). Seven days after the second immunization, the inguinal LN closest to the inoculation site of each mouse was removed and homogenized, and CD8+ CD45.2+ cells were analyzed by flow cytometry. (A) Each contour graph, with the respective cell percentages in each quadrant referred to the lymphocyte gate, corresponds to an individual animal representative of each group. (B) Percentage (mean ± SEM) of CD8+ CD45.2+ cells for each treatment. (C) Contour graphs showing the percentage of CD44high CD62L− cells (CD8+ memory cells) in relation to the lymphocyte gate of an individual mouse representative of each immunized group. Graphs are from the CD8+ CD45.2+ gate. (D) Percentage (mean ± SEM) of CD44high CD62L− cells for each group. Data in (B) and (D) are from a meta-analysis of two independent assays. *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed unpaired t test.

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with 100 m positive controls, respectively. Eight days after the second immunization, 100 (CFSEbright/CFSEdull)vaccinated were determined by FACS. The percentage of specific lysis was calculated as

\[
L_p/OV A/StII (50 \mu g) \times \frac{100}{n = 3}
\]

Results

The inclusion of StII into DPPC:Cho Lp containing OV A en-


cross-presentation.

(45). This experiment demonstrates that, even in the absence of

CD8+ T cells induced by Lp/OV A/StII formulation in the pre-

response. In agreement with the expansion of the OV A-specific

CD8+ T cells after 24 h (19% for PBS group versus 1% for treated

animals) (Fig. 4C). Because a partial recovery was detected at day 4

(8%), we treated animals with the mAb at 4-d intervals during the

experiment. Groups immunized with Lp/OV A/StII exhibited similar

lysis percentages whether or not CD4+ T cells were depleted (66.8

and 74.7%, respectively, p = 0.559) (Fig. 4D). This behavior was

similar to that observed in the group receiving OV A/PIC (p = 0.840),

which was used as control based on the previous demonstration that

induction of CTLs by PIC was independent of CD4+ Th cells (44–46).

To evaluate whether the CTL response induced by the Lp/OV A/StII adjuvant system depends on CD4+ T cell help, we

depicted CD4+ T cells by systemic treatment with an anti-CD4 mAb.

Mice injected with PBS were used as a control group. The effec-

tiveness of depletion was checked by analyzing the residual presence

of CD4+ T cells after 24 h (19% for PBS group versus 1% for treated

animals) (Fig. 4C). Because a partial recovery was detected at day 4

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lysis percentages whether or not CD4+ T cells were depleted (66.8

and 74.7%, respectively, p = 0.559) (Fig. 4D). This behavior was

similar to that observed in the group receiving OV A/PIC (p = 0.840),

which was used as control based on the previous demonstration that

induction of CTLs by PIC was independent of CD4+ T cell help

(45). This experiment demonstrates that, even in the absence of

CD4+ T cells, immunization with Lp/OV A/StII is able to induce high

levels of target cell lysis (∼70%).

OVA-specific Ab titer was measured from immunized mice

serum at the same time as the CTL assays to investigate whether

the inclusion of StII in DPPC:Cho Lp is also able to provide

nonescaped OV A to the MHC class II pathway for priming of

humoral immunity. At 21 d after the first immunization, liposomal

OV A induced significantly higher levels (p < 0.05) of OVA-

specific total IgG, IgG1 (Th2-type), and IgG2c (Th1-type) Ab

responses, regardless of the presence of StII inside Lp, in com-

parison with those observed with the soluble OVA (PBS+OV A)

as control group (Supplemental Fig. 2A–C). This result reveals

that the incorporation of StII into Lp does not cause any addi-
tional effect on the MHC class II OVA-presenting pathway,

which is just enhanced by the very well documented adjuvant

capacity of Lp (47, 48). Additionally, all of the above-described

results indicate that the liposomal Ag combined with StII con-

4-d intervals, starting at day −1. (C) Dot plot graphs of CD4 versus CD3 of

splenocytes from individual mice receiving anti-CD4 mAb or PBS at days

1 and 4 after treatment. Quadrants were individually set at positions where

unstained cells signals were <1%. Values correspond to the percentage of

cells in relation to the total lymphocytes in spleens. (D) Each data point

represents cytotoxic activity in individual experimental mice. (anti-CD4)

and (PBS) indicate animal groups receiving anti-CD4 mAb or PBS, re-

spectively. Statistical significance was calculated using a two-tailed un-

paired t test. The figure shows one representative experiment from three

repetitions with similar results. ns, not significant, p > 0.05.
continues its transit by both Ag-presenting pathways and confirm the ability of Lp to elicit a Th1/Th2 mixed-Ab response (49). Previous results obtained by our group demonstrated that DPPC:Cho (1:1) Lp encapsulating human epidermal growth factor are able to modulate the anti–human epidermal growth factor Ab response in mice to a mixed Th1/Th2 response, but they favored a Th1 pattern (50). The ability of Lp/OVA/StII to induce a Th1/Th2 mixed pattern could be desirable for a candidate vaccine that is addressed not only to intracellular targets (51).

The anti-StII humoral immune response was also analyzed under the experimental conditions described. The total-IgG Ab response elicited by DPPC:Cho Lp containing StII and OVA was compared with the response generated by StII mixed with OVA in PBS at day 21. As shown in Supplemental Fig. 2D, mice immunized with DPPC:Cho Lp presented equivalent titers of anti-StII IgG Abs. These results indicate that StII is an immunogenic protein, but in lower extent than OVA, and it does not interfere in the OVA-specific cellular and humoral responses. In addition, StII immunogenicity is not increased by Lp under the experimental conditions described.

**Immunization with DPPC:Cho Lp encapsulating StII improves an antitumoral immunity partially dependent on CD8+ T cells**

DPPC:Cho Lp containing StII showed to be a promising vaccine candidate to generate cytotoxic CD8+ T cells; however, it was important to evaluate whether that enhanced CTL response was effective in conferring protective immunity. E.G7-OVA thymoma, a murine tumor model expressing OVA, was used to evaluate the ability of Lp/OVA/StII to protect against tumor cells.

First, we explored two routes and doses of Lp/OVA/StII administration in a tumor-protection assay. Two groups of mice received 50 μg of OVA i.m. or s.c., and a third group was treated with 25 μg of OVA s.c. Seven days after the second immunization, animals were challenged with E.G7-OVA cells. All immunization strategies were effective in developing an antitumoral response compared with the PBS group (negative control), which rapidly developed tumors by days 9–10 (Supplemental Fig. 3). Although an apparently higher efficacy (p = 0.012, day 22) in terms of tumor progression was observed with the s.c. route and the higher dose of OVA (50 μg), similar results (p > 0.05) for all tested regimens were clearly evidenced for tumor-free mice and survival percentages. These data demonstrated that the Lp/OVA/StII formulation is able to induce an obvious protection against tumor, even at the half doses used in the CTL assays. It is also relevant that this formulation provided success via the two parenteral routes of immunization described. Therefore, we used both indistinct routes of immunization with the highest doses assessed in the following antitumor assays.

Because Lp/OVA and Lp/OVA/StII showed the ability to stimulate a CTL response, but at different magnitudes (Fig. 4A, 4B), we decided to test the protective efficacy (Fig. 5) of both formulations under different conditions.

**FIGURE 5.** StII coencapsulated into DPPC:Cho Lp with the Ag induces remarkable antitumor prophylactic immunity that decreases upon depletion of CD8+ T lymphocytes. C57BL/6 mice were immunized i.m. twice with Lp/OVA (50 μg OVA) or Lp/OVA/StII (50 μg OVA and 6.25 μg StII) on days 0 and 12. Mice were challenged 7 d later with 3 x 10^5 cells E.G7-OVA tumor cells. A group of mice received PBS as control. Time courses of TV increase (mean ± SEM) (A), tumor grafting (B), and survival curves (C) in experimental groups after tumor challenge. Data are representatives of three independent experiments, each including 7–10 mice per group. Different letters indicate statistical differences among groups based on the Dunnett T3 test (A) and the log-rank test (B and C). The p values are specified in the Results. In another similar antitumoral assay, but immunizing s.c. only with Lp/OVA/StII (OVA 50 μg), mice were depleted of CD8+ T cells by i.p. injection of anti-CD8 mAb (1 mg/mice) 1 d before tumor challenge or they received PBS instead of anti-CD8 (nondepleted group). Time courses of TV increase (mean ± SEM) (D), tumor grafting (E), and survival curves (F) of experimental groups. Statistically significant differences were estimated by the Mann–Whitney U test between the mice groups depleted or not of CD8+ T cells (D) and the log-rank test (E and F). Different letters indicate statistical differences among groups (p values are specified in the Results). Two experiments were performed with similar results. **p < 0.01.
is important in the design of different vaccination strategies. Nevertheless, changes in the immunization schedule, for example, with Lp/OVA, at least under the experimental conditions assessed.

**FIGURE 6.** StII coencapsulated into DPPC:Cho Lp with the Ag elicits a therapeutic antitumoral response. The antitumor therapeutic assay was carried out by challenging the mice (n = 10) with an E.G7-OVA tumor 3 d before immunization. Animals were injected s.c. once per week with Lp/OVA/StII or Lp/OVA (OVA 50 μg) three times. The control group was treated with PBS. Time courses of the IV increase (mean ± SEM) (A) and tumor grafting (B). Different letters indicate statistical differences among groups based on the Dunn test (A) or the log-rank test (B). The p values are specified in the Results. Data represent the result from a meta-analysis of three independent experiments.

4B), their efficacy in prophylactic antitumor activity was also compared. With this purpose in mind, mice were immunized i.m. with Lp/OVA/StII or Lp/OVA before being challenged with the tumor cells. At day 18, both groups immunized with Lp had statistically similar IV averages (p = 0.16), but they were remarkably inferior (p < 0.001) to the PBS group (Fig. 5A). However, significant differences between these two groups were observed with regard to other parameters measured. The tumor appeared in the Lp/OVA/StII group at day 21 after tumor challenge, whereas it appeared at day 11 in the Lp/OVA group (Fig. 5B).

Significantly higher percentages of Lp/OVA/StII-immunized mice remained tumor-free (p = 0.022, Fig. 5B) or survived (p = 0.016, Fig. 5C) compared with Lp/OVA-immunized mice. In summary, immunization with Lp/OVA/StII was able to enhance an Ag-dependent antitumor immunity compared with Lp/OVA, which might be strongly related to the behavior of both liposomal formulations observed in the CTL response.

The role of effector CD8+ T lymphocytes in the antitumoral response was determined by depleting these cells in experimental mice with an anti-CD8 mAb. Mice immunized s.c. with Lp/OVA/StII and depleted of CD8+ T cells. This result confirms that CD8+ T lymphocytes stimulated by Lp/OVA/StII notably contribute to the antitumor response but that other types of cell populations of the immune system also could be influencing this response.

The antitumor effects of Lp/OVA/StII and Lp/OVA were also compared in a therapeutic setting; animals were immunized s.c. three times 3 d after challenge with tumor cells. As shown in Fig. 6, immunization with Lp/OVA did not induce any significant effect compared with PBS in tumor bearing-mice (p > 0.05). In contrast, animals immunized with Lp/OVA/StII exhibited a reduction in IV (p < 0.05) and an increase in the percentage of tumor-free mice (p = 0.016) in relation to the control group, but there was no statistically significant difference compared with the group treated with Lp/OVA, at least under the experimental conditions assessed. However, changes in the immunization schedule, for example, could magnify the difference between both groups. Nevertheless, we could take as fact that StII added to DPPC:Cho Lp improves antitumor activity in preventive and therapeutic scenarios, which is important in the design of different vaccination strategies.

**FIGURE 7.** An irreversibly inactive dimer of rStI coencapsulated into DPPC:Cho Lp with OVA induces a similar Ag-specific cytotoxicity as StII-containing Lp. C57BL/6 mice (three mice per group) were immunized s.c. twice with OVA/PIC (1 mg of OVA, 100 μg of PIC) or with Lp/OVA/Sts (StI, revStI W111C, or irrevStI W111C) (50 μg of OVA and 6.25 μg of St) on days 0 and 12. A group of mice received PBS as control. The CTL assay was performed in vivo as described in Fig. 4. Each data point and the horizontal solid lines represent the cytotoxic activity of individual mice and the average values, respectively. The dashed line indicates the average lysis (%). The role of effector CD8+ T lymphocytes in the antitumoral response was determined by depleting these cells in experimental mice with an anti-CD8 mAb. Mice immunized s.c. with Lp/OVA/StII and depleted of CD8+ T cells. This result confirms that CD8+ T lymphocytes stimulated by Lp/OVA/StII notably contribute to the antitumor response but that other types of cell populations of the immune system also could be influencing this response.

Stimulation of the OVA-specific CTL response by DPPC:Cho Lp encapsulating StII does not depend only on its pore-forming ability

StII and StI exhibit high homology at the sequence and three-dimensional structural levels (18, 19, 52). Mutants containing a cysteine residue in relevant functional regions of rStII were designed and obtained, exhibiting a different ability to form dimeric structures stabilized by disulfide bridges (24, 53). Among them, StI W111C, with a cysteine residue at the membrane-binding region, displayed a high propensity to form a reversibly inactive dimer (revStI W111C) as a result of its inability to bind to membranes (24, 54). This molecular entity was a nice tool to produce an irreversibly inactive dimer by StI W111C conjugation, using the chemical reagent BMH (revStI W111C, Supplemental Fig. 4A). The conjugation yield for the irreversible entity was ∼30% (Supplemental Fig. 4B, lane 3), with a lower amount of the reversible form (∼14% of the total
amount of protein in the reaction). The purification of \( \text{irrevStI} \) \( \text{W111C} \) previously incubated under reducing conditions allowed separation of this inactive dimeric form from the other molecular entities present; its purity was corroborated by SDS-PAGE (Supplemental Fig. 4C, 4D). \( \text{irrevStI} \) \( \text{W111C} \) constituted \( \sim 94\% \) of the total protein (Supplemental Fig. 4D, lane 5), whereas only a small amount of the reversible dimer was present \( \sim 6\% \). As expected, \( \text{irrevStI} \) \( \text{W111C} \) did not exhibit pore-forming ability, as assessed by a hemolytic assay, even in a reducing environment. Under these conditions, the presence of reversible dimer was almost negligible \( (1.5\%) \), at least in terms of its functionality (data not shown).

The coencapsulation efficiency of \( \text{rStI} \), \( \text{rStI} \) \( \text{W111C} \), and \( \text{irrevStI} \) \( \text{W111C} \) with OVA into DPPC:Cho Lp was equivalent to that observed for \( \text{StII} \) in Lp/OVA/StII \( (\sim 57\%) \). In addition, the efficacy of OVA encapsulation was independent of the presence of Sts and was similar to the values reported above.

Surprisingly, in the CTL assay, mice immunized with Lp/OVA/\( \text{irrevStI} \) \( \text{W111C} \) showed an Ag-specific lysis percentage that was equal to mice receiving Lp/OVA/rStI or Lp/OVA/\( \text{rStI} \) \( \text{W111C} \) (Fig. 7). The dashed line indicates the average value of target cell lysis \( (\% \) in animals immunized with Lp/OVA/StII (Fig. 4B) and shows that all experimental groups treated with Lp containing any of the St variants exhibited similar efficacy to this formulation. This result also indicates that \( \text{rStI} \) retains the capability of \( \text{StII} \) to stimulate a strong OVA-specific CTL response. Additionally, \( \text{rStI} \) coencapsulated into Lp with OVA seems to behave as a PFP, which is activated by sulfhydryl groups and is able to enhance the activation of CD8\(^+\) T cells. As mentioned previously, groups treated with OVA/PIC showed lower percentages of target cell lysis.

The coencapsulation of \( \text{rStI} \), \( \text{rStI} \) \( \text{W111C} \), and \( \text{irrevStI} \) \( \text{W111C} \) with OVA into DPPC:Cho Lp showed an Ag-specific lysis percentage that was equal to mice receiving Lp/OVA/rStI or Lp/OVA/\( \text{rStI} \) \( \text{W111C} \) (Fig. 7). The dashed line indicates the average value of target cell lysis \( (\% \) in animals immunized with Lp/OVA/StII (Fig. 4B) and shows that all experimental groups treated with Lp containing any of the St variants exhibited similar efficacy to this formulation. This result also indicates that \( \text{rStI} \) retains the capability of \( \text{StII} \) to stimulate a strong OVA-specific CTL response. Additionally, \( \text{rStI} \) coencapsulated into Lp with OVA seems to behave as a PFP, which is activated by sulfhydryl groups and is able to enhance the activation of CD8\(^+\) T cells. As mentioned previously, groups treated with OVA/PIC showed lower percentages of target cell lysis.

From the results observed with Lp/OVA/\( \text{irrevStI} \) \( \text{W111C} \), we hypothesize that the pore-forming activity of Sts is not crucial for the enhancement of Ag-specific CTL immune responses by DPPC:Cho Lp. Instead of the pore formation in the endosomal membranes, any other mechanism conditioned by the Sts structure is more relevant in the CTL response.

**The PFP StII induces DC maturation**

As has been very well established, activation of naive CD8\(^+\) T cells occurs via stimuli-activated APCs. With the purpose of elucidating whether the maturation of DCs by \( \text{StII} \) is a mechanism involved in the immunomodulatory properties of Sts, we investigated whether LPS-free \( \text{StII} \) could induce this process. The experiment was corroborated using LPS as a positive control in the presence or absence of pmbX, a cationic cyclic lipopeptide that binds the lipid A moiety of LPS and blocks its biological effects \( (55) \). Fig. 8 shows the PFP \( \text{StII} \) induces DC maturation in vitro and could suggest a contribution by this immunomodulatory effect to the immune response mediated by St Lp.

**Discussion**

The development of a vaccine strategy for eliciting functionally robust CTL responses depends primarily on the ability to efficiently introduce exogenous antigenic proteins into the MHC class I pathway of APCs. In this sense, there are interesting vaccine platforms based on several delivery strategies that direct Ags toward the APCs’ cytosol \( (56) \). Bacterial PFPs, particularly LLO from \( L. \) *monocytogenes*, have been used to mediate Ag cytosolic delivery and improve CTL-mediated immune responses \( (13, 14) \).

With regard to this, the potential use of PFPs from other non-bacterial sources as immunomodulators and exhibiting different structural and functional properties, such as tridimensional structures, nature of formed pores, and pH dependence and lipid requirement for the pore formation, among others, could be interesting and promising.

Our results demonstrate that noncationic and non fusogenic lipids–containing Lp, but encapsulating a model Ag together with a PFP classified in the family of actinoporins, are able to induce a potent and functionally protective Ag-specific CTL response. We proved that this liposomal formulation favors Ag cross-presentation and functions as a potent vaccine adjuvant to initiate APC–T cell interactions and to stimulate CD8\(^+\) T cell–mediated immune responses. Moreover, the evidence described in this article point out that the pore-forming activity of Sts is not essential for eliciting an Ag-specific CTL response.

The liposomal lipid composition based on DPPC/Cho, at a 1:1 molar ratio, was chosen to minimize membrane–Sts interactions and, as a consequence, the pore-forming ability of Sts. Because SM is considered a natural membrane acceptor for actinoporins \( (22, 57) \), its exclusion was essential. Based on previous reports \( (58, 59) \), lipid compositions generating lipid phase coexistence, which favors membrane insertion of actinoporins, were also avoided. The absence of permeabilization activity of \( \text{StII} \) in DPPC:Cho LUVs (Fig. 1) indicates the feasibility of encapsulating Sts into these Lp to perform in vivo assays. In fact, the relatively high and equivalent encapsulation efficacy of DPPC:Cho DrVs for all proteins assessed \( (\sim 50\%) \) and their relatively elevated stability, at least in term of their retention capacity during the first 7 d of storage in suspension (Supplemental Fig. 1), confirms this statement. These results agree with those reported previously for DrVs that are characterized by a large internal volume with moderate to high encapsulation and retention efficiency \( (27, 60) \).

Additionally, the low proportion of vesicles carrying at least one molecule of \( \text{StII} \) or OVA on their surface (Fig. 2) supports that most of these proteins are trapped inside the Lp. In summary, the DrVs procedure used to prepare DPPC:Cho Lp, rendering a highly organized membrane resulting from selected lipid composition \( (37, 61) \), indicates that it is possible to administer Lp/OVA/StII even a few days after its reconstitution because these vesicles remain essentially intact.

The large sizes of DrVs carrying OVA, and containing \( \text{StII} \) or not, could contribute to an avid uptake by phagocytic cells, specifically macrophages \( (62) \). However, macrophages do not show an Lp size-dependent pattern of uptake \( (63) \); thus, we could expect a similar degree of internalization for both liposomal formulations by these APCs. In addition, OVA-containing Lp, obtained as described in this article, do not differ from empty Lp in zeta potential, being around \( \sim 25 \) mV \( (Y. \) Cruz-Leal and M.E. Lanzio, unpublished observations). Similar results were reported by Nordly et al. \( (64) \) using cationic Lp carrying OVA adsorbed on their surface. In contrast, in this study, the molar ratio of \( \text{StII} \) and lipids in Lp/OVA/StII is notably lower than that used for OVA; therefore one might presuppose minimal or no influence of \( \text{StII} \) on
the surface potential of Lp. Considering this, we believe that the difference in immunomodulatory properties observed between Lp/ova and Lp/ova/stII could be due to some property of StII, rather than advantageous physicochemical characteristics of Lp/ova/stII in relation to Lp/ova.

The ability of Lp/ova/stII to enhance Ag presentation by MHC class I was studied by following stimulation of the CD8+ T cell population in vivo. The expansion of OVA-specific CD8+ T cells, observed in mice treated with such a liposomal formulation and not in those receiving Lp/ova (Fig. 3B), demonstrated the requirement for StII inside Lp to induce, at least more efficiently, MHC class I–mediated Ag presentation. Consequently, the amount of CD8+ T cells expressing CD44high and CD62L, as markers of memory T lymphocytes (65, 66), elicited by Lp/ova/ StII was higher than that observed with Lp/ova (Fig. 3D). In addition, we presuppose that Lp/ova/stII, as a result of the presence of StII, could also be causing a higher epitope density on APCs than Lp/ova, which results in a higher number of CD8+ memory T cells that is generated and persist in the host, according to Leignadier and Labrecque (67). This ability of Lp/ova/stII could have relevance in future applications, because the development of vaccines capable of inducing long-lived memory CD8+ T cells is desired to establish a stage of immune surveillance.

Because several pathways exist for loading antigenic peptides onto MHC class I molecules (56), we could only hypothesize about a probable internal mechanism followed by Lp/ova/stII that explains its enhancement of MHC class I–mediated Ag presentation. Such a process that should necessarily involve the uptake of Lp by APCs and Lp destabilization in the endosomal compartment is probably followed by StII-mediated endosomal rupture, and the subsequent release of the vesicle content leading to Ag presentation by the MHC class I pathway, as was proposed for LLO-containing Lp (68, 69). Recent results obtained by our group support the relevant role of Lp/ova/stII in mediating Ag cross-presentation in vitro in comparison with Lp/ova and suggest a main role for macrophages in this process (Y. Cruz-Leal, D. Grubaugha, C. Nogueira, I. Lopetegui, A. del Valle, F. Escalona, R. Laborde, C. Mesa, L. E. Fernández, M. Starnbach, D. Higgins, and M.E. Lanio, manuscript in preparation). Indeed, the function of macrophages in the stimulation of naive CD8+ T cells and cross-presenting Ags in vivo also was demonstrated previously (70). The Ag-specific cross-presentation was further confirmed by the highest specific lysis of target cells induced with Lp/ova/stII in the CTL assays in vivo (Fig. 4B). In fact, StII incorporated into Lp was able to enhance the OVA-specific CTL response, and this property was reproduced by the other isoform, StI obtained via recombinant DNA (Fig. 7). Altogether, the results from both assays (CD8+ T cell expansion and in vivo CTL response) indicate that Sts-containing Lp induce a substantial proliferation of OVA-specific CD8+ T lymphocytes that act as CTL precursors, provoking the notable CTL-mediated immune response in mice. A correlation between CD8+ T cell expansion and the number of CTLs was documented (71).

CD4+ Th cells have several important roles during the development and maintenance of CTL responses, including the enhancement of clonal expansion during priming (43). Interestingly, under the described experimental conditions, the OVA-specific CTL immune response enhanced by Lp/ova/stII was independent of CD4+ Th cells (Fig. 4D), as was similarly established for other adjuvants, such as PIC and VSSP (45, 46). It is known that infectious agents can provoke an Ag-specific CTL response, independent of CD4+ Th cells, as a consequence of the induction of strong inflammatory stimuli in the primary response by potent TLR activation of APCs (72). This would explain the indepen-
for PIC, such independence was only observed in the primary response, because the MHC class II–restricted Th cell response was required to generate a memory CTL response (43). A recognized role for CD4+ Th cells in generating functional and sustainable memory CD8+ T cells also was established for inflammatory and noninflammatory agents (73). In contrast, CD4+ T cells were not required for the generation of memory CD8+ T cells in the case of immunization with Lp-coupled OVA peptides mixed with CpG (74). Consequently, it would be necessary to investigate whether the OVA-specific and long-lasting memory CTL immune response enhanced by Lp/OVA/StII depends on the provision of an appropriate Th cell response. Even so, the apparent autonomy of specific CD8+ T lymphocyte responses from Th cells could be pertinent to achieve a CTL response in immunocompromised individuals.

In agreement with the results obtained in the CTL assays in vivo, a significant protection was observed in mice immunized with Lp/OVA/StII and challenged with OVA-bearing tumor cells in terms of tumor-free mice, and survival compared with groups treated with Lp/OVA or PBS, indicating that the Ag-specific CTL response was robust (Fig. 4B), as well as functional (Fig. 5A–C). A comparable correlation was obtained with a pH-sensitive liposomal preparation carrying LLO and OVA (14). Other similar approaches to induce antitumoral protection consist of Lp containing fusogenic proteins from virus (75) or membranotropic peptides (76). Nevertheless, in the present vaccine platform, neither fusogenic nor pH-sensitive Lp were used to carry a nonbacterial PFP. In addition, the contribution of CD8+ T lymphocytes to the antitumor activity induced by Lp/OVA/StII was demonstrated by its significant reduction in the absence of these cells (Fig. 5D–F). However, the antitumor protection was only partially abrogated with CD8+ T cell depletion, suggesting that other immune cells take part in this process. This observation could be also correlated with expansion of the predominantly OVA-non specific CD8+ cell population that was induced by Lp/OVA/StII at the draining LN (Fig. 3A). Both results suggest that this liposomal formulation, in addition to CD8+ T cells, could be stimulating the expansion of other kind of leukocytes, Ag or non-Ag specific, that could contribute to the antitumor response. Clones of OVA-specific CD4+ T cells are activated by Lp/OVA/StII immunization, as was confirmed by the anti-OVA Ab titers induced by these vesicles (Supplemental Fig. 2A–C). These cells probably collaborate with CD8+ T cells in the antitumoral activity similar to that reported by Kim et al. (77) using Lp coencapsulating OVA and CpG. In addition, Lp/OVA/StII and Lp/OVA expanded the CD8+ cell population; therefore, common features of these vesicles could be further modulating such cells. Cells from the innate immune system, such as NK and NKT cells, could be expanded by these vaccinations, as observed under different stimuli, including self-phosphatidylcholine (78) and Lp with immunomodulators exhibiting antitumoral activity (33, 77). Additionally, B1 cells coming from splenocytes could also proliferate under the effect of these Lp. Previous work by our group demonstrated that DPPC-containing Lp encapsulating OVA are internalized by B1 cells and elicit an increase in peritoneal lymphocytes (79, 80). Interestingly, empty DPPC Lp are able to reproduce the effect of those carrying only OVA (80, 81).

The selection of a suitable adjuvant for a therapeutic scenario, as occurs in cancer vaccines, has demands beyond the classical characteristics of an adjuvant for preventive vaccination. In this case, the induction of effective CTL activity is not easy to achieve in the tumor environment because of the variety of immunosuppressive strategies displayed by the tumor to restrict the effectiveness of antitumor responses (82). Despite this, an antitumoral response was observed in tumor-bearing mice treated with Lp/OVA/StII in contrast to those receiving Lp/OVA, reflecting additional properties associated with the former. Our data could suggest a capacity for Lp/OVA/StII to partially overcome the suppressing mechanisms generated by tumors, probably by combining the higher-quality CTL response with the influence on particular immune cells, as mentioned above.

Similar to other PFPs that can be activated by sulphydryl groups (83), revStI W111C encapsulated into DPPC:Cho Lp was able to promote an Ag-specific CTL response equivalent to rStI (Fig. 7), probably as a consequence of its activation in the reducing environment of the endosomal compartment. In turn, organelle disruption by pore formation would lead to Ag delivery to cell cytosol (84). This finding shows that Lp containing revStI W111C could be a safer formulation, because they could only be activated after reaching the endosomal environment. Unexpectedly, revStI W111C coencapsulated with OVA into DPPC:Cho Lp was able to induce an Ag-specific CTL response comparable to that observed with Lp carrying the wild-type protein rStI or its counterpart revStI W111C (Fig. 7), indicating that the pore-forming activity of Sts is not absolutely required for this outcome. In contrast, the effect of LLO incorporated into liposomal vesicles on the enhanced delivery of plasmid DNA was strictly correlated with the endosomolytic activity of this PFP (85, 86).

Effective adjuvants should promote Ag delivery, as well as activate APCs (48). The recognition of bacterial PFPs by TLRs and the induction of APC maturation were documented (55). StII, although evolutionarily distant from bacterial PFPs, interacted with DCs, causing their activation in vitro (Fig. 8). Considering that partial leakage from Lp can occur in vivo as a consequence of their interaction with the biological medium (39, 60), free StII could be found at the inoculation site and initiate the activation of APCs located nearby. StII-containing Lp were not able to induce the maturation of DCs in vitro using serum-free medium (data not shown).

A Toll/TLR protein, as well as likely orthologs of key components of the TLR pathways, were found in anthozoan cnidarians, although the molecular nature of the ligands for these proteins is unknown (87). Actinoporins could be putative ligands for these Toll/TLR proteins, because vertebrate TLRs were suspected of binding to host molecules, including fibrinogen and heat shock proteins (88). StII could interact with other families of TLR proteins, such as those of vertebrates. This idea could account for the ability of StII to mature DCs as a pathogen-associated molecular pattern similar to bacterial PFPs, as well as explain the nonabsolute dependence between the pore-forming ability of Sts and their capacity to enhance an Ag-specific CTL response.

In conclusion, to our knowledge, this is the first report of an adjuvant based on StII encapsulated into Lp for the activation of a robust and functional CTL response with an antitumor effect in preventive and therapeutic scenarios, constituting a promising vaccine platform against cancer. Additionally, the nontotal dependence of the immunomodulatory effects of Sts on their pore-forming activity was demonstrated. The ability of StII to induce DC maturation in vitro might explain this particular behavior. The mechanisms conducive to DC activation and their relevance to the immunomodulatory properties of StII require further investigation.

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