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Listeriolysin O-liposome-mediated cytosolic delivery of macromolecule antigen in vivo: enhancement of antigen-specific cytotoxic T lymphocyte frequency, activity, and tumor protection

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Abstract

Cytotoxic T lymphocytes (CTLs) are primed by peptide antigens that are endogenously processed in the cytosol and presented in the context of major histocompatibility complex I (MHC I) molecules of antigen-presenting cells (APCs). Exogenous soluble protein antigens do not gain efficient entry into the cytosol of APCs, and therefore requires a special cytosolic delivery method. We have developed such a delivery strategy adopting the well-elucidated cytosol-invading listerial endosomal escape mechanism, and report here an efficient delivery of exogenous whole protein antigen into the cytosol in a mouse model. Co-encapsulation of listeriolysin O (LLO) inside liposome (LLO-liposome) was required for delivery of ovalbumin (OVA) into the cytosol of APCs in primary cultures. LLO-liposome-mediated OVA immunization in mice engendered significantly higher OVA-specific CTL activity and increased antigenic peptide-specific CTL precursor (CTLp) frequency as compared to non-LLO-liposome or soluble OVA immunizations. Interferon-γ (IFN-γ) production upon specific stimulation by MHC I-restricted peptide was also significantly stronger by the inclusion of LLO in the liposomes. Rerouting of antigen into the cytosol by LLO-liposomes, however, did not reduce the extent of anti-OVA antibody responses. Moreover, LLO-liposome-antigen vaccination was robust in conferring protection to mice from lethal challenges with antigen-expressing tumor cells. Our study demonstrates a novel delivery system for efficient introduction of exogenous protein into the cytosol in vivo, priming cellular immune responses, which are protective in nature.

Keywords: Liposomal antigen delivery; In vivo animal model; MHC I; CTL vaccine; Endosomolysis; CTLp; ELISPOT; IFN-γ response; Tumor protection

1. Introduction

Development of delivery strategies for efficient introduction of macromolecular therapeutic agents into the cytosolic compartment of cells is a critical issue [1]. Specifically, as one of the important in vivo applications of cytosolic delivery, an efficient introduction of exogenous whole protein antigen is required for induction of an enhanced antigen-specific cytotoxic T lymphocyte activity, which is a key immune response in anti-tumor and anti-viral immune therapy [2]. The cytotoxic T lymphocytes (CTLs) are primed by antigen-derived peptides that are generated in the cytosol via the proteasome-mediated pathway and subsequently presented in the context of major histocompatibility complex I (MHC I) molecules of antigen-presenting cells (APCs) to $CD8^+$ T cells [3]. Exogenous soluble protein antigens used for vaccines, however, typically do not gain efficient entry into the cytosolic compartment of cells. Therefore, development of a method that primes CTLs depends critically on a special cytosolic delivery strategy designed to circumvent endosomal degradation and overcome the membrane barrier.

Several studies have provided various methods and mechanisms leading to the delivery of exogenous proteins for MHC I-mediated presentation [4–9]. With some variations, other strategies also include live attenuated intracellular bacterial strains carrying $CD8^+$ T cell epitopes or proteins into the cytosol [10,11]. These existing approaches, however, often pose unique and inherent concerns regarding their efficacy in vivo, safety, limited ability to carry multiple copies of antigenic macromolecules, or impracticality for pharmaceutical formulation, thus limiting their clinical applications [12].

We have rendered liposomes capable of efficient cytosolic delivery by adopting the mechanism from a unique endosome-escaping, cytosol-invading bacterium, *Listeria mono-*

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cytogenes [13,14]. Liposomes, as potential universal carriers of therapeutic agents, offer several advantages with proven human applications for vaccination [15], but possess limited capability for efficient introduction of macromolecules into the cytosol. This is primarily because most liposomal formulations, unless cationic or pH-sensitive, are taken up into the endosomal compartment of APCs and by default deliver antigens predominantly into the MHC II compartments. The in vitro study by Lee et al. [16] significantly improved the efficiency of liposome-mediated cytosolic delivery by co-encapsulating purified listeriolysin O (LLO) inside liposomes and pH-sensitive liposomes. LLO is the major virulence factor and causative component for phagosomal escape of the facultative intracellular bacteria *Listeria* into the cytosol of host cells [13]. LLO-mediated endosomal escape of exogenous immunogens into the cytosolic space has also been documented using *Listerial* vector [17–20] or engineered *E. coli* [21]. Despite their success in exploiting the efficient membrane pore-forming capability of LLO, the existing LLO-based approaches, other than LLO-liposomes, may not be well suited for a universal delivery strategy because of their safety and toxicity issues, particularly with a goal toward human applications and vaccination [22]. Therefore, extending our previous in vitro observations that elucidated the concept and mechanism of LLO-liposome [16], we report here efficient in vivo cytosolic delivery of a model antigen, ovalbumin (OVA), using LLO-containing, pH-sensitive liposomes with a major focus on the MHC I-mediated induction of naïve CTL precursors for enhanced CTL response. Numerous parameters, such as augmented MHC I-restricted antigen presentation and cytokines, influence the magnitude and character of a primary $CD8^+$ T cell response [23]. To investigate further the role of cytosolic delivery in generating CTL response specific for exogenous antigen, we have monitored the frequency of antigen-specific cytotoxic T lymphocyte precursor (CTLp) by a sensitive enzyme-linked immunospot (ELISPOT) assay, and also examined several functional correlates of the immune responses such as cytokine secretion, antigenic memory, and anti-tumor ability of the CTL in vivo. The results presented here show that LLO-liposome-mediated OVA immunization generates stronger OVA-specific CTL activity, higher MHC class I OVA-peptide-specific CTLp frequency as well as primes stronger and longer-lasting interferon γ (IFN-γ) response. Importantly, we further show that the MHC I-dependent CTL response engendered by the efficient cytosolic delivery is functionally robust in protecting mice against lethal challenge of a tumor cell line expressing the specific antigen. Even though some of the antigens are routed into the cytosol away from the MHC II compartment, the antigen-specific antibody response was not diminished, implying that this delivery system can provide an extremely powerful modality of generating both cellular and humoral immune responses using whole protein antigen.

Our data provide convincing evidence for the applicability of this delivery vehicle in an animal model system, thus proving its general usefulness and suggesting potential of this broadly enabling strategy in clinical settings.

2. Materials and methods

2.1. Purification of LLO and preparation of liposomes containing LLO/OVA

Recombinant LLO (rLLO) was purified from *E. coli* strain BL21 (DE3) transformed with pET29b plasmid (Novagen, WI) carrying six histidine (His6)-tagged LLO gene (from D. Portnoy, University of California, Berkeley) using Ni-NTA affinity resin (Qiagen, CA). The His6-tagged LLO cDNA (1.6 kb) lacking the secretion signal was made by PCR incorporation of nucleotides encoding His6-residues within the 3′ PCR primer immediately before the LLO stop codon [21]. Bacterial culture was induced 4 h for LLO overexpression by isopropyl β-D-thiogalactopyranoside, and afterwards cell pellet was lysed by sonication. Lysate supernatant was adsorbed to Ni-NTA agarose and the matrix was washed extensively with PBS containing 20 mM imidazole, 1 mM PMSF, pH 8.0. His6-tagged rLLO was eluted with PBS containing 500 mM imidazole and dialyzed with 10 mM HEPES containing 140 mM NaCl, 1 mM EDTA, pH 8.3 at 4 °C. Protein purity was analyzed by SDS-PAGE, concentration by BCA protein assay (Pierce, IL). rLLO was immuno-detected by an anti-LLO rabbit serum (obtained from Dr. Portnoy; originally generated in the laboratory of P. Cossart, Pasteur Institute, France [24]), in Western blot, and its membrane pore-forming activity was monitored by hemolysis assay [16]. pH-sensitive liposomes [phosphatidylethanolamine (PE)/cholesteryl hemisuccinate (CHEMS), 2:1 molar ratio (Avanti Polar Lipids, AL, Sigma, MO)] containing OVA (grade VI, Sigma, 20 mg/ml) with or without LLO were prepared, un-encapsulated proteins removed by gel filtration using Sepharose CL-4B (Amersham Pharmacia Biotech, Sweden) as described previously [16], and the encapsulated OVA and LLO concentrations were determined by quantitative SDS-PAGE and densitometry. The size of the liposomes was measured by quasi-elastic light scattering (Nicomp, PA).

2.2. Animals, cell lines, peptides

C57BL/6J (B6) mice ($H\text{-}2^b$, 4–6-week-old female; Jackson Lab., ME) were handled according to the Institutional Guideline. The target cells EL-4 ($H\text{-}2^b$, a B6-derived thymoma) and E.G7-OVA (E.OVA, a subclone of EL-4 stably transfected with pAc-neo-OVA gene) [4] were purchased from the American Type Culture Collection (VA). Cells were grown in complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME (Life Technologies Inc., NY) with 400 μg/ml geneticin for E.OVA. OVA peptide SIINFEKL-specific $CD8^+$ T cell hybridoma (CD8

OVA T1.3, H-2K^b restricted, from Dr. C. Harding (Case Western Reserve University, Cleveland)) was maintained in complete DMEM. Murine CD8-specific monoclonal antibody (anti Lyt-2, 3.155) was obtained from Dr. J. Bluestone (University of California, San Francisco). B16, a B6-derived murine melanoma and MO5 (B16 transfected with pAc-neo-OVA gene) were obtained from Dr. K.L. Rock [25] (University of Massachusetts, Worcester). Cells were grown in complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME (Life Technologies) with 2 mg/ml geneticin plus 60 μg/ml hygromycin B for MO5.

OVA peptide SIINFEKL (amino acids 257–264) and vesicular stomatitis virus nucleoprotein peptide RGYVYQGL (amino acids 325–332) were synthesized (Research Genetics, AL), dissolved in PBS and kept at −80 °C in aliquots.

2.3. In vitro antigen presentation assay

Mouse bone marrow precursor-derived macrophages were cultured in complete DMEM containing macrophage colony-stimulating factor [16], and dendritic cells (DCs) in complete RPMI containing granulocyte-macrophage colony-stimulating factor and interleukin-4, and were enriched on day 6 using a serum column [26]. In vitro antigen delivery experiments in APCs were performed as described [16]. Briefly, APCs were plated (2×10^5/well in 96-well plates) in complete DMEM and were pulsed with liposomal OVA/LLO or OVA alone in serum-free medium for 1 h at 37 °C. Cells were washed, incubated in complete medium for 4 h at 37 °C, and then fixed with 1% paraformaldehyde. 1×10^5 OVA-specific CD8$^+$ T cells, CD8 OVA T1.3, were added to each well in 0.2 ml complete DMEM and further incubated for 24 h at 37 °C in 5% CO_2. Interleukin-2 (IL-2) concentration in the culture supernatant was measured by enzyme linked immunosorbent assay (ELISA) using Duoset IL-2 assay kit (Genzyme Corporation, MA) to monitor antigen presentation.

2.4. ^{51}Cr release assay for OVA-specific CTL activity

Mice (4–6 mice/group) were immunized via subcutaneous (s.c.) or intravenous (i.v.) route on day 0 and day 12 with OVA in various formulations. Amount of injected OVA ranged 35–50 μg over three independent sets of experiments, and was kept constant in the experimental and control groups in each set. Splenocytes (responders) were isolated 9–12 days after boost, and were restimulated in vitro for 5 days with mitomycin-C (50 μg/ml, 4×10^7 cells for 25 min at 37 °C)-treated E.OVA cells (stimulators), as described [4] without exogenous IL-2. Viable T cells separated on a Ficoll gradient were used as effector cells in a standard 4-h CTL assay [4] with ^{51}Cr (Amersham Pharmacia Biotech, IL) labeled E.OVA, EL-4, or EL-4 pulsed with SIINFEKL (10 μg/ml) during ^{51}Cr labeling for 1.5 h at 37 °C, as target cells. Released ^{51}Cr from target cells was measured in a gamma counter and specific cytolytic activity was determined using the formula:

$$\%\ \text{specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

The spontaneous release was routinely between 5% and 15% of the total release of ^{51}Cr from the target cells by detergent lysis.

2.5. ELISPOT assay

Antigen-specific IFN-γ-secreting effector T cells were quantified by an ELISPOT assay [27] using the paired anti-IFN-γ antibodies R4-6A2 for capture and biotinylated XMG1.2 for detection (PharMingen, CA). MAHA-S45 plate (Millipore, MA) was coated with the anti-IFN-γ capture antibody in sterile PBS overnight at 4 °C, and blocked with 2% bovine serum albumin in PBS at room temperature. Splenocytes from immunized mice were depleted of red blood cells with Gey's hemolytic solution and cultured with SIINFEKL or RGYVYQGL (10 μg/ml) for 24 h at 37 °C in the plate. Spots were detected by adding alkaline-phosphatase-conjugated streptavidin (PharMingen) and visualized by adding 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate. The frequencies of IFN-γ-producing cells in each well were determined using a computerized ImmunoSpot Image Analyzer (Cellular Technology, OH).

2.6. Antigen-specific IFN-γ production

Antigen-specific cytokine response was determined by culturing the splenocytes (1×10^7/ml) from the immunized mice in the presence of OVA peptide SIINFEKL (10 μg/ml), nonspecific peptide RGYVYQGL (10 μg/ml), or Con A (5 μg/ml) in 96-well plates for 24 or 48 h [28]. IFN-γ concentration in the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA) performed in triplicate using the paired monoclonal antibodies (PharMingen). IFN-γ concentration was calculated based on the recombinant mouse IFN-γ (Genzyme Corporation, CA) standards and expressed as IFN-γ units/ml. For assessment of antigenic recall memory, IFN-γ secretion was measured upon specific peptide stimulation to the splenocytes harvested from immunized mice 14.5 weeks post-boost.

2.7. OVA-specific antibody titer

OVA-specific IgG titer in immunized mice serum was measured by ELISA using OVA (10 μg/ml)-coated plates (Immulon-II, Nunc, Denmark). Dilutions (1:1000, 1:10 000, 1:20 000) of the serum samples (pre-immune, pre-boost, and

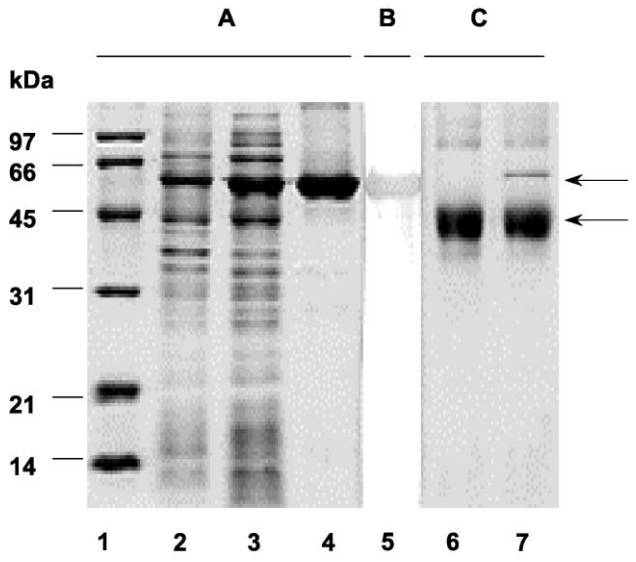

Fig. 1. Purification of rLLO and liposome-encapsulated proteins. (A) SDS-PAGE of Ni-NTA affinity-purified rLLO; (B) immunoblot of purified rLLO; and (C) liposome-encapsulated OVA or OVA/LLO separated by gel filtration chromatography. Lanes: 1, molecular weight markers; 2, bacterial lysate pellet; 3, bacterial lysate supernatant; 4, purified rLLO; 5, immunoblot of rLLO; 6, liposome-encapsulated OVA; 7, liposome-encapsulated OVA/LLO. Upper and lower arrows indicate the position of LLO and OVA, respectively.

post-boost) obtained at different time points were added, and OVA-specific antibody was detected with goat anti-mouse IgG (Fab′)2-biotin (Sigma Immunochemicals, MO). The absorbance (O.D.) was read at 405 nm after developing alkaline phosphatase-streptavidin with *p*-nitrophenyl phosphate (Sigma, MO).

2.8. In vivo tumor protection assay

Antigen-specific tumor protection assay was performed as described [25] with modifications. Mice were immunized s.c. twice at 12-day interval and challenged on day 7 post-boost via intradermal (i.d.) injection bilaterally with 2×10^5 melanoma cells, B16 or MO5 in 100 μl Hanks' balanced salt solution. Tumor growth was monitored every 2–3 days by measuring two largest perpendicular diameters, and the product was recorded as tumor size/area. Mice that became moribund were euthanized following the guidelines, and survival was scored as the percentage of surviving animals.

2.9. Statistical analysis

Wilcoxon rank-sum (Mann–Whitney) test was applied to determine the statistical significance (*P*-value) of the in vivo experimental results among various groups. The Kruskal–Wallist test was used to assess the *P*-value between liposome formulations containing different concentrations of LLO in the in vitro antigen presentation assay.

3. Results

3.1. Protein delivery into APCs in vitro

The LLO-liposome delivery vehicle was characterized and optimized, before testing in mice, using the in vitro antigen presentation assay in primary cultures of murine macrophages and DCs. Affinity-purified, histidine-tagged recombinant LLO migrated at ~ 58 kDa as a major band in SDS-PAGE (Fig. 1 panel A) and used for encapsulation in liposomes. The purification was confirmed by Western blot using a polyclonal anti-LLO serum (panel B) and tested for its functional activity by hemolysis assay (data not shown). Hemolytic activity of purified rLLO per microgram of protein, stored in aliquots at − 20 °C, was retained at similar levels over a period of months and its variability among different batches was minimal (data not shown). Liposome-encapsulated OVA or OVA/LLO was separated from non-encapsulated species by gel filtration chromatography (panel C). On average, the encapsulation efficiency was between 7% and 15% and the mean diameter of the liposomes was in the range of ~ 240–370 nm (SD ± 118–180) as determined by particle size analysis.

The extent of cytosolic delivery of the model antigenic protein OVA by LLO-liposomes was dose-dependent of OVA and concentration-dependent of LLO encapsulated inside

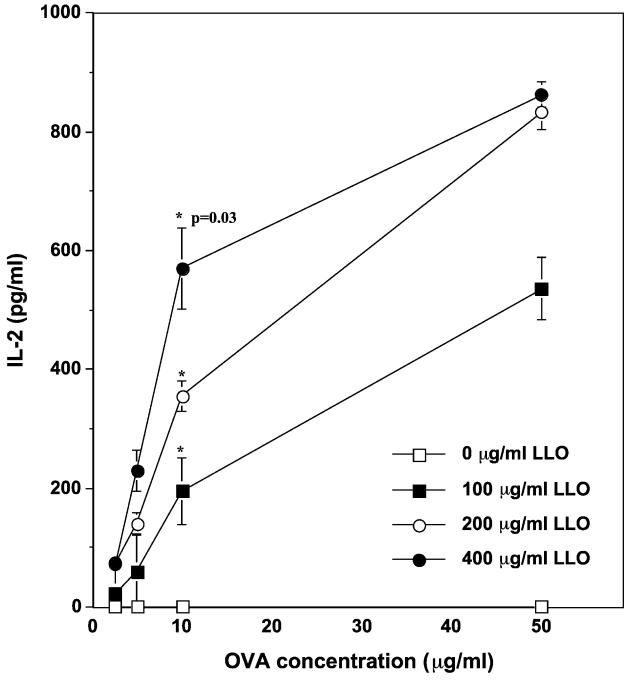

Fig. 2. rLLO-dependent cytosolic delivery of OVA in vitro in APCs. Macrophages were pulsed with liposome-encapsulated OVA containing varying concentrations of LLO (100–400 μg/ml), washed, and incubated for 4 h at 37 °C. Cells were then fixed by 1% paraformaldehyde, and co-cultured with OVA-specific $CD8^+$ T cells for 24 h. The extent of cytosolic OVA-derived peptide presented to the T cells was measured in triplicate by IL-2 ELISA. *P*-value is shown at 10 μg/ml OVA concentration.

liposomes (Fig. 2). With increasing LLO concentrations encapsulated inside OVA-containing, pH-sensitive liposomes, higher levels of MHC class-I (H-2K^b)-binding OVA-peptide, SIINFEKL, were presented by the APCs to CD8$^+$ OVA-specific T cells, as detected by higher levels of IL-2 secreted by the T cells, with an optimum range (200–400 μg/ml) of LLO concentration for efficient cytosolic delivery (Fig. 2). No IL-2 was detected when cells were incubated either with non-liposomal soluble OVA or with OVA encapsulated in non-LLO-liposomes, demonstrating that co-encapsulation of LLO is required for introduction of exogenous OVA into the cytosol of APCs. When macro-

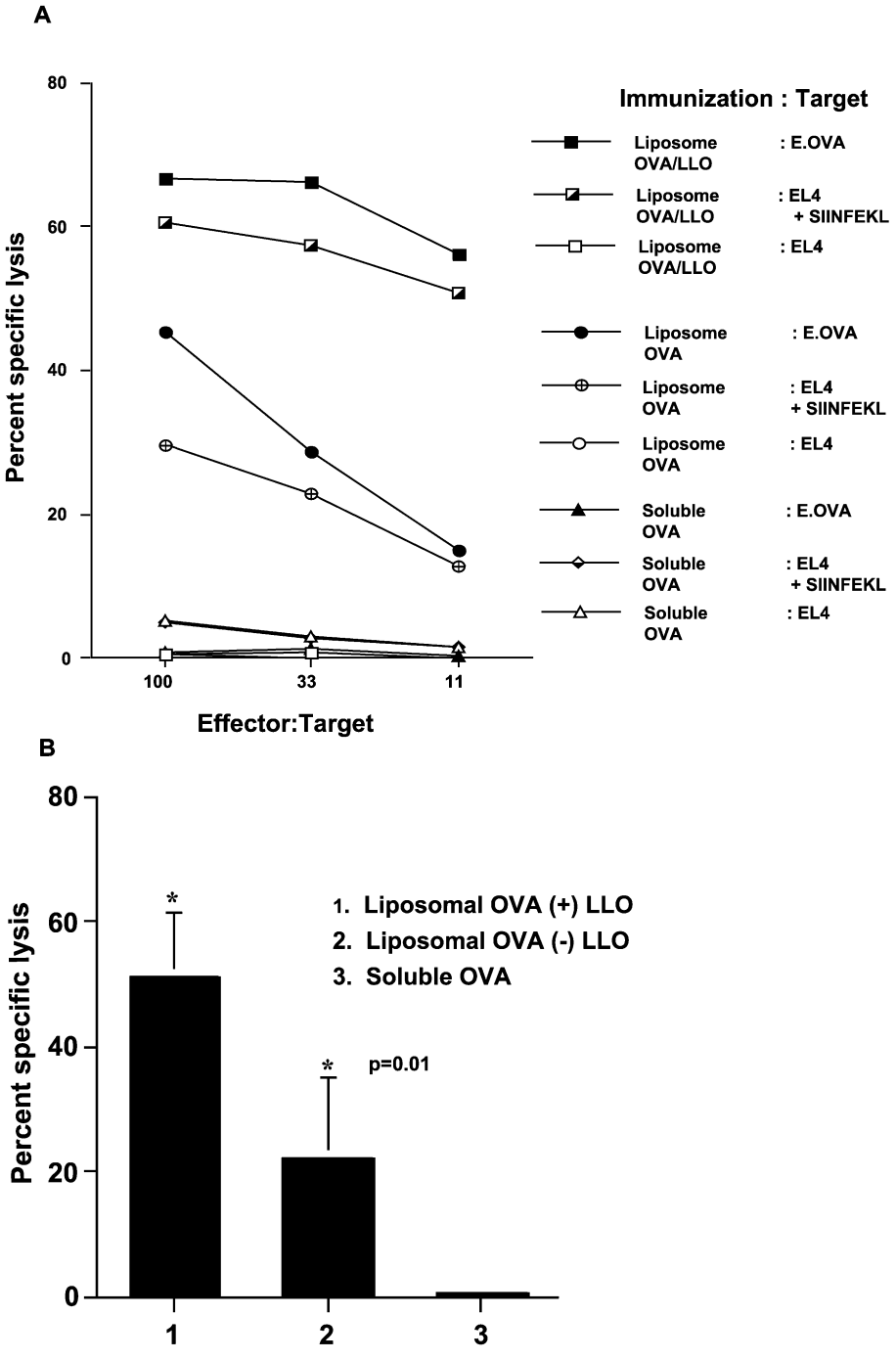

Fig. 3. Strong OVA-specific cytolytic activity generated by LLO-liposome-OVA immunization. (A) Mice were immunized s.c. with liposome-encapsulated OVA, with or without LLO, or free soluble OVA, and monitored for OVA-specific CTL by the standard ^{51}Cr release assay. Each data point represents the mean cytolytic activity of triplicate cultures of the splenocytes pooled from two immunized mice in each group ($n=4$). The representative data are from one set of immunization, and experiments were repeated three times with similar results. (B) Analysis of OVA-specific CTL assay results from three independent sets of s.c. immunization experiments at 11:1 effector/target ratio ($n=12$ per group).

phages derived from BALB/c mice (H-2K^d background) were used in this assay, no OVA presentation was observed (data not shown). Similar LLO-dependent delivery results were observed with DCs; when OVA-pH-sensitive liposomes were tested with and without co-encapsulated LLO at 250 μg/ml concentration, efficient cytosolic delivery was demonstrated in both macrophages and DCs in an LLO-dependent manner albeit with different overall efficiencies (data not shown).

3.2. Enhanced induction of OVA-specific CTL response by LLO-liposome-mediated OVA delivery in vivo

The above in vitro experiments in APCs suggested a hypothesis that the LLO-liposomal delivery system would efficiently introduce exogenous proteins into the cytosolic pathway of antigen presentation in vivo. This was tested by monitoring the induction of antigen-specific CTL responses in mice using OVA-pH-sensitive liposomes with and without LLO. CTL assay results (Fig. 3A) show that s.c. immunizations with OVA in the pH-sensitive, non-LLO-liposomes generated relatively low but detectable levels of OVA-specific CTL over the controls, free soluble OVA immunization or placebo, as has been shown previously [8]. The OVA-specific CTL activity, however, was significantly enhanced using LLO-liposomes over non-LLO-liposomes. The cytolytic activity was MHC I (H-2K^b)-restricted OVA peptide (SIINFEKL)-specific. This significant augmentation upon LLO co-encapsulation ($P=0.01$), as examined in three independent sets of experiments (summarized in Fig. 3B), was in concordance with our hypothesis and the in vitro results. Similar results were obtained with i.v. immunizations (data not shown) demonstrating that LLO-liposome-mediated cytosolic delivery is not restricted to any particular route of administration. Treatment of the effector cells with anti-murine CD8 monoclonal antibody (mAb) during the 4-h incubation period of CTL assay effectively inhibited their cytolytic activity; 87.6 % inhibition was observed at 100:1 effector/target ratio.

3.3. Increased antigen-specific T cell frequency and enhanced antigen-specific cytokine production by LLO-liposome-OVA immunization

We determined the antigen-specific CTLp, after immunization with soluble OVA or liposomal OVA with or without LLO co-encapsulation, using antigen-specific IFN-γ ELISPOT assays. This is to test if the observed higher CTL activation by LLO-liposomal OVA immunization, as monitored by the standard ^{51}Cr release assay, is indeed due to an increase in OVA-specific CTLp frequency. Freshly isolated splenocytes from the immunized mice was stimulated with the immuno-dominant, MHC class I H-2K^b restricted OVA peptide SIINFEKL [4] and analyzed for IFN-γ-secreting cells. A significantly higher frequency of SIINFEKL-specific IFN-γ spots was observed ($P<0.05$) in the splenocytes isolated from the mice immunized with LLO-liposome-OVA as compared to that with non-LLO-liposome-OVA immunization (Fig. 4A).

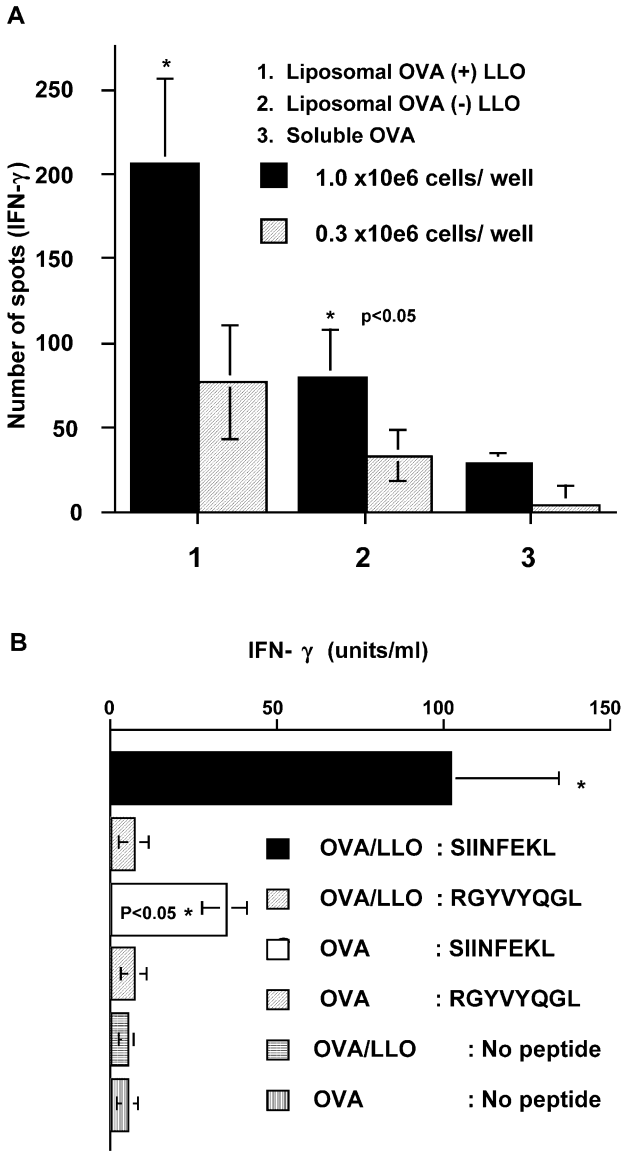

Fig. 4. Higher CTLp frequency (A) and enhanced IFN-γ production (B) generated by LLO-liposome-OVA immunization. (A) Mice were immunized s.c. with liposome-encapsulated OVA, with or without LLO, or free soluble OVA twice at 12-day interval, and monitored for OVA-specific CTLp frequency determined by ELISPOT assay between days 9 and 12 post-boost. Result is shown as IFN-γ-specific spots per 10^6 or 0.3×10^6 cells/well (mean ± S.E.); four mice per group in each experiment, and experiment was repeated twice with similar results. (B) Enhanced production of IFN-γ by the spleen cells from the mice immunized with LLO-liposome-OVA ($*P<0.05$). Mice were immunized i.v. with LLO-liposome-OVA or non-LLO-liposome-OVA twice at 12-day interval. Spleen cells were harvested from the immunized mice, and stimulated in vitro either with OVA peptide (SIINFEKL) or a control peptide (RGYVYQGL), at 10 μg/ml. IFN-γ in the culture supernatant after 48-h culture was measured by ELISA. Some of the wells were also treated with Con A (5 μg/ml) as a positive control (data not shown).

IFN-γ production by the splenocytes harvested from the immunized mice, upon stimulation by the SIINFEKL, was monitored as an additional measure of the cellular immunity generated by LLO-liposome-mediated OVA immunization. Splenocytes from the mice immunized with LLO-liposome-OVA secreted significantly higher ($P < 0.05$) levels of IFN-γ (mean 101.9 SD ± 32.4 units/ml) than those from the non-LLO-liposome-OVA immunized group (mean 34.3 SD ± 6.8

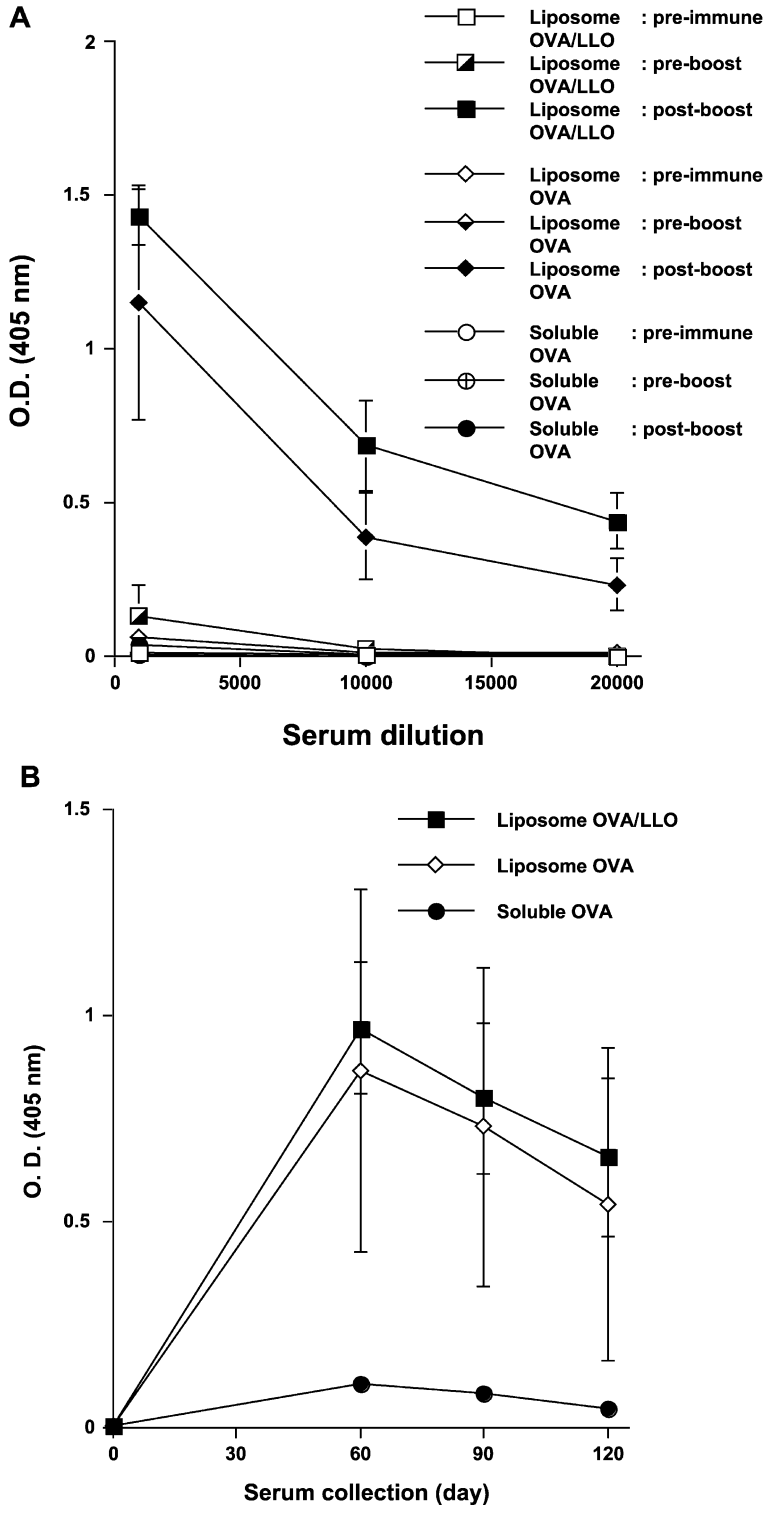

Fig. 5. OVA-specific IgG response. (A) Mice were immunized as described in Fig. 3, and OVA-specific IgG titer in the serum samples of the immunized mice was determined by ELISA. (B) Long-term OVA-specific IgG response post-boost was monitored by ELISA over a 4-month period. Data shown as mean O.D. (± SD) of the serum samples obtained at the indicated time points ($n = 8$ per group, A; $n = 4$ per group, B).

units/ml) (Fig. 4B). Addition of a control peptide, RGYVYQGL, to the splenocytes stimulated very low levels of cytokine produced in both groups, which were similar to the basal level of IFN-γ generated by splenocytes without addition of peptide. Spleen cells from both groups exhibited strong cytokine response when treated with the T cell mitogen, Con A (data not shown). Long-term antigen-specific memory was assessed by monitoring IFN-γ response of the splenocytes harvested at 14.5 weeks post-immunization and stimulated by the specific peptide SIINFEKL ex vivo. A dramatic difference was also observed in the IFN-γ production between LLO-liposome-OVA and non-LLO-liposome-OVA immunized groups, demonstrating the prolonged and superior antigen-specific immunologic memory induced by the LLO-liposomal formulation over the non-LLO-liposomal formulation (data not shown).

3.4. OVA-specific antibody elicited by LLO-liposome-OVA immunization

OVA-specific antibody titer was monitored from the immunized mouse serum samples, concurrently with the CTL assays, to investigate the effect of routing a significant fraction of the internalized antigen from endocytic compartments into the cytosolic pathway of antigen presentation. Liposomal OVA immunizations induced comparably strong

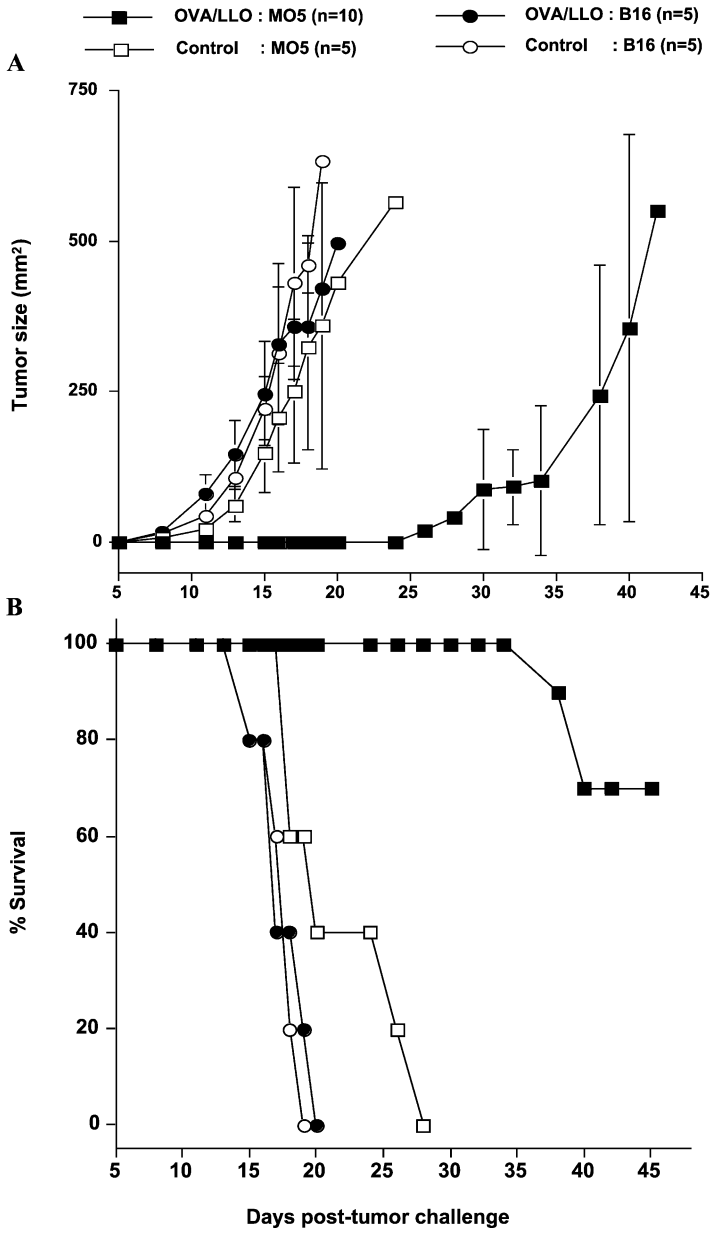

Fig. 6. OVA-specific anti-tumor protective immunity. (A) Mice were immunized as in Fig. 3, and were challenged 7 days later (day 0) with 2×10^5 B16 or MO5 melanoma cells. Data shown as average tumor area (± S.D.) are from one representative experiment of three independent sets. Each set of experiments included 5–10 mice/group. (B) Prophylactic vaccination with LLO-liposome-OVA increases overall survival in response to tumor challenge.

levels of OVA-specific antibody responses regardless of LLO co-encapsulation inside the liposomes (Fig. 5A), in comparison to minimally detectable antibody levels with the non-liposomal, soluble OVA. Fig. 5B shows the long-term anti-OVA IgG response monitored over a period of 120 days post-immunization. Anti-OVA antibody response remained robust during this period for both groups immunized with liposomal OVA.

3.5. LLO-liposomal OVA immunization engenders protective immunity

The ability of LLO-liposome-induced antigen-specific CTL in conferring protective immunity was evaluated in a murine melanoma model. Mice were immunized s.c. with LLO-liposome-OVA and challenged i.d. at multiple lethal doses, approximately 20 times higher, with MO5 melanoma cells, a stable clone of OVA-expressing B16, or with the parental B16 melanoma cells. LLO-liposome-OVA immunization elicited strong OVA-dependent anti-tumor immunity as the immunized mice were protected from MO5 challenge, but not from the parental B16 challenge (Fig. 6A). A significant percentage of LLO-liposome-OVA immunized mice survived and remained tumor-free with MO5 challenge but not with B16 (Fig. 6B), in comparison to placebo immunized control mice, which were equally susceptible to B16 and MO5 challenge. The control groups of mice rapidly developed tumors (by day 12) and most of them died by day 18–20. Furthermore, approximately ~30–40% of the LLO-liposome-OVA immunized mice remained tumor-free and survived until day 150, when the experiment was terminated (data not shown).

4. Discussion

Development of a vaccine delivery strategy for functionally robust CTL responses relies critically on the ability to introduce exogenous antigenic proteins efficiently into the cytosolic compartment of APCs. Our data on antigen-specific CTL responses in mice demonstrate that the unique utilization and formulation of LLO inside antigen-carrying, pH-sensitive liposomes achieves this objective. This new method of cytosolic delivery and consequent CTL induction involves uptake of liposomal exogenous antigen into the endocytic compartment of APCs and subsequent LLO-mediated release of the endosomal contents into the cytosol for classical cytosolic/MHC I pathways of antigen processing and presentation, as previously documented in vitro using a proteasome inhibitor and brefeldin A [16].

Expression of histidine-tagged LLO in *E. coli* and its one-step affinity column purification allowed us to purify the protein in large quantities. The purified recombinant LLO was characterized by SDS-PAGE, Western blot (Fig. 1), and hemolysis assay for its functional activity, which has been also confirmed by Gedde et al. [29]. As *E. coli*-based expression system was used for rLLO used in this study, it was difficult to eliminate the minute amounts of LPS in the rLLO preparations and the liposomes. However, the contaminating level of LPS in the liposomes, with or without encapsulated rLLO in comparison with no treatment, was observed to have no detectable effect on macrophage activation as monitored by cytokine mRNA induction (Lee and Portnoy, unpublished data). As pH-sensitive PE/CHEMS liposomes containing recombinant LLO was characterized and optimized using the in vitro OVA presentation assay in both macrophages and DCs, the extent of cytosolic delivery of OVA by LLO-liposomes was concentration-dependent of LLO encapsulated inside liposomes (Fig. 2). It should be noted, consistent to the previous finding by Lee et al. [16], that the presence of CHEMS in the liposome formulation did not inhibit the effect of LLO although cholesterol has been shown to block the LLO activity [14]. The requirement for LLO in inducing MHC class I-mediated antigen presentation was absolute in both APCs. Moreover, LLO-liposome-mediated cytosolic delivery was highly efficient at low OVA concentrations (~ μg/ml), as compared with the ~ mg/ml antigen concentrations previously reported to be obligate for fluid-phase internalization-mediated, MHC class I-restricted OVA presentation [4,12,30]. Our in vitro data using two major APCs clearly demonstrate LLO as a necessary and sufficient component in pH-sensitive liposomes for efficient cytosolic delivery of exogenous antigenic protein, further supporting our previous report on LLO-liposome-mediated cytosolic delivery of macromolecules [16] and extending its applicability to other cell types.

Subcutaneous immunization, which is a preferred route in a vaccination protocol, generated overall stronger CTL responses than i.v. immunization, although both routes of administration engendered efficient LLO-liposome-mediated antigen delivery. One possible explanation for the differences observed is that the clearance kinetics and biodistribution of PE/CHEMS liposomes depend on the sites of injection. The induced CTLs, predominantly of $CD8^+$ phenotype as evidenced by the anti-CD8 mAb blocking experiment, did not require exogenously added IL-2 for their growth and proliferation ex vivo, suggesting strong adjuvant effects of liposomal immunization [15] in comparison to other previously reported OVA model systems [4,28].

ELISPOT analysis of CTLp frequency through the quantification of MHC I peptide-recognizing T cells allowed a direct comparison of the CTL priming ability between the immunogenic formulations. Previous studies indicated strong correlation between the specific lysis of antigen-expressing targets in a ^{51}Cr release CTL assay and the frequency of MHC I peptide antigen-stimulated, IFN-γ-secreting T cells [31]. Therefore, OVA-specific CTLp frequency was determined by OVA MHC I peptide SIINFEKL-stimulated IFN-γ ELISPOT assay as a functional surrogate for identification of CTLp and to test if the observed higher CTL activation by LLO-liposomal OVA immunization, as monitored by the standard

^{51}Cr release assay, is indeed due to an increase in OVA-specific CTLp frequency. LLO-liposome-OVA was clearly superior to non-LLO-liposome formulation in generating OVA-specific CTLp (Fig. 4A), which is in good agreement with their respective CTL activities monitored (51.5% lysis versus 22.1% lysis; $P = 0.01$, Fig. 3B). Therefore, the results from ELISPOT assays evidence and confirm the enhanced cytosolic delivery of antigen by LLO-liposome in vivo. The observed increase in exogenous antigen-specific CTLp frequency is of particular significance in a disease condition.

Cytokines produced during immune responses, including IFN-γ, play an important role in the induction of immunity, as they are produced by complex interactions of a variety of immuno-competent cells including T cells and APCs. IFN-γ is one of the major cytokines implicated in the priming of T cells contributing to augmented CTL responses [32], thus rendering antigen-specific IFN-γ-producing T cells as one of the critical effector T cell populations [33]. The ability of splenocytes to produce OVA-specific IFN-γ upon SIINFEKL peptide stimulation was dramatically higher and longer-lasting when the splenocytes were from the mice immunized with LLO-containing formulation. This result is in good correlation with and complementary to the superior CTL activity induced by LLO-liposomal OVA immunization, noting that the stimulating peptide SIINFEKL is a MHC I-restricted (H-2K^b) epitope of OVA [4]. Antigenic memory of the splenocytes from the LLO-liposome-immunized mice was well retained over 3 months after the boost (data not shown), demonstrating a very effective and long-lived robust immune response, which is a hallmark of a good vaccine formulation. Although direct evidence is lacking, however, we cannot rule out that the prolonged antigenic T cell memory could also be ascribed to the immunologic activities of LLO other than its major function of endosomolysis. It has been documented that *L. monocytogenes* or LLO induces IL-12, which enhances CTL activity, stimulates IFN-γ production, and augments cell-mediated immunity in mice [33,34], implying a potential adjuvant role of LLO in the LLO-liposome immunized mice in our system.

As our primary objective of LLO-liposome-mediated cytosolic antigen delivery is to augment CTL responses and maintain prolonged cell-mediated immune responses, it is critical to assess whether the antigen-specific antibody response, immediate and long-term, is diminished due to the less amount of antigen being available to the MHC II compartment. LLO-liposomal OVA immunization generated and maintained a strong antibody response, which was comparable to that generated and maintained by non-LLO-liposomal OVA (Fig. 5). This indicates that LLO, at the studied concentration inside pH-sensitive liposomes, was unable to deliver all of the liposomal contents into the cytosol, thereby also providing non-escaped antigens to the MHC II pathway for priming humoral immunity. This dual modality of the liposomal immunization was reported for both pH-sensitive and cationic liposomes as well [8,35], although humoral immunity in these cases was generated more as a default pathway.

The results obtained from CTL and ELISPOT assays evidenced superior immunogenic ability of LLO-liposome-OVA formulation in ex vivo assays. Lethal tumor challenge provided the opportunity to observe in vivo protection in the immunized mice. The observed antigen-specific protection from tumor in LLO-liposome-OVA immunized mice was similar in nature to that of Falo et al. [25], but achieved with less amount of antigen for immunization and with more lethal tumor challenge. A similar level of tumor protection was also reported by Davila and Celis [36] where mice had to be injected repeatedly (nine times) with CpG ODN along with OVA helper T lymphocyte epitope emulsified in incomplete Freund's adjuvant and challenged with 20 times less (5×10^4) B16-OVA cells than our protocol. It has been demonstrated that IFN-γ, a T cell-derived cytokine, promotes tumor rejection in a T cell-dependent mechanism [37]. Mice vaccinated with LLO-liposome-OVA generated significantly stronger CTL, higher CTLp frequency, and OVA-specific IFN-γ responses over controls and were protected from MO5 challenge, thus, indicating a significant role of the OVA-specific CTLs in the in vivo killing of MO5, resulting in the delay of tumor growth and increased survival over control group of mice.

Mice injected with LLO-liposome-OVA did not show any apparent toxicity, monitored over 6 months post-vaccination (data not shown). Thus, along with less potential danger of pathogenic outcomes, LLO-liposome-OVA would be superior to other documented use of *L. monocytogenes* as a delivery vehicle that has the safety concerns regarding chromosomal integration [22]. While other existing methods for the induction of CTL responses against exogenous protein antigen depend on either somewhat ill-defined 'leakage' or nonspecific 'disruption' of endosomes or plasma membranes, or 'regurgitation' of a fraction of endosomal antigenic peptide contents and thus require relatively high concentrations of antigen [12,35], our current study demonstrates an efficient strategy for the cytosolic delivery of protein. This unique strategy exploits a well-defined endosomal escape and cytosol-invading mechanism that has been developed by the facultative intracellular bacterium *L. monocytogenes* through evolution to achieve a pathogenic niche in the cytosol of cells.

In conclusion, the results of the present study strongly demonstrate the unique potential and efficacy of LLO-liposome as a vaccine delivery system that is efficient in introducing exogenous antigenic proteins into both MHC classes I and II pathways of antigen presentation, thus inducing both arms of immunity. This is the first in vivo report utilizing recombinant LLO inside pH-sensitive liposomes as exogenous antigen delivery system, proving its utility and superior ability in animal models over the best currently available liposomal formulation for cytosolic delivery of macromolecules and demonstrating its potential as tumor vaccine delivery vehicle. This nonviral/nonbacte-

rial delivery vehicle is able to carry exogenous proteins, thus making it an attractive, universal carrier for vaccine-based immunotherapy against intracellular pathogens or cancers, as well as for cytosolic delivery of macromolecular therapeutic agents.

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