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Cytosolic Delivery of Viral Nucleoprotein by Listeriolysin O-Liposome Induces Enhanced Specific Cytotoxic T Lymphocyte Response and Protective Immunity

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Abstract: Cytotoxic T lymphocytes (CTLs) are capable of conferring protection against intracellular pathogens and tumor. Protective antiviral immunity, mediated by the activation of antigenic epitope-specific CTL, can be achieved by delivering exogenous antigen into the cytosol of antigen-presenting cells. Cytosolic introduction of vaccine antigen, however, requires a specialized delivery strategy due to the membrane barrier limiting the access of macromolecules to the cytosol. In this study, we have investigated the potential ability of listeriolysin O-containing liposomes (LLO-liposomes) to deliver lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP), harnessing the intracellular invasion mechanism of *Listeria monocytogenes*, to stimulate a NP-specific CTL response. We have analyzed the ability of LLO-liposomes to induce an enhanced CTL response and determined the extent of CTL-mediated protection using an *in vivo* infection model. Mice immunized with LLO-liposomes containing NP generated a higher frequency of NP-specific $CD8^+$ T cells with greater effector activity than the control groups immunized with either non-LLO-liposomal NP or LLO-liposomes containing control protein. Moreover, LLO-liposomal NP-immunized mice were completely protected against a lethal intracerebral challenge with a virulent strain of LCMV and were capable of clearing a chronic LCMV infection. Our study demonstrates that LLO-liposomes can be used as an efficient vaccine delivery system carrying a viral antigenic protein to generate protective antiviral immunity.

Keywords: Viral antigen delivery; antiviral immunity; listeriolysin O; liposomes; CTL response; CTL frequency; LCMV infection; protection

Introduction

Adaptive immunity mediated by cytotoxic T lymphocytes (CTLs) is important and often essential in the protection and clearance of viral infections and tumors. Thus, it is critical that vaccines are capable of inducing CTLs as a part of the immune response. Antigen-specific CTLs are induced and activated by recognizing antigenic peptide fragments in complex with major histocompatibility complex I (MHC I) molecules on the surface of professional antigen-presenting cells (APCs). These antigenic peptides are typically generated via the cytosolic pathway of antigen processing and presentation,[1,2] and thus are derived primarily from endogenous proteins, but under unique situations from exogenous proteins delivered to the cytosol. Immunizations with exogenous protein vaccine antigens fail in most cases to induce strong CTL responses because of the limited accessibility of the protein to the cytosol; protein antigens must circumvent the plasma and endosomal membrane barrier to gain efficient

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access to cytosolic antigen processing and presentation. Therefore, one key focus for protein vaccine development, with the goal of generating the requisite robust CTL response for protection, must be the development of a nonbacterial, nonviral, nonreplicative, safe and efficient vaccine delivery strategy that can be readily adapted to the clinical setting and can overcome this limitation of cytosolic delivery.[3−5]

By adopting the phagosome escape mechanism mediated by the endosome-disrupting protein listeriolysin O (LLO) of *Listeria monocytogenes* (LM),[6−8] we have developed a listeriolysin O-containing liposome (LLO-liposome)-based cytosolic delivery system that can be used to deliver antigenic macromolecules to the MHC class I processing pathway. The utility of the LLO-liposomes as vaccine carriers has been demonstrated both *in vitro* and *in vivo*. LLO-liposomes efficiently introduced exogenous antigen into MHC I/cytosolic processing and presentation in macrophages[9] and generated a strong antigen-specific CTL response in mice that ultimately conferred protection against antigen-expressing lethal melanoma challenge.[4] Whole protein antigen-based immunization combined with such a delivery strategy offers several advantages over peptide-based immunization for CTL activation: the identification of MHC-restricted peptide epitopes is not necessary; vigorous polyclonal responses can be generated toward a broad antigenic repertoire, while synthetic peptides are poor immunogens with a short serum half-life.[10]

The study presented here was designed to test the hypothesis that LLO-mediated cytosolic delivery of viral antigen *in vivo* augments CTL responses and consequently confers protection against lethal and chronic LCMV infections. The mouse LCMV infection model used in our study has been well-characterized and extensively used to study vaccine-induced CTL-mediated protection and viral clearance.[11−13] It should also be noted that priming LCMV-NP-specific $CD4^+$ T cell response or LCMV-specific antibody response alone does not confer protection against LCMV challenge,[14] thus providing the mechanistic basis of utilizing this model to assess the protective ability of cytosolic delivery-mediated enhanced CTL induction. Our results demonstrate that LLO-liposomes carrying LCMV-NP are capable of inducing significantly enhanced NP-specific CTL activity and frequency that conferred complete antiviral protective immunity in mice, which is in good correlation with the enhanced NP-specific CTL response. Recombinant LCMV-NP encapsulated in LLO-liposomes efficiently induced a NP-specific CTL response in mice that resulted in survival following intracerebral challenge with LCMV and clearance of virus after a chronic infection with LCMV. Thus, LLO-liposomes constitute a promising nonviral and nonreplicating vaccine delivery strategy capable of cytosolic introduction of antigen with a defined LLO-based endosomolytic mechanism that can be readily adapted to carrying various vaccine candidate molecules and results in the induction of a strong and protective CTL response.

Experimental Section

LCMV-NP Expression, Purification, and Encapsulation in Liposomes. A truncated LCMV-NP was expressed as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli*. DNA encoding truncated LCMV-NP (amino acid residues 82−173) fused to GST was obtained from D. Portnoy (University of California, Berkeley, CA) and used for this study. This was generated by polymerase chain reaction (PCR) amplification of a full-length LCMV-NP

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gene.[13] The primers used for PCR were as follows: CTGCACTTGGGATCCACATCAAAGAAGAATGTTTTGAAAGTG for the forward primer containing a *Bam*HI restriction site (underlined) and CCATGAGCGAATTCTGCCAAATTGATTGTTCAAAAGTCATGA for the reverse primer containing an *Eco*RI site (underlined). The PCR product was digested with *Bam*HI and *Eco*RI and inserted into the carboxy terminus of glutathione *S*-transferase (GST) in the pGEX-2T vector (Amersham Pharmacia Biotech, Piscataway, NJ) using *Bam*HI to *Eco*RI sites, and the sequence was confirmed by DNA sequencing. *E. coli* strain BL21(DE3) (Novagen, Madison, WI) transformed with the plasmid was induced at 30 °C with 0.1 mM isopropyl β-D-thiogalactoside for 3.5 h. Bacteria were harvested, resuspended in 50 mM Tris and 100 mM NaCl (pH 8.0) containing 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A, and lysed in a French press. The cell lysate was centrifuged at 39000*g* for 20 min, and the supernatant was adsorbed to a glutathione-Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) affinity matrix at 4 °C for 1 h with continuous mixing. After the mixture had been extensively washed with Tris buffer containing 300 mM NaCl, GST-NP was eluted from the matrix with 10 mM reduced glutathione (Sigma, St. Louis, MO) in the same buffer. Purified protein was analyzed by SDS-PAGE, quantified by the BCA protein assay (Pierce, Rockford, IL), and dialyzed with 10 mM HEPES and 140 mM NaCl (pH 8.4). Recombinant six-histidine-tagged LLO expressed in *E. coli* was purified by Ni-NTA affinity chromatography as described previously.[4] pH-sensitive liposomes [2/1 phosphatidylethanolamine/cholesteryl hemisuccinate molar ratio (Avanti Polar Lipids, Alabaster, AL, and Sigma)] containing GST-NP (8−10 mg/mL) with LLO (250 μg/mL), GST-NP without LLO, or GST (10−12 mg/mL) with LLO were prepared and purified following the method used previously.[4]

Liposomal Immunization and CTL Assay. Female BALB/cByJ (BALB/c) mice (*H-2*^{d}, 6−8 weeks old, The Jackson Laboratory) were immunized subcutaneously with liposomal antigen (5−10 μg of GST-NP/mouse, 10−15 μg of GST/mouse) with or without LLO twice with a 12−14 day interval. Immunized mice were bled and sacrificed for removal of spleen 9−12 days after the booster. Splenocytes were cultured for 5 days at a concentration of 4×10^6 cells/mL with 1 μg/mL NP peptide, amino acid residues 118−126, RPQASGVYM (NP118−126, MHC I *H-2*^{d}-restricted, immunodominant CD8$^+$ T cell epitope of NP, obtained from Research Genetics), and these effector cells were tested for their cytotoxic activity in a standard 5 h ^{51}Cr release assay, as described previously.[4,15] The target cells were murine mastocytoma P815 (*H-2*^{d}) cells pulsed with LCMV-NP118−126 peptide (10 μg/mL) and labeled with 200 μCi of ^{51}Cr (Amersham Pharmacia Biotech). Nonspecific lysis was evaluated using ^{51}Cr-labeled P815 target cells without a peptide pulse. P815 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 μM 2-mercaptoethanol (Gibco BRL).

Tetramer Binding Assay and Enzyme-Linked Immunospot (ELISPOT) Assay of NP118−126-Specific T Cell Frequency. Mice were bled retro-orbitally 11 days postboost, and peripheral blood lymphocytes (PBLs) were isolated using a histopaque gradient (Sigma). Cell surface staining was performed as previously described.[16] Briefly, PBLs were stained with anti-CD8 antibody (CD8α clone 53-6.7) and with MHC-peptide tetramer, L^d-NP118−126 tetramer in phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 0.2% sodium azide [fluorescence-activated cell sorting (FACS) buffer]. Cells were washed with FACS buffer and then fixed with 2% paraformaldehyde in PBS. Samples were analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson). Antibodies were purchased from PharMingen.

Gamma interferon (IFN-γ)-releasing spleen cells from immunized mice were quantified, after being stimulated with LCMV-NP peptide at 5 μg/mL for 24 h, by a cytokine-specific ELISPOT assay using the paired anti-IFN-γ antibodies R4-6A2 for capture and biotinylated XMG1.2 for detection (PharMingen) in MAHA-S45 plates (Millipore) and counted in an automated fashion with an ImmunoSpot reader (Cellular Therapeutics) as described previously.[4]

Anti-GST-NP Antibody Assay. Preimmune and postboost serum samples obtained from mice were analyzed for anti-GST-NP antibody by the enzyme-linked immunosorbent assay (ELISA). Dilutions of serum samples were added to ELISA plates coated with 10 μg/mL GST-NP. Biotin anti-mouse IgG (Sigma) used as a secondary antibody was detected by the alkaline phosphatase-streptavidin system and read at 405 nM with a plate reader (Molecular Devices) as reported previously.[4]

Lethal and Chronic LCMV Challenge. Fifteen days postboost, four immunized mice from each liposome group were challenged intracerebrally (ic) with 100 plaque-forming units (pfu) of LCMV Armstrong CA1371.[15,17] Mice were checked daily for 30 days following the challenge for survival. Forty-three days postboost, four to five immunized mice per group were challenged intravenously (iv) with 2×10^6 pfu of LCMV clone 13.[17] Mice were bled retro-

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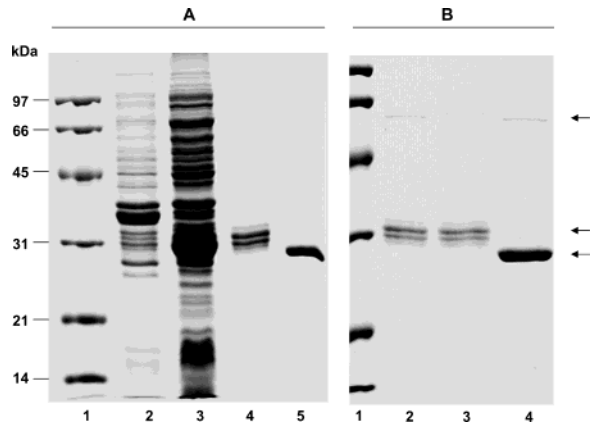

Figure 1. Expression, purification, and encapsulation of GST-NP and GST inside liposomes. (A) Coomassie blue-stained SDS-PAGE of purified GST-NP and GST using a glutathione-Sepharose 4B affinity matrix: lane 1, molecular weight standards; lane 2, bacterial pellet; lane 3, bacterial supernatant; lane 4, purified GST-NP; and lane 5, purified GST. (B) SDS-PAGE of liposome-encapsulated immunogens and LLO after Sepharose CL-4B gel purification: lane 1, molecular weight standards; lane 2, liposomal GST-NP with LLO; lane 3, liposomal GST-NP; and lane 4, liposomal GST with LLO. Top, middle, and bottom arrows indicate the positions of LLO, GST-NP, and GST, respectively.

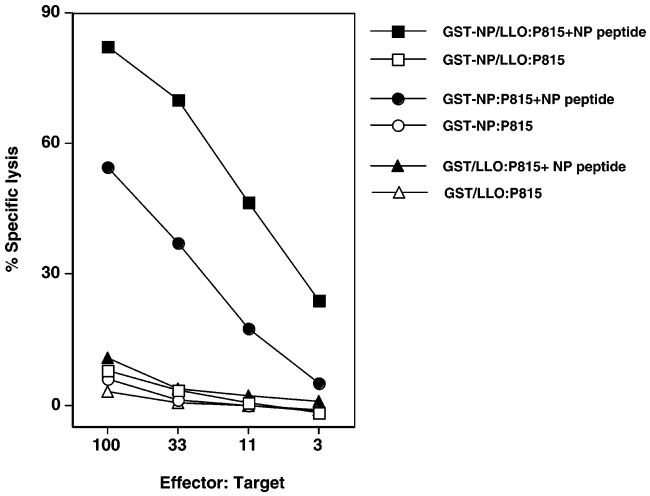

Figure 2. Enhancement of the LCMV-NP-specific CTL response mediated by LLO-liposome-based immunization. Mice were immunized sc with liposome-encapsulated GST-NP and LLO, GST-NP alone, or GST and LLO, and NP-specific CTL responses in the isolated splenocytes were measured by the standard ^{51}Cr release assay after *in vitro* restimulation. Data represent the averages from four mice by calculating the mean cytolytic activity of triplicate cultures of the splenocytes. Splenocytes were pooled after *in vitro* restimulation from two immunized mice in each group that consisted of four mice ($n = 4$). The representative data are from one set of immunization, and the experiments were repeated twice with similar results.

orbitally 5, 8, 15, and 30 days after the challenge, and viral levels in sera were quantitated on Vero cell monolayers as previously described.[17,18]

Results and Discussion

Expression, Purification, and Encapsulation of NP in LLO-Liposomes. A truncated LCMV-NP gene encoding amino acid residues 82−173, which contains the *H-2*^{d}-restricted, immunodominant CD8$^+$ T cell epitope (NP118−126), was expressed as a GST fusion protein and purified in a single step by GST affinity chromatography. The majority of the recombinant protein was located in the insoluble pellet, and only a fraction was recovered from the supernatant with some degradation, which is commonly observed for GST fusion systems[19] and was specifically reported for recombinant adenylate cyclase toxin carrying the LCMV-NP CTL epitope.[20]

Purified GST-NP was encapsulated inside liposomes with or without recombinant LLO, and unencapsulated protein was separated from liposomes by gel filtration chromatography. Figure 1A shows the SDS-PAGE analysis of purified GST-NP or GST, and Figure 1B shows SDS-PAGE analysis of liposome-encapsulated purified GST-NP with LLO, GST-NP, or GST with LLO.

LLO-Liposome Immunization Induces Vigorous Antiviral CTL Responses. Mice were immunized with three liposomal vaccine formulations, GST-NP with LLO, GST-NP, and GST with LLO, and LLO-mediated enhanced cytosolic delivery of NP *in vivo* was monitored by measuring NP-specific CTL activity. NP-specific CTL activity in the splenocytes of the immunized mice, after a secondary *in vitro* stimulation, was monitored using ^{51}Cr-labeled P815 target cells coated with NP118−126 peptide. As shown in Figure 2, spleen cells harvested from the mice immunized with LLO-liposomal GST-NP efficiently lysed NP peptide-pulsed P815 targets, and its activity was significantly higher than that observed with spleen cells from the mice immunized with the liposomal GST-NP formulation without LLO. Mice immunized with LLO-liposomal GST showed no NP peptide-specific killing activity. The higher cytotoxic killing activity exhibited by the effector cells from the LLO-liposome GST-NP group demonstrated LLO-mediated augmented cytosolic introduction of NP in APCs of the immunized mice and a consequent stronger CTL response.

Immunization with LLO-Liposomal GST-NP Generates Higher NP118−126-Specific T Cell Frequency. The effect of LLO-liposome-mediated delivery of NP in augmenting the frequency of antigen-specific CD8$^+$ T cells was determined by L^d-NP118−126 tetramer staining on PBLs

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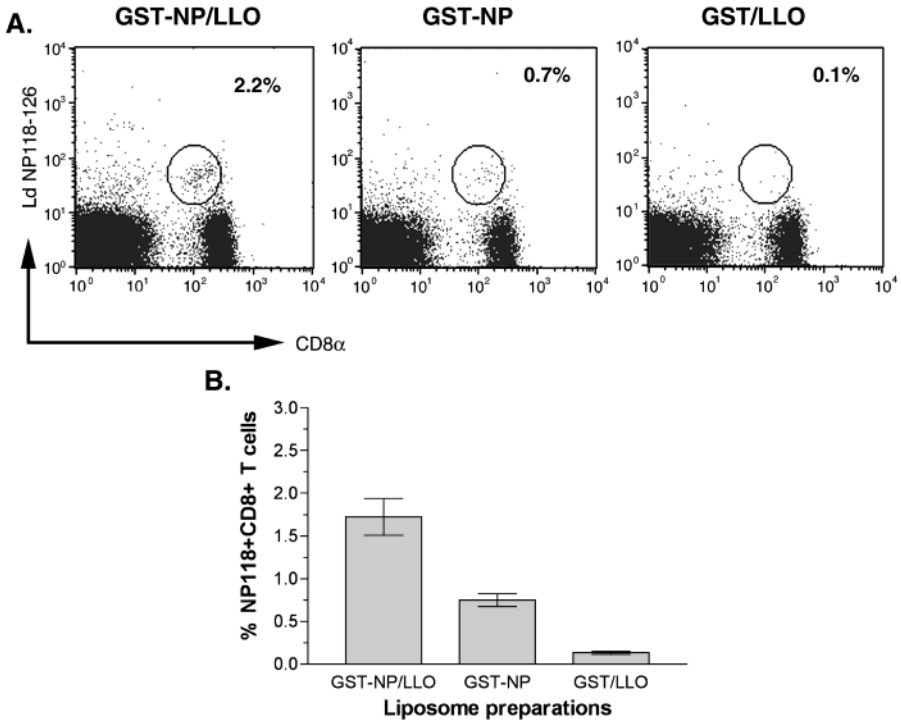

Figure 3. LLO-liposomes enhance the frequency of NP118−126 peptide-specific $CD8^+$ T cells. BALB/c mice were immunized with liposomal GST-NP with LLO, GST-NP without LLO, or GST with LLO. Twelve days postboost, the frequency of NP118−126-specific $CD8^+$ T cells in PBLs was determined by MHC I tetramer staining. (A) Representative FACS plots for each group of immunized mice show the NP118−126 population of $CD8^+$ T cells (circled). (B) Average percentage of NP118−126-specific $CD8^+$ T cells after immunization with liposomes containing GST-NP with LLO, GST-NP only, or GST with LLO. Each group represents an average of nine mice, and error bars represent standard deviations.

of immunized mice. Figure 3A shows representative flow cytometry plots for each liposome formulation-immunized group. The average percentage of NP118−126 peptide-specific $CD8^+$ T cells for each group 12 days postboost is presented in Figure 3B. Mice immunized with LLO-liposomal GST-NP had 1.7% tetramer-positive $CD8^+$ T cells in circulation, an approximately 15-fold increase over the frequency (0.1%) found in the control mice immunized with LLO-liposomal GST, while liposomal GST-NP immunization without LLO generated 0.7% NP118−126 $CD8^+$ T cells. A similar trend of a 2−3-fold increase in the level of NP118−126-specific cells upon inclusion of LLO in liposomes was observed when an IFN-γ ELISPOT assay was performed on spleen cells stimulated with NP118−126 peptide from immunized mice (data not shown) with no significant NP-specific $CD8^+$ T cell response following immunization with LLO-liposomal GST.

Quantitative analysis of NP-specific CTL precursor (CTLp) frequency by the sensitive tetramer binding assay provided the information about the magnitude of the CTL response induced by liposomal immunization and LLO-mediated enhancement in CTLp. The average percentage of $CD8^+$ T cells binding to L^d-NP118−126 (1.7%) achieved with LLO-liposomal GST-NP was as high as or better than those generated by other vaccination approaches: recombinant vaccinia virus (VV) carrying the same NP epitope (NP118−126 responsive intracellular cytokine staining, ∼1%),[11] immunization with plasmid DNA encoding full-length LCMV-NP (NP118−126-specific intracellular cytokine staining, ∼1.5%),[21] or a single immunization using the recombinant *L. monocytogenes* (rLM) vector (∼0.1%).[22] Interestingly, Harrington et al.[11] observed that the $CD8^+$ T cell response to the NP epitope was coordinately regulated with the response to the VV vector, yet at a much lower magnitude compared to the $CD8^+$ T cell response to the VV vector itself (25% of the $CD8^+$ T cells). Using the LLO-liposome delivery system in our study, however, the LLO90−91 peptide (immunodominant epitope in the *H-2*^{d} background)-responsive T cell frequency was approximately $^1/_3$ of the T cell frequency specific for NP118−126, as determined by an ELISPOT assay (data not shown). Our observation demonstrates (1) the efficient cytosolic delivery capability of the LLO-liposome that is comparable or more effective than viral or bacterial vaccine delivery systems and (2) the weak vector-specific host immune response as compared with that of the viral vector/delivery system.

Humoral Immune Response. GST-NP-specific antibody responses were similarly robust as determined with an ELISA on serum samples obtained from the immunized mice

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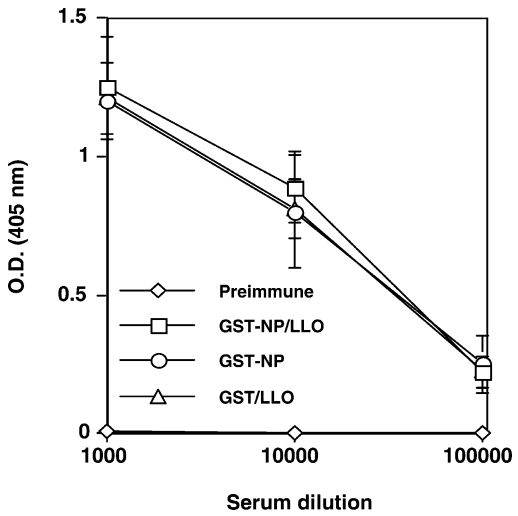

Figure 4. Antigen-specific antibody response to liposomal GST-NP with LLO, GST-NP alone, or GST with LLO. GST-NP-specific IgG titer was determined by an antibody capture ELISA in serum samples obtained from the immunized mice 9−12 days postboost. Data shown as the mean OD (±standard deviation) of the serum samples obtained from two sets of experiments ($n = 8$ per group).

irrespective of the inclusion of LLO in the liposomal formulations (Figure 4). As the ELISA plates were coated with GST-NP, anti-GST antibody was also detected by this assay in the serum of the mice immunized with GST-NP fusion protein or with GST alone in the liposomes. The results from the wells coated with GST alone were similar to those using the GST-NP-coated wells (data not shown) as GST constitutes two-thirds of the total molecular mass of the GST-NP fusion protein and a significant fraction of the detected antibodies is expected to bind to the GST moiety accordingly. Thus, an efficient antibody response to the liposomal immunogen was generated regardless of LLO inclusion in the liposomes, although the apparent strength of the GST-NP-specific humoral response was not modulated by LLO. In contrast, LCMV-NP-specific antibody responses in general are not easily achieved by typical vaccine formulations such as DNA vaccine[23] and VV-NP immunization.[24]

Immunization with LLO-Liposomal GST-NP Confers Complete Protection against a Lethal or Chronic LCMV Infection to Mice. To determine whether CTL responses to NP primed by liposomal immunization are capable of conferring protection against lethal LCMV infection, immunized mice were challenged ic with 100 pfu of the virulent LCMV Armstrong strain per mouse 15 days postboost, and their survival was monitored. Liposomal immunization with GST-NP and LLO conferred complete protection (100% survival monitored up to day 30 postinfection), while only partial protection (50% death within 7−8 days postchallenge) was achieved with the immunization using liposomal GST-NP without LLO. No protection was seen with immunization with LLO-liposomal GST (100% death within 7−8 days postchallenge). The extent of protective antiviral immunity generated by the liposomal GST-NP was correlated to the level of $CD8^+$ NP118−126-specific T cell frequency described in the previous section. The protection against and clearance of LCMV infection mediated by priming NP-specific CTL have been extensively reported by using other modalities of immunization, although at different levels of protection.[13,23,25,26] Indeed, it has been observed that mainly the CTL precursor frequency, and to some extent cytolytic activity following secondary stimulation, are the correlates of protection.[25] In our study, LLO-mediated enhanced cytosolic delivery of NP generated an increased level of CTLp as determined by tetramer binding and ELISPOT assays; moreover, complete protection was conferred upon the immunized mice as compared with control vaccine formulations, thus supporting the previous observations on the correlates of protection.

In the chronic LCMV infection model, immunized mice were challenged iv with LCMV clone 13 (2×10^6 pfu/mouse) 43 days after the boost, and the level of virus in the serum was assayed on days 5, 8, 15, and 30 postinfection. LCMV clone 13 causes a systemic infection with viremia that can be detected for up to 3 months in normal unimmunized mice.[17,27] In the case of chronic LCMV challenge, similar levels of viral control were achieved by immunizations with liposomal GST-NP; both the liposomal GST-NP/LLO- and GST-NP-immunized mice showed serum viral titers of approximately 10^2-10^3 pfu/mL around day 5 after infection and cleared the virus from the serum by day 15 postinfection. In contrast, a high level of viremia of approximately 10^4 pfu/mL was observed in the control group immunized with GST encapsulated in LLO-liposome by day 5 postinfection that persisted past day 15. The level of detection for the viral plaque assay was 40 pfu/mL of serum. This clearance of the viral infection achieved by the liposomal GST-NP is considerably faster than that by

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recombinant *Listeria* expressing the full-length or the single CTL epitope of NP.[22]

The viral challenge study utilized a limited number of animals to demonstrate a proof of the concept for our vaccine delivery strategy. The results show CTL-mediated antiviral protection and strongly suggest that GST-NP with coencapsulated liposomal LLO immunization is required for conferring complete protection against LCMV infection.

Conclusion

Antigen delivery strategies are an important aspect of vaccine development, which dictate the resulting immune responses qualitatively as well as quantitatively. Induction of virus-specific CTL is critical both to the control of LCMV infection and to the effective protective immunity. In this report, we have utilized LLO-mediated enhancement of liposomal protein antigen delivery into the cytosolic pathway of antigen presentation, and demonstrated an efficient activation of CTL specific against viral nucleoprotein. The results show that, while the humoral responses were induced to similar extents, the CTL response to viral nucleoprotein was significantly augmented by the inclusion of LLO in the liposomes. This augmentation in the specific CTL activity and $CD8^+$ T cell frequency was shown to be translated into, and correlated to, a significantly improved outcome of vaccination in terms of protection against acute and lethal viral infection in the mouse model of LCMV. This strategy of using whole protein-based immunization combined with a cytosolic delivery system is superior to peptide immunization-based activation of CTL, as the identification of appropriate peptide epitopes is not required. In addition, the LLO-liposome formulation possesses the potential to carry multiple species of protein antigens to APCs and activate robust polyclonal CTL responses in a polymorphic MHC population as well as the versatility of readily carrying additional adjuvants. As shown in this report, the LLO-liposome-based immunization is superior and required for efficient activation of antigen-specific CTL, providing protective immunity without compromising strong antibody response.

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