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Complexing TLR9 agonists such as plasmid DNA to cationic liposomes markedly potentiates their ability to activate innate immunity. We therefore reasoned that liposomes complexed with DNA or other TLR agonists could be used as effective vaccine adjuvants. To test this hypothesis, the vaccine adjuvant effects of liposomes complexed to TLR agonists were assessed in mice. We found that liposomes complexed to nucleic acids (liposome-Ag-nucleic acid complexes; LANAC) were particularly effective adjuvants for eliciting CD4+ and CD8+ T cell responses against peptide and protein Ags. Notably, LANAC containing TLR3 or TLR9 agonists effectively cross-primed CD8+ T cell responses against even low doses of protein Ags, and this effect was independent of CD4+ T cell help. Ag-specific CD8+ T cells elicited by LANAC adjuvants were functionally active and persisted for long periods of time in tissues. In a therapeutic tumor vaccine model, immunization with the melanoma peptide trp2 and LANAC adjuvant controlled the growth of established B16 melanoma tumors. In a prophylactic vaccine model, immunization with the Mycobacterium tuberculosis protein ESAT-6 with LANAC adjuvant elicited significant protective immunity against aerosol challenge with virulent M. tuberculosis. These results suggest that certain TLR agonists can be combined with cationic liposomes to produce uniquely effective vaccine adjuvants capable of eliciting strong T cell responses against protein and peptide Ags. The Journal of Immunology, 2006, 176: 7335–7345.

The only vaccine adjuvants currently licensed for use in humans (aluminum hydroxide, salts, MF59, and virosomes) augment immune responses largely through enhancing Ag delivery (6, 7). Both aluminum hydroxide and MF59 stimulate primarily humoral immune responses (8). In contrast, other common vaccine adjuvants, such as Freund’s adjuvant (FA), monophosphoryl lipid A (MPL) adjuvant, and CpG oligonucleotides, function primarily as activators of innate immunity (9). For example, FA and MPL adjuvant both activate innate immunity via activation of TLR2 or TLR4, whereas the adjuvant effects of CpG oligonucleotides are dependent on TLR9 activation (8–16).

Cationic liposomes have been shown previously to markedly potentiate activation of innate immunity by TLR9 agonists (bacterial DNA and CpG oligonucleotides) (17–21). The ability of cationic liposomes to potentiate activation of innate immunity by DNA is due to protection of the DNA from extracellular degradation and to enhanced entry of DNA into the endosomal compartment, where TLR9 is selectively expressed (19, 22, 23). The ability of cationic liposomes to promote entry into the cell via the endosomal compartment may also be important to activation by TLR3 agonists such as poly(I:C), inasmuch as TLR3 is also expressed primarily in the endosomal compartment (2–5, 24). Liposomes have also been used to introduce protein Ags into the cytosol and the MHC class I pathway for generating CD8+ T cell responses (10, 25, 26). However, cationic liposomes alone are relatively inert in terms of activating innate immune responses (16, 27, 28).

Currently available vaccine adjuvants are generally effective at eliciting Ab responses, but few nonreplicating vaccine adjuvants are able to generate strong CD8+ T cell responses against protein Ags (8). Immune stimulating complexes are probably the most effective adjuvants developed to date for generating cellular immune responses, including CD8+ T cell responses, against protein...
Ags (29, 30). Immune-stimulating complex adjuvants are thought to elicit cell-mediated immunity primarily by enhancing Ag delivery to APC. Use of CpG oligonucleotides as vaccine adjuvants can also elicit cross-priming when high doses of Ag are administered (14, 31, 32). However, the most efficient and robust CD8+ T cell responses are currently generated by immunization with replicating vaccines, including both viral and bacterial vectored vaccines (7, 8, 11, 33–39).

Therefore, there remains a need for new nonreplicating vaccine adjuvants capable of eliciting strong cellular immune responses against purified Ags. Based on prior observations that cationic liposomes complexed to CpG oligonucleotides or plasmid DNA could greatly augment activation of innate immunity, we wondered whether liposome-nucleic acid complexes could also be used as effective vaccine adjuvants. Previous reports have indicated that complexes of liposomes with encapsulated CpG oligonucleotides or with protamine-DNA complexes could be combined with protein or peptide Ags to elicit effective antitumor immunity in vivo (40–42). In addition, it was reported previously that plasmid DNA and liposome complexes could be used as vaccine adjuvants (43). Therefore, we conducted studies to further investigate the adjuvant properties of liposome-DNA complexes and to extend the earlier observations. Our studies have now also included an examination of the adjuvant properties of other TLR agonists when complexed to liposomes. We also investigated the effectiveness of these adjuvants in therapeutic and prophylactic vaccination models.

We found that adjuvants consisting of cationic liposomes complexed to TLR9 or TLR3 agonists were particularly effective in generating strong CD8 and CD4 T cell responses. In contrast, liposomes complexed to other TLR agonists were much less active as vaccine adjuvants. Vaccination with liposome–TLR9 agonist adjuvants elicited functional and long-lived T cells in tissues and generated therapeutic antitumor immunity and significant protective immunity against aerosol challenge with Mycobacterium tuberculosis. Thus, the liposome–TLR agonist adjuvant system described here represents a novel and potentially clinically effective nonreplicating vaccine adjuvant system for generating strong cellular immune responses against subunit Ags.

Materials and Methods

TLR agonists and Ags

TLR agonists, including LPS, zymosan, R848, poly(I:C), and CpG oligonucleotides were purchased from InvivoGen. All TLR agonists except R848 were prepared by dissolving at a final concentration of 3 mg/ml in sterile PBS, whereas R848 was prepared by dissolving at a final concentration of 1 mg/ml in ethanol. Low endotoxin content plasmid DNA was prepared by Alltech Technologies. The plasmid used in these studies (pMB75.6) did not contain a coding gene (44). OVA was purchased from Sigma-Aldrich and was prepared as a 1 mg/ml solution in PBS. The so-

Preparation of cationic liposomes and vaccines

Liposomes were prepared by dissolving the cationic lipid octadecenoloxylethyl-2,3-dihydroxyethyl imidazolium chloride (Sigma-Aldrich Chemical) and cholesterol (Avanti Polar Lipids) in chloroform and adding equimolar concentrations to round-bottom, 15-ml glass tubes to a final concentration of 2 mM. The solution was then dried overnight in a vacuum desiccator to a thin film. The lipids were rehydrated in 5% dextrose in water at 50°C for 50 min, followed by incubation for 2 h at room temperature. The liposomes were then extruded through a series of 1-, 0.45-, and 0.20-μm filters to form the final liposomes, as described previously (45). To formulate the vaccines, complexes of liposomes and TLR agonists were prepared by first diluting the liposomes in 5% dextrose in water at a concentration of 100 μl of liposomes per 1 ml of dextrose solution. Next, TLR agonists were added with gentle pipetting to the liposome solution at a final concentration of 100 μg of Ag per milliliter of liposome solution. In the case of complexes prepared with R848, the R848 in methanol was first dried down along with lipids, then rehydrated together with the liposomes. Next, peptide or protein Ags were added to the preformed liposome–TLR agonist complexes and mixed by gentle pipetting. Vaccines were prepared at room temperature and administered within 30 min of preparation.

Mice and immunizations

Female C57BL/6 mice ages 8–12 wk were used for most experiments. All mice were purchased from Harlan Sprague Dawley or The Jackson Laboratory. CD4−/− mice on the C57BL/6 background were provided by E. Gelfand and P. Marrack (National Jewish Medical and Research Center, Denver, CO). Female MyD88−/− mice on a C57BL/6 × 129 background were provided by R. Titus (Colorado State University (CSU), Fort Collins, CO). Control female wild-type control mice on the C57BL/6 × 129 background were purchased from The Jackson Laboratory. Protocols for the animal experiments described in this study were approved by Institutional Animal Care and Use Committees at National Jewish Medical and Research Center and at Colorado State University. For most experiments, mice were immunized with 5 μg of peptide or protein Ag, based on prior dose-titration experiments. Mice were immunized with 100 μl of liposome–TLR agonist vaccine for i.v. immunization and 200 μl for s.c., i.p., and i.m. immunization. For most experiments, mice were immunized once and then boosted 7–10 days later, and cells were collected for analysis on days 4–5 after the boost. In some experiments, mice were primed and boosted then left untreated and several weeks to 4–6 months before being rechallenged with the Ag.

Immune responses to immunization with conventional vaccines were also assessed. One group of mice was immunized s.c. with 5 μg of OVA8 peptide in CFA (Sigma–Aldrich) and boosted 1 wk later with peptide in IFA. Other mice were immunized with peptide-pulsed or protein-pulsed DC. Briefly, bone-marrow cells were propagated in high levels of GM-CSF for 6 days, and the nonadherent cells were collected (>85% CD11c and MHC class II+). After 16 h of IFNγ and cytokine-mimic peptide-pulsed overnight with 10 μM OVA8 peptide (or 10 μg/ml OVA protein), then activated with 1 μg/ml LPS for the last 4 h (46). Mice were each immunized with 1 × 106 Ag-pulsed DC by either the s.c. or i.p. routes, then boosted 7 days later, and spleen cells were analyzed by flow cytometry 5–7 days after that. Another group of mice was immunized i.v. with 107 PFU of vv-OVA (vaccinia virus encoding full-length OVA protein), then spleen cells were analyzed 7 days later by flow cytometry. Mice were also immunized with plasmid DNA encoding the OVA protein. For plasmid DNA vaccination, mice each received 100 μg of OVA plasmid DNA injected at several sites i.m. in the cranial tibiais muscles bilaterally and were then boosted 2 wk later, and T cells were analyzed 1 wk after the boost.

Cell preparation

Single-cell suspensions of spleen cells were prepared by mechanical dissection and screening through a 100-μm nylon mesh screen (BD Biosciences), followed by NH4Cl lysis. Lymph node cells were prepared by mechanical screening through a 100-μm nylon mesh screen. Liver and lung lymphocytes were isolated by first mincing the tissues, then digesting in a solution of 2 mg/ml collagenase (type IA; Sigma–Aldrich) plus soybean trypsin inhibitor (100 μg/ml) and DNase (500 IU/ml) for 1 h at 37°C, followed by mechanical disruption through an 18-gauge needle, as described previously (47). The cells were then washed twice with RPMI 1640 containing 10% FBS (Invitrogen Life Technologies) before analysis. Abs and flow cytometric analysis

Directly conjugated Abs used for flow cytometric analysis were purchased from either BD Pharmingen or eBioscience. The following Abs were used: anti-CD8a (APC; clone 53-6.7.1), anti-CD4 (APC; clone RM4-5), anti-CD44 (FITC; clone IM7), anti-CD62L (PE/Cy5; clone Mel-14), anti-CD69 (PE/Cy7; clone H1.2F3), anti-I-A/I-E (MHC class II, biotin, or PE; clone M5/114.15.2), followed by either SA-pe/cy5 or SA-Alexa-350 (Molecular Probes), anti-CD11b (PE-Cy5 or APC-Cy7; clone M1/70), anti-CD11c (PE or APC; clone N418), anti-Gr-1 (PeCy7; clone RB6-8C5), B220 (APC-Cy7; clone RA3-125), and CD11b (PE-Cy5 or APC-Cy7; clone M1/70). Nonspecific binding of Abs was blocked by preincubation of cells in normal mouse serum with 40% supernatant from rat anti-FcRIII hybridoma 24-G2, plus 0.2 μg/ml human IgG. Abs were

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diluted in FACS buffer (PBS with 2% FBS and 0.1% sodium azide) for staining. Staining (except for tetramers) was done at 4°C for 20 min, followed by washing in FACS buffer. In most cases, cells were fixed in 1% paraformaldehyde for 30 min and stored in FACS buffer at 4°C before analysis. Flow cytometry was performed using either a BD FACSCalibur cytometer (BD Biosciences) for four-color analysis or a Cyan MLE cytometer (DakoCytomation) for six- to seven-color analysis.

For analysis of CD8+ T cell responses, gates were drawn to include live lymphocytes, based on forward- and side-scatter characteristics of spleen cells. For analysis of tetramer-positive cells, the total CD8+ T cell population was first gated, and then the percentage of tetramer-positive cells as a percentage of total CD8+ or CD4+ T cells was calculated. A minimum of 200,000 total events were collected for tetramer experiments. Data analysis was done using either CellQuest Software (BD Biosciences) or Summit Software (DakoCytomation).

MHC-peptide tetramers

A soluble MHC Kb tetramer was prepared as described previously (48). Tetramers were loaded with the OVA8 peptide SIINFEKL or the trp-2, peptide SYVYDFFVWL. Single-cell suspensions from spleen, lymph nodes, lung, or liver (5 × 10^3 to 1 × 10^6 cells in 100 μl of complete medium) were incubated with tetramer at 37°C for 1–1.5 h. Splenocytes from OT-1 mice (OVA8-specific TCR transgenic mice; provided by T. Potter, National Jewish Medical and Research Center, Denver, CO) were used as positive controls for tetramer staining. Negative controls included spleens from naive mice and use of tetramers prepared with irrelevant peptides. For analysis of Ag-specific CD4 T cells, MHC class II tetramers were produced with a covalent linkage to the gp61–80 peptide of LCMV (I-Ab-gp61) as described previously (49). After incubation with tetramers, cells were washed and stained with Abs to surface determinants, then washed and stained with appropriate streptavidin conjugates for 15 min, then washed and reconstituted in FACS buffer or fixed in 1% paraformaldehyde before analysis.

Tracking uptake of fluorescent liposome-DNA complexes in vivo

To track cellular uptake of liposome-DNA complexes by APCs in vivo, liposomes were labeled with the fluorescent dye BODIPY and used to prepare complexes. The labeled liposome-DNA complexes were injected by the i.v., s.c., and i.p. routes, and, at various time points postinjection, spleen cells, peritoneal cells, and relevant draining lymph node cells were collected, and single-cell suspensions were prepared. In the case of analysis of spleen and lymph node APC, the cell suspensions were also digested in 1 mg/ml collagenase for 20 min at room temperature to release DC. Cells were then immunostained with Abs to cell surface Ags and analyzed by flow cytometry. Analysis gates were set on all live cells, including those with high side-scatter and forward-scatter characteristics to enumerate all cell types that might have bound the labeled liposomes.

CD8+ T cell assays

Cytotoxic activity of CD8+ T cells elicited by vaccination was assessed by in vivo assay, as described previously (48). Briefly, equal numbers of autologous spleen cells from naive donor mice were pulsed for 2 h at 37°C in vitro with 10 μg OVA8 peptide, or were incubated but unlabeled. After peptide pulsing and washing, the peptide-pulsed and unpulsed spleen cell populations were labeled with either 50 μl CFSE (Molecular Probes) or 10 μg/ml CFSE for 15 min, respectively. Immediately before tail vein injection, equal numbers of the two spleen cell populations were mixed. Recipient vaccinate and control mice were each injected with equal numbers of the two spleen cell populations. The injected mice were sacrificed 18 h later, and single-cell suspensions were prepared from the spleen. The cells were evaluated by flow cytometry, and the number of CFSEhigh and CFSELow populations was determined. The ratio of CFSEhigh/CFSELow was calculated to determine the amount of in vivo lysis of peptide-pulsed target cells.

Cytokine assays

Cytokine production by CD8+ spleen cells and lung cells from immunized mice was assessed by determination of Ag-specific cytokine release into supernatants and by intracellular cytokine staining. Single-cell suspensions of spleen cells were cultured overnight at a concentration of 2 × 10^6 in complete medium in the presence or absence of 1 μM OVA8 peptide. Supernatants were collected 18 h later and assayed for release of IFN-γ, using commercial ELISAs (R&D Systems). Controls included spleen cells from nonimmunized mice and wells incubated without peptides. For intracellular cytokine assay, spleen cells were plated in complete medium with 10 μg/ml Brefeldin A, then stimulated with 10 μM OVA8 peptide for 5 h at 37°C. Cells were then stained for cell surface determinants, then fixed and permeabilized for detection of intracellular IFN-γ, according to manufacturer’s directions (BD Pharmingen). Controls included incubated without peptide and cells from nonimmune mice incubated with and without OVA8 peptide.

B16 tumor model

Mice (5/group) were injected s.c. with 1 × 10^6 B16.F10 cells (provided by I. Fidler, MD Anderson Cancer Center, Houston, TX) and vaccination was initiated 7 days later, at which time most mice had palpable tumors. Mice were treated by injection of 5 μg of trp-2 peptide in liposome-plasmid DNA complexes, given by the s.c. or i.p. routes. Treatments were continued weekly for 4 wk or until the mice were euthanized. Tumor growth was monitored by calipers every 2–3 days, and the tumor surface area was determined by multiplying two perpendicular tumor dimensions.

M. tuberculosis aerosol protection model

Mice (5 animals/treatment group) were immunized with 10 μg of recombinant ESAT-6 protein in liposome-DNA complexes by the i.p. or s.c. routes. Another group of mice was immunized ESAT-6 protein in MPL adjuvant (Corixa). Recombinant ESAT-6 Ag was prepared as part of the National Institutes of Health tuberculosis vaccine contract at CSU. Mice were primed, then boosted at 2 and at 4 wk. Negative control groups included unvaccinated mice and mice given liposome-DNA complexes without Ag, also by the i.p. route. Mice were challenged by aerosolization of 100 CFU of M. tuberculosis, Erdman strain, 4 wk after the last vaccination. Thirty days after aerosol challenge, mice were sacrificed, and the number of viable organisms in lung tissues was determined by homogenizing lung tissues and plating serial dilutions of the homogenates on Middlebrook agar plates. The log_{10} protective titer was calculated by subtracting the observed titer from the titer obtained in mice treated with saline only.

Statistical analyses

In experiments with multiple groups of mice, statistical differences between treatment groups were compared using ANOVA and Tukey’s multiple means comparisons test. For comparisons between two treatment groups, Student’s t test was used. Statistical analyses were done using GraphPad software. A P value <0.05 was considered statistically significant for these analyses.

Results

Immunization with liposome-Ag-nucleic acid complexes (LANAC) elicits strong CD8+ and CD4+ T cell responses

The ability of liposome-DNA complexes to function as vaccine adjuvants and elicit CD8+ or CD4+ T cell responses was first assessed by immunizing mice with OVA8 or gp61 peptides, respectively. The vaccines were formulated using cationic liposomes complexed to noncoding plasmid DNA, which we demonstrated previously could elicit marked activation of innate immunity (20, 21). The complexes used in these studies also had a net positive charge, which likely facilitated binding of protein or peptide Ags to the complexes. Mice were immunized with LANAC formulated with noncoding plasmid DNA and with low doses (typically 5 μg) of Ag. Dose ranging studies revealed that strong T cell responses could be elicited when mice were immunized with LANAC containing as little as 1 μg of peptide per mouse, whereas doses above 10 μg per mouse did not increase the efficiency of vaccine responses (data not shown). Mice were immunized by the s.c., i.m., i.p., and i.v. routes to assess the efficiency of vaccination by different routes. Mice were immunized twice, 7–10 days apart, and cells were analyzed 5 days after the last immunization using Kb-OVA8 or I-Aβ-gp61 tetramers.

Immunization i.p. with 5 μg of OVA8 peptide in LANAC elicited a significant (p < 0.01) increase in the percentage of OVA8-specific CD8+ T cells in spleens of immunized mice when compared with control mice (Fig. 1A). For example, immunization with OVA8 peptide in LANAC generated an average of 5.4% of OVA8-specific CD8+ T cells in the spleens of mice. Immunization by the i.p. route was the most efficient route of immunization, although immunization by the s.c. or i.m. routes also elicited large
Thus, the LANAC adjuvant was extremely effective in eliciting OVA8-specific CD8+ T cell responses to immunization with LANAC. In fact, CD8+ T cell responses in mice immunized with OVA8 peptide in LANAC were significantly greater than those elicited by conventional peptide vaccines, including immunization with peptide in FA and immunization with peptide-pulsed bone marrow-DC, as described in Materials and Methods. The mean (±SD) OVA8-specific CD8+ T cell responses were calculated following a prime and boost immunization (4 animals/group) and plotted. As a control, another group of mice was immunized with liposome-nucleic acid complexes without peptide Ag (LANAC). Immunization with LANAC elicited significantly greater CD8+ T cell responses (p < 0.01) than control mice. These results were pooled from two independent experiments. CD4+ T cell responses were quantitated in C57BL/6 mice (4/group) immunized with gp61 peptide with LANAC adjuvant, using I-Aβ-gp61 tetramer. A representative FACS plot is presented for a control mouse and a mouse immunized with gp61. D, The mean gp61-specific CD4+ T cell response (±SD) in the spleen of mice (4/group) immunized with gp61 peptide in LANAC was compared with that elicited by infection with live LCMV. In LCMV-infected mice, the T cells were analyzed at the predicted peak of the T cell response to live virus infection (7 days postinoculation). Immunization with gp61 LANAC elicited a significantly greater (p < 0.05) CD4+ T cell response than LCMV infection, and both gp61 vaccination and live virus infection elicited significantly greater CD4+ T cell responses than observed in control mice. Similar results were obtained in one additional experiment. * Denotes significant differences (p < 0.05) between gp61 LANAC-vaccinated mice compared with control mice, as determined by ANOVA, followed by Tukey multiple means comparison, whereas ** denotes significant differences (p < 0.05) between gp61 LANAC-vaccinated mice and LCMV-infected mice.

LANAC vaccines elicit effective cross-priming

One of the major drawbacks to immunization with nonreplicating vaccines and recombinant Ags is the relative inability of these vaccines to elicit CD8+ T cell responses. Therefore, the ability of the LANAC vaccine to generate CD8+ T cell responses against protein Ags was assessed. In these experiments, C57BL/6 mice were immunized with 5 μg of intact OVA protein, using the same immunization protocol described above, and CD8+ T cell responses were quantitated using Kβ-OVA8 tetramers. Remarkably, the LANAC adjuvant was extremely effective in eliciting OVA8-specific CD8+ T cell responses following immunization with whole OVA (Fig. 2). In fact, CD8+ T cell responses to immunization with OVA in LANAC consistently exceeded those elicited by immunization with equivalent doses (by weight) of OVA8 peptide. For example, mice immunized with OVA generated an average of 10.2% OVA8-specific CD8+ T cells per total CD8+ splenic cells, compared with 5.4% OVA8-specific CD8+ T cells per total CD8+ in mice immunized with OVA8 peptide in LANAC. Immunization with LANAC also appeared to be more efficient in cross-priming CD8+ T cell responses than immunization with OVA-pulsed DC, with a viral vectored vaccine (vv encoding full-length OVA), or with plasmid DNA encoding full-length OVA (Fig. 2).

numbers of OVA8-specific CD8+ T cells, whereas smaller numbers were elicited by vaccination by the i.v. route (data not shown). Large increases in the numbers of OVA8-specific CD8+ T cells were also observed in peripheral lymph nodes of immunized mice (data not shown). Immunization with liposome-DNA complexes did not elicit OVA8-specific CD8+ T cell responses. Immunization with peptide in LANAC also appeared to be more efficient than immunization with peptide in FA or immunization with peptide-pulsed bone marrow-DC, as described in Materials and Methods. Briefly, total CD8+ T cells were gated for analysis (after excluding MHC class II+ cells), and the percentage of Kβ-OVA8+ cells was plotted vs CD44+ expression. A, Representative FACS plot of OVA8-specific T cells elicited by vaccination with peptide in LANAC adjuvant is shown. This result is representative of >6 independent experiments. B, CD8+ T cell responses to peptide vaccination with LANAC was compared with those elicited by conventional peptide vaccines, including immunization with peptide in FA and immunization with peptide-pulsed bone marrow-DC, as described in Materials and Methods. The mean (±SD) OVA8-specific CD8+ T cell responses were calculated following a prime and boost immunization (4 animals/group) and plotted. As a control, another group of mice was immunized with liposome-nucleic acid complexes without peptide Ag (LANAC). Immunization with LANAC elicited significantly greater CD8+ T cell responses (p < 0.01) than control mice. These results were pooled from two independent experiments. C, CD4+ T cell responses were quantitated in C57BL/6 mice (4/group) immunized with gp61 peptide in LANAC with that elicited by infection with live LCMV. In LCMV-infected mice, the T cells were analyzed at the predicted peak of the T cell response to live virus infection (7 days postinoculation). Immunization with gp61 LANAC elicited a significantly greater (p < 0.05) CD4+ T cell response than LCMV infection, and both gp61 vaccination and live virus infection elicited significantly greater CD4+ T cell responses than observed in control mice. Similar results were obtained in one additional experiment. * Denotes significant differences (p < 0.05) when LANAC-vaccinated mice were compared with control mice, as determined by ANOVA, followed by Tukey multiple means comparison, whereas ** denotes significant differences (p < 0.05) between gp61 LANAC-vaccinated mice and LCMV-infected mice.
some-DNA complexes, we found that OVA-specific CD8 T cell responses were compared with those elicited by immunization with intact LANAC (data not shown). Immunization with OVA administered s.c., followed by administration of liposome-DNA complexes i.p. did not elicit detectable CD8 T cell responses (data not shown).

Therefore, it appeared that physical association of all three components of the vaccine was required for optimal efficiency of cross-priming and generation of CD8 T cell responses. Similarly, the three-part liposome-DNA-Ag complex was also required for optimal generation of CD8 T cell responses against peptide Ags (data not shown). Using labeled OVA and labeled liposomes and fluorescence resonance energy transfer analysis, we also observed that the OVA protein was in fact physically associated with the liposome-DNA complexes (T. Anchordoquy, unpublished data). These results suggest that it is likely that the three components of the LANAC vaccine may physically enter the same APC to elicit CD8 T cell responses.

**Adjuvant activity of LANAC is dependent on signaling via a MyD88-dependent pathway**

Inclusion of TLR3 or TLR9 agonists with charged liposomes was able to elicit optimal induction of CD8 T cell responses in our experiments. Therefore, to determine whether activation of innate immunity was critical to the activity of LANAC adjuvants formulated with plasmid DNA, mice lacking the MyD88 adaptor protein (MyD88-/-) were immunized with OVA and LANAC, and CD8 T cell responses were compared with those elicited in wild-type C57BL/6 mice. We observed that Ag-specific CD8 T cell responses were almost completely abrogated in vaccinated MyD88-/- mice, compared with responses in wild-type mice (Fig. 3B). For example, the mean percentage of OVA8-specific CD8 T cells in immunized MyD88-/- mice was only 0.37% (±0.54%) following immunization with LANAC, whereas immunized wild-type mice on the same background (C57BL/6 x 129) mounted CD8 T cell responses equivalent to those of C57BL/6 mice (data not shown). Thus, activation of innate immunity via a MyD88-dependent pathway was critical to the ability of the LANAC adjuvant to elicit efficient CD8 T cell responses.

**Cross-priming by LANAC is independent of CD4 help**

We observed that immunization with OVA protein consistently elicited stronger CD8 T cell responses than immunization with the same amount of peptide (see Figs. 1 and 2). The CD8 T cell responses to immunization with protein Ag may therefore have been augmented by CD4 T cell help provided by MHC class II epitopes contained within the full-length OVA cDNA (DNA), with vv-OVA, or with 5 μg of OVA in LANAC adjuvant, as described in Materials and Methods. A representative FACS plot of spleen cells from a control mouse (left panel) and a mouse immunized twice with LANAC containing 5 μg of OVA (right panel). Total CD8 T cells were gated for analysis (after excluding MHC class II cells), and K0-OVA8 T cells were plotted vs CD44 expression by total CD8 T cells. This result is representative of >6 independent experiments done with OVA protein. B. Comparison of the mean percentage (±SD) of K0-OVA8 CD8 T cells in nonimmunized control mice (4/group), as compared with mice immunized twice with bone marrow DC pulsed overnight with OVA (DC), with plasmid DNA encoding the full-length OVA cDNA (DNA), with vv-OVA, or with 5 μg of OVA in LANAC adjuvant, as described in Materials and Methods. This data was pooled from two separate experiments. *, Denotes values significantly different (p < 0.05) when LANAC-immunized mice were compared with control mice, as assessed by ANOVA and Tukey multiple means comparison.

**Generation of CD8 T cell responses by LANAC vaccines is optimal when the three vaccine components are physically associated**

Studies were conducted next to elucidate the contribution of the individual components of the LANAC adjuvant to overall vaccine efficacy. Mice were therefore immunized with OVA plus liposomes only or OVA plus plasmid DNA only, and the CD8 T cell responses were compared with those elicited by immunization with all three components of the complex (OVA plus liposomes plus noncoding plasmid DNA). Mice immunized with OVA plus liposomes alone or OVA plus plasmid DNA alone failed to generate significant CD8 T cell responses against the OVA8 peptide, whereas mice immunized with the three-part complex of liposomes, DNA, and OVA (LANAC) generated strong CD8 T cell responses (Fig. 3A).

To further evaluate the need for physical association of the components of the LANAC vaccine for optimal immunization, we performed experiments where the Ag was administered separately from the liposome-DNA complexes. When mice were injected i.p. with 5 μg of OVA, followed 5 min later by i.p. injection of liposome-DNA complexes, we found that OVA8-specific CD8 T cell responses were elicited, but with only ~50% the efficiency with which LANAC elicited CD8 T cell responses (data not shown).

When the order of injection was reversed and liposome-DNA complexes were injected first, followed by injection of OVA, the magnitude of the CD8 T cell response was only ~10% of that following immunization with intact LANAC (data not shown). Immunization with OVA administered s.c., followed by administration of liposome-DNA complexes i.p. did not elicit detectable CD8 T cell responses (data not shown).
these experiments, liposomes labeled with the fluorescent dye BODIPY were used along with flow cytometry to track the distribution of LANAC complexes. Within 1 h of i.p. injection of labeled LANAC, virtually all cells in the peritoneal cavity contained BODIPY⁺ liposomes (data not shown). By 4 h after injection, BODIPY⁺ cells could also be detected by flow cytometry in cells in the mediastinal lymph nodes, but not in cells in the spleen or other lymph nodes (Fig. 4). The majority of labeled LANAC in the mediastinal lymph node were present in cells that were CD11b⁺ or Gr-1⁺ (Fig. 4B). Most (90%) BODIPY⁺ Gr-1⁺ cells coexpressed CD11b⁺ (consistent with neutrophils or immature monocytes), whereas 40% of BODIPY⁺ CD11b⁺ cells did not express Gr-1⁺ (most consistent with macrophages). In contrast, labeled complexes were relatively rare in F4/80⁺ or CD11c⁺ cells. These results suggest that early after immunization, LANAC were taken to draining lymph nodes primarily within macrophages and neutrophils and possibly monocytes, rather than within classical DC. However, most of the BODIPY⁺/CD11b⁺/Gr-1⁻ macrophages were also F4/80⁻, indicating that they were probably not derived from resident peritoneal macrophages, which were strongly F4/80⁺ (data not shown). The second population of CD11b⁺/Gr-1⁻ cells was most likely comprised of both neutrophils and inflammatory monocytes (50). The presence of neutrophils in the draining lymph nodes was also confirmed by cytologic examination (data not shown). The Gr-1⁻/CD11b⁺ cells observed here resembled in some respects a recently described population of unique APC present in the peritoneal cavity that are associated with Ag presentation following vaccination with aluminum hydroxide adjuvant (51).

Additional experiments were done using BODIPY-labeled liposomes complexed to other TLR agonists, including poly(I:C), zymosan, and LPS, to assess uptake by APC in draining lymph nodes. Important differences in cell uptake were not observed when uptake of labeled complexes with other TLR agonists was compared with that observed using plasmid DNA (data not shown). Therefore, the initial uptake of LANAC appeared to be mediated primarily by the liposome component, because altering the TLR agonist did not affect cellular uptake. These results suggested that macrophages and possibly inflammatory monocytes, rather than classical DC, were the primary APC responsible for transport of LANAC to lymph nodes soon after immunization. However, these observations do not exclude the possibility that Ags were transferred later to other APC in the lymph nodes, or that the phenotype of the earliest APC transporting LANAC to lymph nodes changed over time to resemble cells more consistent with typical DC, as has been reported previously with inflammatory monocytes (50).

$T$ cells elicited by LANAC vaccination are functionally active and long-lived

Although some vaccines can generate large numbers of Ag-specific T cells, in some cases these T cells are nonfunctional (52). Therefore, we conducted experiments to assess the functionality of CD8⁺ T cells elicited by LANAC immunization. In an in vitro $^{51}$Cr-release assay using effector cells derived from spleens of immunized mice and restimulated in vitro, high levels of peptide-specific killing were observed (data not shown). An in vivo CD8⁺ T cell assay was also used to directly assess cytotoxic activity. Following immunization with OVA in LANAC, very high levels of specific CD8⁺ T cell activity were observed in spleen cells (Fig. 5A). The mean R value (ratio of nonpulsed to peptide-pulsed target cells) for LANAC-immunized mice was 52.3 (±3.7), compared with 1.2 (±0.1) for nonimmunized control mice. Thus, LANAC immunization elicited CD8⁺ T cells with functional cytolytic activity.

The ability to generate IFN-γ was also assessed in CD8⁺ T cells elicited by LANAC adjuvant. After in vitro restimulation, spleen cells from mice immunized with OVA-vaccinated mice released large quantities of IFN-γ into the supernatant following overnight
Acute viral infections typically elicit large expansions of CD8⁺ T cells, but these T cells may be relatively short-lived (53, 54). Therefore, experiments were done to assess the survival and tissue distribution of Ag-specific CD8⁺ T cells elicited by immunization with LANAC. CD8⁺ T cell responses in lung and spleen tissues were analyzed 5 days and 3 mo after immunization with 5 μg of OVA in LANAC. Large numbers of OVA-specific CD8⁺ T cells were present in the lungs at 3 mo, although the percentage of Ag-specific CD8⁺ T cells had declined by ~50% during this time period (from 66% of total CD8⁺ T cells to 32% of total CD8⁺ T cells; Fig. 5C). The phenotype of these Ag-specific CD8⁺ T cells (CD44⁺CD62L⁻) was consistent with that of memory effector CD8⁺ T cells, which have been described as residing for long periods of time in tissues following systemic viral infection (53). In contrast, OVA8-specific CD8⁺ T cells were relatively rare in the spleen at 3 mo (data not shown). Thus, LANAC vaccination appeared to elicit large numbers of long-lived memory effector CD8⁺ T cells.

**Adjuvants prepared with TLR9 or TLR3 agonists effectively cross-prime CD8⁺ T cell responses**

The preceding results indicated that a TLR9 agonist (plasmid DNA) could elicit efficient cross-priming when combined with cationic liposomes. To determine whether other TLR agonists complexed to liposomes were capable of eliciting similar responses, a series of liposome-TLR agonist complexes were prepared and assessed for their ability to elicit CD8⁺ T cell responses following immunization with 5 μg of OVA. The liposome-TLR agonist complexes evaluated included zymosan (TLR2), poly(I:C) (TLR3), LPS (TLR4), R848 (TLR7/8), and CpG oligonucleotides or plasmid DNA (TLR9). The adjuvants were all prepared using the same relative amounts of cationic liposome and TLR agonist. Mice were immunized twice i.p., and spleen, lung, and liver cells were analyzed by tetramers to quantitate CD8⁺ T cell responses. We found that only adjuvants prepared using TLR9 agonists (plasmid DNA, CpG oligonucleotides) or TLR3 agonists (poly(I:C)) were able to efficiently generate strong Ag-specific CD8⁺ T cell responses (Fig. 6). Each of these three adjuvants elicited large numbers of Ag-specific CD8⁺ T cells in spleen, lung, and liver tissues following immunization with OVA. Of the other adjuvants evaluated, only liposome-zymosan complexes elicited a significant increase (p < 0.05) in OVA8-specific CD8⁺ T cells, although the response was still much less than that elicited by CpG-, DNA-, and poly(I:C)-containing adjuvants. Thus, TLR3 and TLR9 agonists appeared to be uniquely effective as vaccine adjuvants when formulated with liposomes.

Several recent reports indicate that the ability to cross-prime CD8⁺ T cell responses is linked to induction of type I IFN production (48, 55, 56). Therefore, to determine whether a similar association was true with LANAC-based vaccines, we examined the ability of different liposome-TLR agonist complexes to induce production of IFN-α in vivo. We found that only liposome-TLR agonist adjuvants that contained TLR9 or TLR3 agonist elicited substantial production of IFN-α in vivo (Ref. 21 and data not shown). These results are consistent with previous reports and suggest that the ability of vaccine adjuvants comprised of liposomes and TLR3 or TLR9 agonists to cross-prime CD8⁺ T cell responses may depend in part on induction of type I IFNs.

**LANAC vaccination against trp2 elicits antitumor activity in mice with established melanoma**

Generation of T cell responses against shared tumor Ags such as the endogenous melanoma Ag (trp2) is typically very difficult in mice with established tumors (57). Therefore, we assessed the ability of LANAC vaccines to elicit therapeutic antitumor activity in mice with established B16 melanomas. The melanoma Ag (trp2) was used as the target for immunization. Mice with day 7 established tumors were vaccinated with the trp2 melanoma Ag weekly for three immunizations beginning on day 7, by either the s.c. or i.p. routes of immunization. Tumor responses were quantitated by serial tumor measurements. Vaccination with trp2 peptide (5 μg of peptide per mouse per immunization) in LANAC administered by
LANAC vaccination elicits protective immunity against aerosol challenge with M. tuberculosis

The ability of LANAC vaccines to elicit protective immunity was also assessed in an aerosol M. tuberculosis challenge model. For these studies, the ESAT-6 Ag of M. tuberculosis was used, because this Ag has been shown previously to elicit protective immunity against M. tuberculosis (58–60). Mice were each immunized twice by the s.c. or i.p. routes with 10 ug of recombinant ESAT-6 protein formulated in LANAC. The liposomal MPL adjuvant has been used previously to successfully vaccinate mice against M. tuberculosis, so another group of mice was immunized with ESAT-6 protein in the MPL adjuvant. Controls included mice injected with saline only. Mice were subjected to aerosol challenge with virulent M. tuberculosis (Erdman strain) 3 wk after the last vaccine, and titers in lung tissues were determined 30 days after challenge. We found that immunization with ESAT-6 in LANAC elicited significant protection (p < 0.05) from challenge, compared with nonvaccinated control mice and mice immunized with ESAT-6 in MPL adjuvant (Fig. 8). Thus, immunization with LANAC adjuvant and a recombinant protein Ag was able to generate significant protective immunity in a rigorous tuberculosis aerosol infection model.

Discussion

The major findings to emerge from these studies are that 1) certain TLR agonists can be combined with cationic liposomes and Ags to produce very potent vaccines capable of eliciting both CD4 and CD8 T cell responses; 2) liposomes complexed to TLR3 and TLR9 agonists are uniquely effective at cross-priming CD8 T cell responses in vivo; 3) the full activity of liposome-TLR agonist adjuvants requires their physical association with the Ag; and 4) liposome-TLR agonist adjuvants can be used to generate effective therapeutic antitumor immunity and protective immunity against aerosol challenge with M. tuberculosis. These results suggests that coupling vaccine delivery using liposomes with activation of innate immunity using specific TLR agonists represents an effective

![Image](http://www.jimmunol.org/)

**FIGURE 5.** Immunization with LANAC adjuvant elicits functional CD8 T cells. Experiments were conducted to assess the functionality and long-term survival of CD8 T cells elicited by vaccination with LANAC. A, CD8 T cell cytolytic activity was assessed in vivo using adoptive transfer of peptide-pulsed and unpulsed CFSE-labeled spleen cells into naive control mice or mice vaccinated twice with OVA in LANAC (3 mice/group), as described in Materials and Methods. Eighteen hours later, spleen cells were harvested from the adoptively transferred mice, and the relative proportions of the two populations of CFSElow cells were assessed. Representative FACS plots of CFSElow-transferred target cell populations (peptide-pulsed CFSEhigh and unpulsed CFSElow cells) in spleen of a control mouse (top panel) and an OVA-vaccinated mouse (bottom panel) are shown. The mean ratio (±SD) of CFSElow (unpulsed) to CFSEhigh (peptide pulsed) -transferred cells in the spleens of OVA LANAC-vaccinated mice was significantly greater (p < 0.05) than in unvaccinated control mice 18 h after adoptive transfer of target cells (data not shown). Similar results were obtained in one additional experiment. B, Spleen cells from unvaccinated control mice (left panel) and mice (4/group) immunized twice with OVA in LANAC (right panel) were analyzed by flow cytometry for intracellular production of IFN-γ following in vitro restimulation with 1 µM OVA8 peptide, as described in Materials and Methods, and a representative FACS plot is shown. Similar results were obtained in one additional experiment. C, Spleen cells from unvaccinated mice, mice vaccinated twice with 5 µg of OVA8 peptide in LANAC, and mice vaccinated with 5 µg of OVA in LANAC were restimulated in vitro with 1 µM OVA8 peptide for 18 h, and supernatants were collected and analyzed for production of IFN-γ by ELISA, as described in Materials and Methods. The mean (±SD) IFN-γ concentration was plotted for each treatment group (4 animals/group). Mice immunized with OVA8 or OVA both produced significantly more (p < 0.05) IFN-γ than unvaccinated control mice, as denoted by *. Similar results were obtained in two additional experiments. D, The long-term survival of OVA8-specific CD8 T cells elicited by LANAC vaccination in peripheral tissues (lung) was assessed in mice (4/group) 44 days after the first immunization and compared with the percentage of OVA8-specific CD8 T cells present 14 days after the first immunization. The percentage of OVA-specific CD8 T cells in lung at 44 days declined by approximately half compared with day 14 mice, but there were still significantly (p < 0.01) more Ag-specific CD8 T cells in lung tissues at day 44 than in control mice, as denoted by *. Similar results were obtained in one additional experiment.
Vaccine adjuvants are generally classified as either vaccine delivery systems or as immune potentiators, depending on their primary mode of action (8). Vaccine delivery systems (e.g., liposomes or microparticles) deliver the Ag to appropriate APC and in some cases prolong the duration of Ag presentation. Immune potentiators (e.g., bacterial products or cytokines) function by activating innate immune responses, leading to enhanced Ag presentation. The effectiveness of the liposome-TLR agonist adjuvants described in this study probably results from the combination of enhanced Ag delivery and potent stimulation of innate immunity. For example, liposomes have been previously used as vaccine adjuvants, primarily by facilitating delivery of Ags to APC in vivo (25, 61–63). Liposomes also facilitate cross-priming by promoting entry of protein Ags into the MHC class I pathway for presentation to CD8⁺ T cells (64). In addition, cationic liposomes have been shown to markedly enhance the immune stimulation elicited by bacterial DNA and CpG oligonucleotides (18–20). The fact that CD8⁺ T cell responses were largely eliminated in MyD88⁻/⁻ mice provided additional evidence of the importance of simultaneous Ag delivery and activation of APCs for efficient generation of T cell responses (see Fig. 3).

One of the most notable properties of the liposome-TLR agonist complex adjuvants, particularly those formulated with TLR3 or TLR9 agonists, was their ability to stimulate efficient cross-priming in vivo. This property appeared to be independent of CD4 help (see Fig. 3). Thus, immunization with the LANAC adjuvant probably activated CD40 on APC directly, thereby bypassing the requirement for CD4⁺ T cells for initial generation of Ag-specific CD8⁺ T cells (65, 66). The efficiency of cross-priming may have
been due in part to the ability of cationic liposomes to facilitate the interaction of TLR3 and TLR9 agonists with their endosomal receptors and thereby promote immune activation (2, 67). In addition, complexes of liposomes with TLR3 or TLR9 agonists stimulated production of type I IFNs, including IFN-α, and the association between induction of type I IFNs and cross-priming has been noted previously (55, 68).

The relative inability of other TLR agonists (e.g., TLR2, TLR4, and TLR7/TLR8 agonists) complexed to cationic liposomes to function as effective vaccine adjuvants may have several explanations (see Fig. 6). For example, TLR2 and TLR4 are expressed primarily on the cell surface and not within endosomes (2). Therefore, they may not receive a strong activating stimulus when their ligands are delivered as liposome complexes. However, failure to activate innate immunity is probably not the only explanation, because we observed that all the liposome-TLR agonist complexes evaluated in these studies were capable of activating innate immunity when administered in vivo (data not shown). It is also possible that signaling via TLR2, TLR4, and TLR7/8 agonists may have delivered qualitatively different signals to the APCs, which would have affected the magnitude of the T cell responses that were elicited.

The liposome-TLR agonist adjuvants evaluated in this study targeted an unusual population of APC in draining lymph nodes, which may have played an important role in adjuvant effectiveness. At early time points after immunization withLANAC, the complexes were primarily associated with CD11b+/Gr-1− cells and were rarely present in CD11c+ cells (Fig. 4). Therefore, the cells responsible for much of the early trafficking of LANAC vaccines to APC in draining lymph nodes were primarily neutrophils, macrophages, and inflammatory monocytes, rather than classical DC (50). Inflammatory monocytes, which can differentiate into DC in lymph nodes, may therefore play a key role as APC for liposome-TLR agonist-based vaccine adjuvants.

The results of these studies suggest additional strategies for developing more effective vaccine adjuvants based on the liposome-TLR agonist platform. For example, other pattern recognition receptors could be incorporated in the liposomal delivery system, in addition to TLR ligands. Moreover, coupling of targeting molecules such as Abs to DEC-205 into the liposomes may also prove effective for retargeting of the complexes to more classical DC, as has been demonstrated recently (69, 70). Other applications of the adjuvant platform include mucosal immunization, where we have found that the liposome-TLR agonist adjuvants are effective (S. Dow, unpublished data). The LANAC adjuvants may also be useful clinically, because we showed recently that vaccination of pet dogs with refractory atopic dermatitis with allergens complexed to LANAC adjuvants was effective in reducing clinical signs and reversing some Th2 abnormalities (71). Therefore, the liposome-TLR agonist adjuvant system described in this study may be a useful addition to the list of currently available vaccine adjuvants capable of eliciting strong T cell responses against protein and peptide Ags.

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Disclosures

A patent has been filed covering the adjuvant formulation described in this manuscript, and Steven Dow is listed as one of the coinventors.

References