Sticholysins, pore-forming proteins from a marine anemone can induce maturation of dendritic cells through a TLR4 dependent-pathway

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ABSTRACT

Sticholysins (Sts) I and II (StI and StII) are pore-forming proteins (PFPs), purified from the Caribbean Sea anemone Stichodactyla helianthus. StII encapsulated into liposomes induces a robust antigen-specific cytotoxic CD8+ T lymphocytes (CTL) response and in its free form the maturation of bone marrow-derived dendritic cells (BM-DCs). It is probable that the latter is partially supporting in part the immunomodulatory effect on the CTL response induced by StII-containing liposomes. In the present work, we demonstrate that the StII’s ability of inducing maturation of BM-DCs is also shared by StI, an isoform of StII. Using heat-denatured Sts we observed a significant reduction in the up-regulation of maturation markers indicating that both PFP’s ability to promote maturation of BM-DCs is dependent on their conformational characteristics. StII-mediated DC maturation was abrogated in BM-DCs from toll-like receptor (TLR) 4 and myeloid differentiation primary response gene 88 (MyD88)-knockout mice but not in cells from TLR2-knockout mice. Furthermore, the antigen-specific CTL response induced by StII-containing liposomes was reduced in TLR4-knockout mice. These results indicate that StII, and probably by extension StI, has the ability to induce maturation of DCs through a TLR4/MyD88-dependent pathway, and that this activation contributes to the CTL response generated by StII-containing liposomes.

Abbreviations: Sts, Sticholysins; PFPs, pore-forming proteins; BM-DCs, bone marrow-derived dendritic cells; BM-Mφs, bone marrow-derived macrophages; DPPC, dipalmitoyl phosphatidylcholine; Cho, cholesterol; Lp/OVA/StII, liposomes encapsulating OVA and StII; TLRs, toll-like receptors; MyD88, myeloid differentiation primary response gene 88; CTL, cytotoxic CD8+ T lymphocytes.

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1. Introduction

Naive CD8⁺ T lymphocytes need to be primed by previously activated professional antigen-presenting cells (APCs), usually dendritic cells (DCs), before they can exert their cytotoxic effector functions (Kurts et al., 2010). Viral and bacterial pathogens bear ligands, called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), CpG DNA, double-strand RNA, single-strand RNA, lipoteichoic acid, flagellin, etc., that bind to a family of toll-like receptors (TLRs) expressed by DCs, macrophages (Mφs), and other cell types. Binding of PAMPs can trigger a diversity of inflammatory responses and mediate the activation of APCs, particularly DCs (Williams and Bevan, 2007).

DCs in peripheral tissues are in an immature state but enter the process of maturation and migrate to lymphoid organs, where T cells are found, upon challenge with antigens and upon exposure to PAMPs and inflammatory products (e.g., interleukin [IL]-1, tumor necrosis factor-α) (Mellman, 2013). Immature DCs are specialized for antigen capture and processing but weakly stimulate T cells. Only mature DCs expressing high levels of immunostimulatory molecules, such as major histocompatibility complex class II (MHC-II) molecules, co-stimulatory molecules (e.g., B7.1 [CD80], B7.2 [CD86], CD40) and cytokines (IL-12, type I interferons), can efficiently stimulate T cell activation (Arens and Schoenberger, 2010; Mellman, 2013).

Additionally, DCs have the ability of processing exogenous antigens for presentation on MHC class I (MHC-I) molecules to CD8⁺ T lymphocytes, process termed cross-presentation. Cross-presentation is important for the generation of cytotoxic CD8⁺ T lymphocytes (CTL) immunity to a variety of antigens, particularly those associated with viral infection and tumorigenesis as well as DNA vaccination (Heath et al., 2004; Kurts et al., 2010). DC stimulation through TLRs results in quantitative and qualitative changes in antigen presentation and cellular activation. Consequently, the incorporation of TLR ligands into vaccines could improve their efficacy. This is supported by studies that have demonstrated that TLR ligands promote the acquisition of cross-presenting properties by DCs (Lutz and Schuler, 2002; Datta et al., 2003). In particular, CpG was shown to not only induce the expression of co-stimulatory molecules required for CD8⁺ T cell activation, but also to regulate the intracellular mechanisms of cross-presentation such as antigen degradation rates in pre-CD8⁺ DCs (de Brito et al., 2011).

Incorporation of monophosphoryl lipid A (MPLA) into glycoliposomes which were targeted to DCs, significantly enhanced the antigen cross-presentation of the melanoma tumor antigen gp100280-288 peptide to CD8⁺ T cells and induced tumor antigen-specific CD8⁺ T cell responses (Boks et al., 2015). Gram-positive bacterial pore-forming proteins (PFPs) such as Listeriolysin O (LLO) have been reported to act as a PAMP in a TLR4-dependent manner (Park et al., 2004). Remarkably, a non-hemolytic form of LLO fused to or simply mixed with a human papillomavirus type-16 E7 recombinant protein enhanced the antitumor immune response and facilitated tumor eradication (Wallecha et al., 2017).

Sticholysins (Sts) I and II (StI and StII) are PFPs produced by the Caribbean Sea anemone Stichodactyla helianthus and belong to the actinoporin protein family (Lanio et al., 2001; Alvarez et al., 2009). They exhibit a high structural similarity, affinity by sphingomyelin (Tejuca et al., 1996; Martínez et al., 2007), and can permeabilize cellular and model membranes by forming oligomeric pores of ~2 nm of diameter (Tejuca et al., 2001; Lanio et al., 2001; Vesely et al., 2011). However, Sts differ in their cytolytic ability with StI being less active than StII (Martínez et al., 2001). We previously reported that liposomes comprised of dipalmitoyl phosphatidylcholine and cholesterol (1:1 molar ratio) co-encapsulating ovalbumin (OVA) and StII (Lp/OVA/StII) induced a robust CTL response with antitumor function in mice (Lobo et al., 2017). Interestingly, the CTL response was uncoupled from the StII pore-forming activity and the protein was also able to induce maturation of CD8⁰ cells. Therefore, we propose that the CTL generation by StII-containing liposomes could be supported in part by the StII-mediated maturation of DCs. More recently, we also demonstrated that the Lp/OVA/StII formulation promotes antigen cross-presentation in Mφs through the vacuolar pathway and that these cells play a pivotal role for the CTL response observed in vivo (Cruz-Leal et al., 2018). The isoform StI encapsulated in liposomes with OVA also promoted similar CTL response in vivo (Laborde et al., 2017).

In this work we extend the studies of the immunomodulatory effects of Sts. Firstly, we demonstrated that like StII, StI also activated DCs and that this ability depended on their native state. Secondly, StI-mediated DC maturation was dependent on TLR4 and myeloid differentiation primary response gene 88 (MyD88) molecules, but not on TLR2. Lastly, the OVA-specific CTL response induced by Lp/OVA/StII was significantly reduced in the absence of TLR4.

2. Materials and methods

2.1. Proteins, reagents and cell lines

StI (Swiss Protein Data Bank P07845) was purified from the sea anemone Stichodactyla helianthus and characterized as previously described by Lanio et al. (Lanio et al., 2001). StI was obtained by recombinant way according to the procedure described by Pazos et al. (Pazos et al., 2006). The protein concentration was determined using the absorption coefficients of 1.87 and 2.13 mL/mg/cm at 280 nm for StI and StII respectively (Lanio et al., 2001; Pazos et al., 2006), and their hemolytic activities (HA) were monitored as previously described (Laborde et al., 2017). The proteins were stored at –20 °C until use. Chicken egg ovalbumin (OVA) grade V, LPS (0.3 mg/mL) from Escherichia coli O111:B4 and polymyxin B (pmxB) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). The immunodominant OVA257-264 peptide (SIINFEKL) was synthesized and provided by the Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba). It was dissolved in phosphate-buffered saline (PBS: 9.6 mM Na₂HPO₄, 13.7 mM NaCl, 2.7 mM KCl, 1.47 mM KH₂PO₄, pH 7.4), and stored at –20 °C in aliquots until use. Recombinant murine granulocyte macrophage colony-stimulating factor (rmGM-CSF) was purchased from PeproTech (Rocky Hill, NJ, USA). Invitrogen (Paisley, UK) supplied the carboxy-fluorescein dialcetate succinimidyl ester (CFSE) and Sigma-Aldrich (Gibco™, Waltham, MA, USA) supplemented with 10 % fetal calf serum (FCS) (Gibco™), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1 mg/mL sodium pyruvate, penicillin (100 U/mL) and streptomycin (100 μg/mL) (Life Technologies Inc., Grand Island, NY, USA). The L929 murine cell line (ECACC, Wiltshire, UK), which secretes the macrophage colony-stimulating factor (M-CSF) (Weischedel and Porse, 2008), was cultured in RPMI-1640 medium (Gibco™, Waltham, MA, USA) supplemented with 10 % fetal calf serum (FCS) (Gibco™), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1 mg/mL sodium pyruvate, penicillin (100 U/mL) and streptomycin (100 μg/mL) (Life Technologies Inc., Grand Island, NY, USA). The B3Z T cell line, a CD8⁺ T-cell hybridoma specific to the H-2K²/ SIINFEKL complex expressing β-galactosidase (LacZ-inducible gene) under the transcriptional control of the IL-2 enhancer (Karttunen et al., 1992), was used. This cell line was cultured in RPMI-1640 medium (Gibco™, Waltham, MA, USA) supplemented with 10 % fetal calf serum (FCS) (Gibco™), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1 mg/mL sodium pyruvate, penicillin (100 U/mL) and streptomycin (100 μg/mL) (Life Technologies Inc., Grand Island, NY, USA). The L929 murine cell line (ECACC, Wiltshire, UK), which secretes the macrophage colony-stimulating factor (M-CSF) (Weischedel and Porse, 2008), was cultured in RPMI-1640 medium (Gibco™) with the above supplements.

2.2. Mice and ethics statement

Female wild type (WT) C57BL/6 mice (H-2b) were obtained from the National Center for Laboratory Animals Production (CENPABA, Cuba) or from the Development Center of Experimental Models for Medicine and Biology (CEDEME) at the Federal University of Sao Paulo (UNIFESP, Brazil). Male or female OT-I [CD8⁺ T cell receptor (TCR) transgenic mice expressing the TCR recognizing SIINFEKL in H-2K²] mice were produced at the Center for Molecular Immunology (CIM, Cuba). Male or female TLR4-, TLR2- and MyD88-deficient mice (knockout mice) used in the experiments were obtained from CEDEME. Mice aged 8–12 weeks were kept in specific-pathogen-free conditions at CIM or UNIFESP animal.
facilities. Experiments were performed in accordance with the respective institutional guidelines. All procedures were performed in compliance with the protocols approved by the Institutional Committee for the Care and Use of Laboratory Animals of the CIM (CICUAL, 0017/2008) and the special Ethics Committee of UNIFESP (ID number: 1210/2008).

2.3. Heat inactivation of Sts

Sts were inactivated through heat treatment for 2 h at 80 °C as described by Martínez et al. (Martínez et al., 2001). Lack of HA as a consequence of the loss of native conformation was corroborated by hemolysis assay. These Sts variants were named denatured Sts (dSt or dStII).

2.4. Liposomes preparation

The procedure based on dehydration and rehydration of vesicles developed by Kirby and Gregoriadis (Kirby and Gregoriadis, 1984) was used to obtain multilamellar liposomes of DPPE, an equimolar quantity of Cho, and co-encapsulating OVA with or without StII as described previously (Laborde et al., 2017). Briefly, small unilamellar vesicles (SUrs) were obtained by ultrasonication and then mixed with the proteins in the following proportion: 16 μmol of total lipids per 80 μg of OVA and 10 μg of StII. The mixture of SUrs and proteins was subjected to freeze-dry for 24 h. The rehydration step was performed with distilled water (50 μL water/16 μmol phospholipid) and followed by incubation for 30–45 min at 45 °C. Non-entrapped components were removed by centrifugation and pellets were finally re-suspended in an appropriate volume of PBS. This procedure rendered vesicles of around 2 μm in diameter with very similar encapsulation efficiencies for both proteins (around 50 %) (Laborde et al., 2017).

2.5. Generation of bone marrow-derived dendritic cells and macrophages

Bone marrow-derived DCs (BM-DCs) were generated as described in Lutz et al. (Lutz et al., 1999). Briefly, BM cells were harvested from the femur and tibia of WT C57BL/6 mice, and TLR4, TLR2 and MyD88 knockout mice. Single-cell suspensions were cultured at a density of 2 × 10^6 cells/well in 6-well plates (BD Falcon, Oxford, UK), were pulsed with the stimuli for 3 h at 37 °C and 5 % CO₂, and counted on day 6. BM-derived MDCs were cultured in complete RPMI plus 10 % FCS) supplemented with 200 unit/mL (20 ng/mL) of rmGM-CSF (DC medium). An additional 5 mL of DC medium was added after 9 days, and the cultures were incubated for 18–24 h in conditions similar to those described in the DC maturation assay. BM-DCs from WT C57BL/6 mice were incubated with StII (1 μg/mL) plus OVA (10 μg/mL) in 96-well flat bottom plates (BD Falcon) for 96 h, at 37 °C and 5 % CO₂. Non-stimulated BM-DCs co-cultured with OT-I CD8⁺ T cells in the presence of SIINFEKL peptide (1 μM) were used as positive control. As a measure of CD8⁺ T cell activation the expression of CD69 molecule was determined by flow cytometry.

In some experiments, a B3Z hybridoma T cell line was used as source of OVA-specific CD8⁺ T effector cells. In this case, BM-DCs as well as BM-Møs (2.5 × 10⁵ cells) from WT C57BL/6 mice were exposed to OVA (25 μg/mL) alone and OVA plus different concentrations of StII or StI (0.03–0.5 μg/mL) in 96-well flat bottom plates (BD Falcon). BM-Møs were also stimulated with Lp/OVA/StII or liposomes without StI (Lp/OVA) containing the following protein concentrations: OVA 0.1–0.8 μg/mL, StI 0.01–0.1 μg/mL. The incubation conditions were similar to what has been described above. After stimulation, cells were washed and co-cultured with 1 × 10⁶ B3Z T cells (1:1 ratio). After 18–24 h of incubation at 37 °C and 5 % CO₂ the supernatant was withdrawn and 100 μL of LacZ buffer containing 0.15 mM CPRG (Sigma-Aldrich) in PBS, 0.13 % Nonidet P-40, 9 mM MgCl₂ and 0.1 mM β-mercaptoethanol, was added to each well. The plates were incubated in the dark for 5 h at room temperature. Absorbance was measured at 570/620 nm using a Spectra Max Plate Reader (Tecan, Crailsheim, Germany) and the optical density was rectified by subtracting the values of B3Z cells incubated with non-stimulated APCs. B3Z T cells exposed to APCs pulsed with SIINFEKL peptide (1 μM) were included as reference. The APCs viability after incubating with the Sts was 70–100 %, measured by the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Goteborg, Sweden).

2.6. Dendritic cells maturation assays

BM-DCs from WT C57BL/6 mice, and TLR4, TLR2 and MyD88 knockout mice suspended in RPMI at 2 × 10⁶ cells/well in 6-well plates (BD Falcon, Oxford, UK), were pulsed with the stimuli for 3 h at 37 °C and 5 % CO₂. The stimuli consisted of StI, StII, dStI or dStII, and LPS (positive control) at 1 μg/mL, mixed or not with pmxB (10 μg/mL). BM-DCs in medium plus pmxB were used as negative control. Further incubation of the cells for 18–20 h with complete RPMI (the presence of serum inactivates Sts (Basalto et al., 2006)) was performed. Subsequently, cells were analyzed by flow cytometry to determine the surface expression of the maturation markers CD80, CD86 and CD40.

2.7. Detection of MHC-I antigen-presentation

BM-DCs from WT C57BL/6 mice were incubated with StI (1 μg/mL) plus OVA (10 μg/mL) in the same conditions as described above. Cells cultured in complete RPMI alone (non-stimulated) were used as a negative control. BM-DCs were harvested and the OVA257–264-presentation on MHC-I molecules was analyzed by flow cytometry using a monoclonal antibody (mAb) specific to the H-2Kb/SIINFEKL complex (AF6–88.5, BioLegend, San Diego, CA, USA).

2.8. Antigen-specific CD8⁺ T cell activation assays

Samples comprised of OVA (10 μg/mL) alone, mixed with StII (1 μg/mL) or encapsulated into StII-containing liposomes (Lp/OVA/StII), with OVA and StII at the same concentrations than when assayed in non-encapsulated form, were added to BM-DCs from WT C57BL/6 mice and incubated for 18–20 h in similar conditions as described in the DC maturation assay. CD8⁺ T cells, purified from OT-I mice by negative selection using a CD8a⁺ T Cell Isolation Kit II (Miltenyi Biotec, GBH, Germany), were co-cultured (1 × 10⁶ cells) with the stimulus-pulsed BM-DCs at 1:1 ratio in complete RPMI in 96-well round bottom plates (BD Falcon) for 96 h, at 37 °C and 5 % CO₂. Non-stimulated BM-DCs co-cultured with OT-I CD8⁺ T cells in the presence of SIINFEKL peptide (1 μM) were used as positive control. As a measure of CD8⁺ T cell activation the expression of CD69 molecule was determined by flow cytometry.

Cells were stained with specific mAbs using conventional protocols to analyze the expression of surface molecules. In BM-DCs assays, cells were stained with the following anti-mouse mAbs: CD11c/PE, CD86/FITC, CD40/FITC or CD80/PE (eBiosciences, San Diego, CA, USA) or anti-mouse CD11c/PE and CD40/PE Txed (BD Biosciences, San Jose, CA, USA). A live/dead fixable aquea dead cell stain kit was used to exclude dead cells (Invitrogen) and the cell viability was between 93.5–98.3 %. Doublets were excluded from the total acquired cells through the analysis of Forward Scatter-High (FSC-H) vs. Forward Scatter-Area (FSC-A) parameters. The flow cytometry gating strategy for evaluating maturation markers on BM-DCs is shown in Figure S1. For detecting OVA257–264-presentation on MHC-I molecules of BM-DCs a biotin-conjugated mAb anti-H-2Kβ/SIINFEKL complex followed by FITC-conjugated Streptavidin, both purchased from eBiosciences, were used. CD8⁺ T lymphocyte activation was evaluated with the anti-mouse...
mAbs CD8/PE and CD69/PECy5 from eBiosciences. Cells were acquired using FACSCalibur (BD Biosciences), Gallios (Beckman Coulter, Miami, FL, USA) or Attune Acoustic Focusing (Applied Biosystems, Waltham, MA, USA) flow cytometers and analyzed with FlowJo7.6.1 (Tree Star Inc., USA) software.

2.10. Antigen-specific CTL assay in vivo

CTL assays in vivo using WT C57BL/6 and TLR4 knockout mice were carried out as previously described (Laborde et al., 2017). Briefly, mice were immunized with Lp/OVA/StII twice at 12-day intervals through a subcutaneous (s.c.) injection of 0.2 mL/mouse into the inferior right limb. The doses of each component per injection per mouse were as follows: DPPC:Cho 10 μmol, OVA 50 μg, and StII 6.25 μg. Eight days after the last immunization total splenocytes of naïve C57BL/6 mice were pulsed (target cells) or not (internal control) with 1 μM of SIINFEKL in PBS/0.02 % FCS for 60 min at 37 °C and 5 % CO2. Target cells and internal control cells were also labelled with 5 μM (CFSEbright) or 0.33 μM (CFSEdull) of the fluorescent dye CFSE in PBS, respectively, for 5 min at 37 °C. CFSEbright and CFSEdull cells were mixed at an 1:1 ratio and 60 x 10^6 cells were injected intravenously (i.v.) into each immunized mouse. Sixteen hours later, the total events corresponding to CFSEbright and CFSEdull cells from the inguinal lymph node closest to the immunization site were determined by flow cytometry. The target-cell lysis

![Flow Cytometry](image-url)
percentage for each mouse was calculated as:

\[
\text{% Lysis} = 100 \times \frac{\text{CFSE}^{\text{bright}}/\text{CFSE}^{\text{null}}_{\text{vaccinated}}}{\text{CFSE}^{\text{bright}}/\text{CFSE}^{\text{null}}_{\text{PBS}}}.
\]

2.11. Statistical analysis

Data normal distribution was analyzed by Kolmogorov Smirnov test followed by the unpaired t-test (two-tailed) or Mann-Whitney’s U test. Data regarding the in vitro or in vivo activation of CD8+ T lymphocytes by StII or liposomes were analyzed using the one-way ANOVA and Tukey test as a post-test for multiple means comparison. Statistical analyses were conducted using the statistical software GraphPad Prism 5, version 5.01 (GraphPad Software, Inc.). A probability value of \( p < 0.05 \) was considered as statistically significant.

3. Results

3.1. Sticholysin isoforms share the ability of inducing DC maturation

Previously, we reported that Sts encapsulated into liposomes were able to induce a robust antigen-specific CTL response even uncoupled from their pore-forming ability. Particularly, free StII promoted the maturation of BM-DCs in vitro (Laborde et al., 2017). As a consequence, the contribution of the latter to the StII-containing liposomes’ ability of eliciting a potent antigen-specific CTL response was hypothesized. Since both isoforms, StI or StII, carried by analogous liposomes exhibited similar capacities of enhancing CTL responses (Laborde et al., 2017), we decided to also explore the potentiality of StI to activate BM-DCs such as has been demonstrated for StII.

StII induced a significant increase in the percentages of cells expressing maturation markers, the co-stimulatory molecules CD86, CD80 and CD40, when compared to the negative control (Fig. 1A, \( p < 0.01 \)). In agreement with the high structural homology reported for StII and StI (93%) (Huerta et al., 2001; Alvarez et al., 2009), such an increase was similarly observed for StI for cell percentages and mean fluorescence intensity (MFI) of the CD40 and CD80 markers (Fig. 1A and B). PmxB was used to demonstrate that the maturation of DCs was not due to the potential endotoxin contamination since Sts promote DC maturation even in the presence of this very well-known LPS-sequester (Domingues et al., 2012; Lu et al., 2017). The addition of pmbxB reduced the effect of LPS to the level of medium, which is consistent with our previous results (Laborde et al., 2017). Moreover, the DC maturation was also confirmed with the Sts purified by a Detoxi-Gel Endotoxin Removing Gel column prepurified (Supplementary Fig. S2). The endotoxin content determined by Limulus amebocyte lysate (LAL) test was lower than 20 EU/mg for both Sts purified.

In order to elucidate the importance of the tridimensional (3D) structure of Sts in their ability to induce DC maturation, we carried out a similar experiment with heat-denatured Sts (StI and StII). The efficacy of denaturation had been previously verified by the near-UV circular dichroism spectra and determination of the hemolytic activity for both proteins (Martinez et al., 2001). In the present work the Sts denaturation was confirmed by a hemolysis assay as illustrated for StII (Supplementary Fig. S3). The addition of StI and StII, mixed with pmbxB to BM-DCs significantly reduced the amount of DCs expressing the co-stimulatory molecules CD40 and CD80 when compared to the DCs treated with the native proteins. The values were equal to those obtained with LPS plus pmbxB (Fig. 1C, \( p < 0.001 \)). These results corroborate the relevance of the Sts’ 3D structure for this function.

Interestingly, the prophylactic antitumor activity previously observed for StII encapsulated into liposomes (Laborde et al., 2017) was significantly decreased in terms of tumor volume increase and tumor-free mice after tumor challenge when StI was used (Supplementary Fig. S4). This result confirms the importance of the 3D structure of Sts for their immunomodulatory properties. The remaining antitumor activity of the preparation containing StII is likely due to the adjuvant properties of this liposomal formulation containing only OVA as has been previously observed (Laborde et al., 2017). In summary, the results indicate that both proteins, StI and StII, per se share the property of stimulating BM-DCs in vitro and that this could be related to the induction of the antigen-specific CTL response by St-containing liposomes in vivo (Laborde et al., 2017).

3.2. Free Sts mixed with antigen can mediate the antigen cross-presentation on MHC-I molecules in DCs and the activation of antigen-specific CD8+ T cells

In addition to the maturation of BM-DCs, other crucial events that precede the activation of CTL are antigen processing and presentation on MHC-I molecules on these APCs (Jung et al., 2002; Williams and Bevan, 2007). We have previously shown that the presence of Sts in liposomes enhances the antigen cross-presentation by Mφs in vitro (Cruz-Real et al., 2018) and antigen-specific CTL immune responses in vivo (Laborde et al., 2017). Furthermore, non-encapsulated Sts are able to promote maturation of BM-DCs (Fig. 1 and Laborde et al., 2017). However, the capacity of these PFPs simply mixed with OVA to promote the cross-presentation of this antigen in BM-DCs in vitro has not been explored yet. Thus, we examined the presence of the OVA peptide SIINFEKL in the context of MHC-I molecules of BM-DCs from C57BL/6 mice pulsed with StII and OVA. BM-DCs incubated with this PFP plus the antigen led to the presentation of MHC-I/SIINFEKL complexes at the surface (percentage of positive cells, 16.2 ± 3.5%) which was not the case for non-stimulated cells (Fig. 2A and B, \( p < 0.01 \)). The magnitude was comparable to that promoted by the adjuvant VSSP (very small size particles) mixed with antigen (data not shown). The capacity of this nanoparticle adjuvant to facilitate this process has been well described (Mesa et al., 2006; Fernández et al., 2014).

Other essential stage for generating CTL responses that can be the consequence of the exogenous antigen-presentation by an endogenous MHC-I pathway is the priming of CD8+ T lymphocytes by APCs among other mechanisms (Kurts et al., 2010). Therefore, we evaluated whether StII simply mixed with the antigen could facilitate the activation of OVA-specific CD8+ T cells by DCs. BM-DCs previously exposed to StII plus OVA and then co-cultured with OT-I CD8+ T cells induced a higher activation of these T lymphocytes, measured as the percentage of cells expressing CD69 molecules on their surface, compared to BM-DCs that were only incubated with the antigen (Fig. 2C and D, \( p < 0.05 \)). In fact, the CD8+ CD69+ T cells percentage due to stimulation of BM-DCs with StII and OVA was equivalent to the value of the positive control (BM-DCs pulsed with SIINFEKL). This indicates that StII per se besides facilitating the exogenous antigen-presentation on MHC-I molecules in DCs promotes the priming of antigen-specific CD8+ T lymphocytes.

In order to extend the study to BM-Mφs as well as other APCs that are able to cross-prime CD8+ T cells (Asano et al., 2011) and to the isoform StI, we performed other assays employing the CD8+ T cell hybridoma B3Z cell line (Karttunen et al., 1992). After stimulating BM-DCs and BM-Mφs with the different stimuli, these APCs were co-cultured with B3Z T cells and the β-galactosidase activity was registered as an indicator of the B3Z hybridoma activation. The relative effectiveness (%) of different concentrations of StII or StI, simply mixed with a fixed concentration of OVA, of inducing the OVA-specific CD8+ T cell activation by BM-DCs or BM-Mφs is shown in Fig. 2A and B, respectively. The StII behavior observed in the experiment using OT-I CD8+ T cells (Fig. 2C and D) was reproduced with these experimental conditions. The presence of StII significantly increased the B3Z hybridoma activation mainly for the concentrations of 0.06 and 0.13 μg/mL, when compared to BM-DCs stimulated with OVA alone (discontinuous line) (Fig. 2A). StI showed an equivalent effect to StII. However, both Sts were not able to promote the activation of B3Z T cells mediated by BM-Mφs (Fig. 2B). In contrast to free Sts, liposomes encapsulating StII promoted the B3Z T cell
line activation only by BM-Mφs (Cruz-Leal et al., 2018). In summary, these results demonstrated that free Sts can mediate the activation of antigen-specific CD8⁺ T lymphocytes in vitro through BM-DCs as APCs, but not through BM-Mφs.

3.3. The immunomodulatory effect of StII is mediated through the TLR4 pathway

Considering that the Sts led to the maturation of BM-DCs as well as promoted the presentation of exogenous antigens in the context of MHC-I and that both processes are favored by the interaction of some TLRs with their respective ligands (Datta et al., 2003; de Brito et al., 2011), we hypothesized that the immunomodulatory effect of these PFPs might be associated with the activation of one or more TLRs. Therefore, we carried out maturation assays with BM-DCs from TLR2- and TLR4-knockout mice (TLR2 KO and TLR4 KO, respectively) that were incubated with StII and SIINFEKL (1 μM, positive control). Primed CD8⁺ T lymphocytes (CD8⁺ CD69⁺) were evaluated by flow cytometry. (C) Dot Plot graphs represent the percent (%) of the CD69⁺ CD8⁺ cells from a CD8⁺ gate induced by each stimulus. (D) Mean ± SD of percent (%) of the CD69⁺ CD8⁺ cells (n = 3). Statistical analysis was carried out by Tukey’s test (p < 0.05) and different letters on each bar indicate significant differences among the groups (a > b). Each experiment was performed two times with similar results.
C57BL/6 mice and TLR2 KO mice exhibited equivalent values which CD86 molecules on their surface. In contrast, the BM-DCs from WT mice were not different from those obtained with TLR4 KO mice expressing CD40 and CD86. The BM-DCs from TLR4 KO mice expressing CD40 and CD86 were significantly superior to those observed with TLR4 KO cells ( Fig. 4C ). Since LPS is able to induce signaling by TLR4 through both the MyD88-dependent and MyD88-independent/TRIF-dependent pathways ( Lu et al., 2008 ; Cannova et al., 2015 ), it did not elicit any change in the relative percentage of CD86-expressing BM-DCs from MyD88 KO mice. The results obtained with STI support the idea that Sts-mediated BM-DCs maturation occurs through the MyD88-dependent TLR4 pathway. However, we cannot completely discard the connection of the TRIF-dependent pathway with Sts-mediated DC maturation which could probably occur in a less efficient manner because in the absence of MyD88 this effect was not practically observed.

The evidence of the involvement of TLR4 in the Sts-mediated maturation of BM-DCs might presuppose its contribution to the exogenous antigen-presentation on the MHC-I promoted by free Sts ( Fig. 5A ) or encapsulated into liposomes ( Lanio et al., 2014 ; Laborde et al., 2017 ). Therefore, the impact of TLR4-dependence by STI in the CTL immune response generated by liposomes containing this PFP in vivo was also investigated. Indeed, immunization of TLR4 KO mice with Lp/OVA/STI provoked a considerably lesser cytotoxicity than that observed in WT mice ( Fig. 4D and E ), in which the CTL activity generated by this liposomal formulation was equivalent to what has previously been reported ( Laborde et al., 2017 ). These results show that TLR4 and consequently the outcome of its activation exert an influence on the CTL immune response induced by liposomes co-encapsulating STI and the antigen.

4. Discussion

An optimal vaccine-induced CTL immune response requires the introduction of exogenous antigen into the MHC-I presenting pathway of APCs and also sufficient danger signals provided by the immunogen to trigger innate immune responses including the activation of DCs, subsequent signaling from the TCR, co-stimulatory molecules, and cytokines ( Yong et al., 2012 ). In a previous work we showed that liposomes comprised of DPPC:Cho (1:1 molar ratio) and containing STI together with the antigen OVA, induced a powerful functional CTL immune response in a tumor setting in mice. Furthermore, the ability of STI to induce the BM-DCs maturation has been previously described ( Laborde et al., 2017 ). In the present work we observed that this ability is shared by the STI isoform and is dependent on the 3D-structures of these PFPs. We also showed that BM-DCs maturation by STI takes place through the TLR4 pathway and that this process has a direct influence on the CTL response induced by liposomes carrying STI.

The capacity of bacterial PFPs to induce the maturation and activation of APCs (up-regulation of co-stimulatory molecules, cytokines secretion, etc.) has been very well documented ( Park et al., 2004 ; Peng et al., 2004 ; Inden et al., 2009 ; Wallecha et al., 2013 ). In spite of the STs being evolutionary distant from bacterial PFPs ( Dal Peraro and van der Goot, 2016 ), both STI and STL were able to interact with BM-DCs, causing the maturation of these APCs in vitro in a similar manner ( Fig. 1 ). These findings are evidence of the immunomodulatory properties of STs and suggest those probably play a role in the previously reported CTL response induced by the STs-containing liposomal formulations ( Laborde et al., 2017 ). The high sequence homology (93 %) ( Alvarez et al., 2009 ) between STs could explain their similar behavior towards BM-DCs. The difference in their functionality, with STI being more active than STII in terms of cytolytic activity ( Martinez et al., 2001 ), doesn’t seem to have a real impact on the effect on BM-DCs. This is likely because the sublytic concentrations that are employed in the maturation assays are too low and the stimulation period is too long (hours, probably in a steady state) when compared to the temporal course of pore formation induced by both PFPs (min) ( Martinez et al., 2001 ; Cabezas et al., 2017 ; Soto et al., 2018 ). However, the pore formation by these PFPs was not indispensable for the CTL immune response elicited by STs-liposomes in vivo ( Laborde et al., 2017 ), we cannot completely discard its contribution to the STs.

A

B

Fig. 3. STI similar to STII simply mixed with OVA mediates activation of OVA-specific CD8+ T cells by BM-DCs but not by BM-Møs. BM-DCs and BM-Møs were stimulated with ST or STII at different concentrations (0.03-0.5 μg/ ml), mixed with OVA (25 μg/mL) and kept at 37 °C and 5% CO2 in serum-free medium. Cells pulsed with SIINFEKL (1 μM) or treated only with OVA were used as a reference. After 3 h, the APCs were co-cultured with 1 × 10^5 B3Z T cells (1:1 ratio) for 18-24 h and subsequently exposed to β-galactosidase substrate in a buffer lysis for 5 h at room temperature and in the dark. The absorbance was measured at 570 nm and 620 nm as reference. The relative efficacy (%) of each stimulus to mediate the OVA-specific CD8+ T cell activation in relation to the positive control (APCs exposed to SIINFEKL) was calculated. BM-DCs and BM-Møs only with medium (negative control) exhibited relative efficacies of 11.6 ± 0.19 and 14.4 % ± 0.23 (mean ± SD), respectively. (A) Efficacy (mean ± SD) of STs in BM-DCs. (B) Efficacy (mean ± SD) of STs in BM-Møs. Asterisks indicate significant differences between the values corresponding to each stimulus and that of OVA alone (discontinuous line) as determined by a two-tailed unpaired t-test (*p < 0.05, **p < 0.01, ***p < 0.001). Data is from a single experiment representative of two independent assays each performed in triplicate with similar results.

STII ( Fig. 4A and B ). As expected, LPS, a TLR4 ligand ( Hoshino et al., 1999 ; Poltorak et al., 1999 ) which was used as a control, did not increase the proportion of BM-DCs from TLR4 KO mice expressing CD40 and CD86 molecules on their surface. In contrast, the BM-DCs from WT C57BL/6 mice and TLR2 KO mice exhibited equivalent values which were significantly superior to those observed with TLR4 KO cells ( Fig. 4A and B ). These results and particularly the similar behavior of STII to LPS suggest the probable involvement of TLR4 in the maturation process of BM-DCs generated by these PFPs.

Upon activation of TLR4 two signaling pathways become involved, one dependent and the other independent of the adaptor molecule MyD88 ( Lu et al., 2008 ; Cannova et al., 2015 ). To elucidate the contribution of this adaptor protein to the DCs maturation induced by STI, we also stimulated the BM-DCs from MyD88 knockout (MyD88 KO) mice with STII plus pmxB. A significant decrease of the CD86 expressing BM-DCs from the mutant mice was detected, when compared with the BM-DCs from WT mice ( Fig. 4C ). Since LPS is able to induce signaling by TLR4 through both the MyD88-dependent and MyD88-independent/TRIF-dependent pathways ( Lu et al., 2008 ; Cannova et al., 2015 ), it did not elicit any change in the relative percentage of CD86-expressing BM-DCs from MyD88 KO mice. The results obtained with STII support the idea that Sts-mediated BM-DCs maturation occurs through the MyD88-dependent TLR4 pathway. However, we cannot completely discard the connection of the TRIF-dependent pathway with Sts-mediated DC maturation which could probably occur in a less efficient manner because in the absence of MyD88 this effect was not practically observed.

The evidence of the involvement of TLR4 in the Sts-mediated maturation of BM-DCs might presuppose its contribution to the exogenous antigen-presentation on the MHC-I promoted by free Sts ( Fig. 5A ) or encapsulated into liposomes ( Lanio et al., 2014 ; Laborde et al., 2017 ). Therefore, the impact of TLR4-dependence by STII in the CTL immune response generated by liposomes containing this PFP in vivo was also investigated. Indeed, immunization of TLR4 KO mice with Lp/OVA/STII provoked a considerably lesser cytotoxicity than that observed in WT mice ( Fig. 4D and E ), in which the CTL activity generated by this liposomal formulation was equivalent to what has previously been reported ( Laborde et al., 2017 ). These results show that TLR4 and consequently the outcome of its activation exert an influence on the CTL immune response induced by liposomes co-encapsulating STII and the antigen.
immunomodulatory properties. The relevance of the Sts conformational properties in the maturation of BM-DCs (Fig. 1C) could point to an additional role of the Sts native structures and/or functional activity in their immunomodulatory properties.

The immunomodulatory effects of Sts simply mixed with OVA were also supported by their ability to mediate antigen-presentation on MHC-I molecules (Fig. 2A and B) and the subsequent antigen-specific CD8 T cells activation (Fig. 2C, 2D and 3A) by BM-DCs. In fact, the CD8 T cells activation by these APCs was corroborated through two different approaches; using OVA-specific CD8 T lymphocytes from OT–I mice (Fig. 2C and D) and the B3Z T cell hybridoma (Fig. 3A). In contrast to BM-DCs, BM-MΦs that were stimulated using a mixture of free Sts plus OVA did not enhance the activation of OVA-specific CD8 T cells when compared to OVA alone (Fig. 3B). Results obtained by our group (manuscript submitted) show that contrary to Sts, LLO mixed with OVA is able to enhance the activation of antigen-specific CD8 T cells through both kinds of APCs. These findings could be suggesting different mechanisms of priming CD8 T cells for these two PFPs. An improved antigen delivery to the cytols following the classical route of processing by proteasome has been considered as an essential mechanism mediated by different forms of LLO including simple mixture with the antigen or carried by liposomes (Provoda and Lee, 2000; Dietrich et al., 2001; Mandal et al., 2004; Sun and Liu, 2013), and without underestimating its direct effect on maturation of APCs (Wallecha et al., 2013). In fact, the potential use of LLO to design a cytols-delivery system of nucleic acids based on its pore forming capacity in the endosomal compartment has also been reported (Kulberg et al., 2014; Kim et al., 2015).

Interestingly, StI in the liposomal formulation promoted the B3Z T cell line activation by BM-MΦs, although this did not happen with BM-DCs that were pre-incubated with these liposomes (Cruz-Leal et al., 2018). The inability of BM-DCs to cross-present antigen carried by this liposomal formulation has been mainly explained by the significantly lower uptake of liposomes by these cells. In summary, these observations could explain the ability of Lp/OVA/StII of improving the antigen-specific CTL immune response in vivo and are in agreement with the key role of BM-MΦs in the immune response elicited by these liposomes which has been previously described (Cruz-Leal et al., 2018). Additionally, different to what has been reported for the LLO formulation (Lee et al., 1996; Provoda and Lee, 2000; Dietrich et al., 2001; Mandal et al., 2004; Sun and Liu, 2013) the vacuolar pathway was established as the route of action for the liposomes carrying StII in BM-MΦs. A restricted degradation of the antigens and lower levels of expression of cathepsins in the endocytic compartment of BM-DCs were also considered as possible causes of the inability of these cells to cross-present OVA contained in the liposomal formulation (Cruz-Leal et al., 2018).

We do not yet have a detailed understanding of the behavior of the free Sts simply mixed with OVA in BM-DCs and BM-MΦs, but we have been able to observe the activation of antigen-specific CD8 T cell by BM-DCs upon stimulation with Sts in solution (Fig. 2 and 3A). This could be connected to the limited antigen degradation in the endocytic pathway of DCs which has been correlated with efficient antigen cross-presentation (Joffre et al., 2012). In this scenario, we could assume that Sts mixed with the antigen facilitate in some way the antigen delivery to the cytosol following the classical route of processing by proteasome has been considered as an essential mechanism mediated by free Sts in BM-DCs.
Since bacterial PFPs seem to behave as agonists of TLRs (Park et al., 2004; Inden et al., 2009; Wallecha et al., 2013) they are able to provoke the maturation of DCs and this latter effect is reproduced by Sts (Fig. 1). Therefore, an interaction with BM-DCs mediated by a specific receptor (or receptors) could also be hypothesized for these PFPs. This interaction could produce a very potent stimulus, converting these cells in fully activated and competent cells which are able to prime efficient T-cell responses (Sporri and Reis e Sousa, 2005; Kratky et al., 2011). In fact, experiments with TLR4 KO and TLR2 KO mice showed that DCs maturation by StII was dependent on TLR4 but not on TLR2 (Fig. 4A and B). Moreover, MyD88, which is the canonical adaptor for inflammatory signaling pathways downstream of members of the TLR and IL-1 receptor families and critically involved in the TLR4 signaling pathway (Deguine and Barton, 2014; Cannova et al., 2015), intervenes in the effect exerted by StII (Fig. 4C). Although we can assume a direct interaction between StII and TLR4, a TLR4-signaling pathway activation derived from a coalescence of lipid micromembranes for the first time the relation between an actinoporin and a TLR. In line with our previous proposal (Laborde et al., 2017), Sts could be binding for the first time the relation between an actinoporin and a TLR. In line with our previous proposal (Laborde et al., 2017), Sts could be binding to Toll/TLR proteins or orthologs of components of the TLR pathways found in antherozoan cnidarians (Hemmrich et al., 2007). Furthermore, we obtained important evidence that TLR4 and its derived signals contribute to the CTL response induced by Lp/OVA/StII, as this response was notably reduced in TLR4 KO mice (Fig. 4D and E). The inclusion of TLR4 ligands such as those of the TLR4 (Nordly et al., 2011), TLR3 was notably reduced in TLR4 KO mice (Fig. 4D and E). The inclusion of TLR4 ligands such as those of the TLR4 (Nordly et al., 2011), TLR3 (Varypataki et al., 2015) and TLR9 (Zaks et al., 2006) into liposomal vesicles with the purpose of enhancing the efficacy of these vesicles to stimulate competent CTL responses has been explored. The presence of TLR4 ligands in liposomes has allowed not only a noticeable CD8+ T cells activation, but also an improvement of their functionality in terms of therapeutic antitumor activity and long-term persistence, as well as an dependence of CD4+ T cell help (Zaks et al., 2006). Similar results have been formerly reported in immunized mice with Lp/OVA/StII (Laborde et al., 2017), which suggests that StII could therefore also behave as immunomodulatory molecule.

 Trafficking pathways that delay early endosome maturation and/or sorting of early endosome cargo to slowly maturing vesicles are a requirement for efficient cross-presentation. TLR4 engagement can induce a Rab34-dependent re-organization of the lysosomal distribution which delays antigen degradation and transiently enhances cross-presentation, thereby optimizing the priming of CD8+ T cell responses against pathogens (Allotti et al., 2015). As recently demonstrated, active Rab14 triggered by innate immune stimuli can recruit the kinesin KIF16b to endosomes which causes the retention of incoming material in the cellular periphery thereby delaying fusion with acidic degradative vesicles and ultimately regulating cross-presentation (Weimershaus et al., 2018). Furthermore, Nair-Gupta et al. provided some insights on MHCI recruitment from Rab11+ ERC to antigen-containing phagosomes in mouse BM-DCs upon TLR2 and TLR4 activation via the MyD88 signaling pathway (Nair-Gupta et al., 2014). Our Lp/OVA/StII formulation certainly exhibited the capacity of leading antigenic peptides to MHCI-molecules, independently of the pore-forming capacity of Sts (Laborde et al., 2017), and mainly by the vacuolar pathway at least in Mϕs (Supplementary Fig. S4 and (Cruz-Leal et al., 2018)). Both processes, the activation of TLR4 on APCs by StII released from liposomes and the enhanced capture of liposomes, carrying StII and antigens, by Mϕs (Cruz-Leal et al., 2018) likely to occur at the immunization site could synergistically improve CTL immune responses against antigens and increase the effectiveness of vaccines.

The main role of Mϕs in the CTL response induced by Lp/OVA/StII in vitro and in vivo (Cruz-Leal et al., 2018), begs the question about the role of DCs stimulated by Sts. In this scenario, a cross-talk between Mϕs and DCs could be considered as previously described by Zhang et al. (Zhang et al., 2012). Further studies are required to answer this question and fully clarify the mechanism of the StII: TLR4 connection that leads to DC maturation and the priming of CD8+ lymphocytes. In order to do so, StII mutants that cannot bind to lipids or oligomerize are currently being produced by our group for this purpose.

In conclusion, our work here has deepened our understanding of the immunomodulatory properties of Sts and further elucidated the possible ways through which these PFPs, carried by liposomes, acts. The dependence on TLR4 for DC maturation mediated by Sts and the OVA-specific CTL response induced by Lp/OVA/StII was demonstrated for the first time. The immunomodulatory properties of Sts, i.e. PFPs from non-prokaryotes organisms, make these proteins attractive tools for the development of vaccine platforms.

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CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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the activation of regulated intracellular responses that anticipates cell death.

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