

The combination of stabilized plasmid lipid particles and lipid nanoparticle encapsulated CpG containing oligodeoxynucleotides as a systemic genetic vaccine

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Abstract

Background DNA vaccines offer unique potential for generating protective and therapeutic immunity against infectious and malignant diseases. Unfortunately, rapid degradation and poor cellular uptake has significantly limited the efficacy of 'naked' plasmid DNA vaccines. We have previously described stabilized plasmid lipid particles (SPLP) as effective nonviral gene delivery vehicles for the transfection of tumours at distal sites following intravenous administration. Based on their low toxicity and favourable transfection profile following systemic administration, we investigate SPLP as gene delivery vehicles for the generation of a systemically administered genetic vaccine.

Methods The uptake of SPLP and their ability to transfect splenic antigen presenting cells (APC) following systemic administration is assessed through fluorescently-labelled SPLP in combination with phenotype markers and a very sensitive flow cytometry-based assay for the detection of the transgene, beta-galactosidase. The priming of antigen-specific adaptive and humoral immune responses following vaccination with SPLP alone or in combination with liposomal nanoparticle encapsulated CpG-ODN containing oligodeoxynucleotides (LN CpG-ODN) is characterized through the use of antigen-specific cytotoxicity assays, interferon- γ secretion assays and enzyme-linked immunosorbent assay.

Results We demonstrate that SPLP are taken up by and transfect APC in the spleen following intravenous administration and that, in the presence of a strong immunostimulatory signal provided by LN CpG-ODN, are able to prime transgene-specific humoral and cellular immune responses.

Conclusions SPLP represent an effective candidate for the nonviral delivery of a systemic genetic vaccine when combined with additional immune stimulation provided by LN CpG-ODN. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords CpG-oligodeoxynucleotides; genetic vaccine; liposomal nanoparticle; plasmid DNA; stabilized plasmid lipid particles

Introduction

Currently, a wide variety of approaches are being investigated for the generation of vaccines against infectious and malignant disease. DNA vaccines

provide an important advantage over other vaccine strategies in that they mimic natural viral infection in their expression and processing of foreign proteins in a manner that induces CD8+ T-cell responses as well as humoral responses. Following early work which demonstrated the ability of naked plasmid DNA (pDNA) to elicit both cellular and humoral immune responses and provide subsequent protection from a targeted disease in animal models [1–3], attempts have been made to use plasmid-based immunization to generate protective immunity against a wide variety of infectious, malignant, autoimmune and allergic diseases [4–6]. Although naked DNA vaccines have proven effective in small animal models, they have often been found to prime sub-optimal immune responses in human clinical studies. This can be attributed in part, to low *in vivo* transfection efficiencies stemming from the rapid degradation of the phosphodiester backbone [7,8] as well as inefficient uptake of free DNA by antigen-presenting cells.

Considerable effort is now being focused on developing ways to improve the efficacy of DNA vaccines, and it is well recognized that the route and method of DNA vaccine administration strongly influences both the strength and nature of the resultant immune response [9]. Although systemic administration of genetic vaccines possesses the advantage of direct access to the relatively large number of antigen presenting cells (APC) in the spleen and has, in a number of instances, demonstrated the potential to induce antigen-specific humoral and cellular responses [10–13], the instability of naked pDNA in the blood makes this approach very inefficient. It is clear that nucleic acid-based vaccines for the immunotherapy of infectious disease and cancer would benefit from the development of vector systems that can be safely and effectively administered systemically. Cationic lipid-based delivery systems, which partially protect the nucleic acid payload from nuclease degradation and improve uptake by APC [14–16], can dramatically enhance the potency of DNA vaccines following administration via a number of routes [17,18]. However, cationic lipid-based delivery systems have a limited ability to protect pDNA from degradation by serum nucleases and the systemic administration of these complexes is often associated with significant toxicity [19,20].

We have previously described stabilized plasmid lipid particles (SPLP) as nonviral gene delivery vehicles for the delivery of pDNA to distal tumour sites following systemic administration [21–27]. However, as a result of their ability to protect pDNA from nuclease degradation, their small size (140 ± 50 nm), net neutral charge and low toxicity following systemic administration, combined with the fact that these particles are naturally cleared from the circulation by APC [14,28,29], SPLP represent a prime candidate for the delivery of systemic genetic vaccines. In the present study, we investigate the ability of SPLP to be taken up by and transfect APC following systemic administration, and the ability of SPLP-mediated delivery of a transgene to APC to induce transgene-specific humoral and cellular immune

responses. Furthermore, we characterize the resultant immune response following vaccination with SPLP alone or in the presence of liposomal nanoparticle encapsulated CpG-ODN containing oligodeoxynucleotides (LN CpG-ODN), a potent vaccine adjuvant previously demonstrated to promote immune cell activation and the induction of adaptive cellular responses against co-administered peptide or protein antigens [28–30].

Materials and methods

Materials

1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), whereas cholesterol was obtained from Sigma (St Louis, MO, USA). 1,2-dioleoyloxy-3-N,N-dimethylaminopropane (DODMA) and polyethylene glycol-dimyrystol glycerol (PEG-DMG) were provided by Tekmira Pharmaceuticals Corporation (Burnaby, BC, Canada). The fluorescently-labelled lipid 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Di-I) was purchased from Invitrogen Molecular Probes (Burlington, ON, Canada). The pCMVluc and pCMV β gal plasmids, encoding luciferase (luc) and beta-galactosidase (β gal) reporter genes, respectively, under the control of the cytomegalovirus promoter, were propagated in *Escherichia coli* strain DH5 α and purified by standard alkaline lysis with two rounds of cesium chloride density gradient centrifugation. Endotoxin levels in pDNA were less than 10 EU/mg as determined by the limulus amoebocyte lysate chromogenic endpoint assay as per the manufacturer's instructions (Charles River Laboratories, Wilmington, MA, USA). INX-6295, a 16-mer phosphorothioate ODN (5'-TAACGTTGAGGGGCAT-3') containing unmethylated cytosine residues in the CpG motif, was synthesized by Trilink Biotechnologies (San Diego, CA, USA).

Animals and cell lines

Female, 6–8-week-old BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were quarantined for at least 2 weeks prior to use. All procedures involving animals were performed in accordance with the guidelines established by the Canadian Council on Animal Care. The macrophage cell line, RAW264.7 and the parental and β gal expressing colon carcinoma cell lines, CT26 and CT26.CL25, respectively, were obtained from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin-G and 100 μ g/ml streptomycin sulphate at 37°C with 5% CO₂. CT26 and CT26.CL25 cells were cultured in RPMI 1640

medium supplemented with 10% FBS, 2 mM L-glutamine, 1.0 mM sodium pyruvate and adjusted to contain 4.5 g/l glucose. CT26.CL25 media also contained 0.4 mg/ml of the selection antibiotic G418. Splenocytes, isolated from BALB/c mice, were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin-G, 100 µg/ml streptomycin sulphate, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 mM HEPES and 5×10^{-5} M β -mercaptoethanol. All cell culture reagents were obtained from Invitrogen (Burlington, ON, Canada).

Preparation of SPLP and LN-CpG ODN

Plasmid DNA was encapsulated in LN consisting of DSPC/cholesterol/DODMA/PEG-DMG at a molar ratio of 20/45/15/10 using an ethanol dialysis procedure, as previously described [31,32]. SPLP were characterized with respect to plasmid entrapment using a PicoGreen assay (Invitrogen Molecular Probes), and mean particle diameter was determined using a submicron quasi-elastic light scattering particle sizer (Nicomp, Santa Barbara, CA, USA). SPLP formulations were 145 ± 50 nm ($\chi^2 = 0.3$) in diameter, had a plasmid-to-lipid ratio of 0.04–0.05 (w/w) and a maximum of 5–8% untrapped plasmid following purification.

Unmethylated phosphorothioate ODN (16-mer, 5'-TAACGTTGAGGGGCAT-3'; Trilink Biotechnologies) were encapsulated in LN consisting of POPC/cholesterol/DODMA/PEG-DMG at a molar ratio of 25/45/20/10 using an ethanol dialysis procedure, as previously described [29,32]. The ODN-to-lipid ratio was typically 0.1 (w/w) and particle size was 100 ± 25 nm.

Uptake and transfection of APC by SPLP

In vitro and in vivo uptake of SPLP by APC

For *in vitro* studies, splenocytes isolated from BALB/c mice were prepared as single cell suspensions by passage through a sterile 100 µm nylon mesh filter (BD Biosciences, Mississauga, ON, Canada) and red blood cells were lysed in ACK buffer [0.1 M ammonium acetate, 10 mM potassium bicarbonate, 70 µM ethylenediaminetetraacetic acid (EDTA)] on ice. Cells were plated at a density of 5×10^6 cells/ml and were incubated at $37^\circ\text{C} + 5\%$ CO₂ overnight. Cells received 4 µg/ml of SPLP (luc) formulated to contain the fluorescently-labelled lipid Di-I (Invitrogen Molecular Probes; Di-I-SPLP) at a final lipid concentration of 0.5 mole percentage and were incubated at 37°C or 4°C for the indicated time-points. For *in vivo* studies, 8–10-week-old BALB/c mice were injected intravenously (i.v.) with 5 mg/kg Di-I-SPLP. Mice were euthanized by a terminal dose of 3.2% (v/v) ketamine/0.8% (v/v) xylazine 1, 4, 12 and 24 h after administration and lymph nodes and spleens were harvested. Single cell suspensions were prepared and stained for phenotype markers (CD11c for DCs, CD11b and Mac3

for macrophages, B220/CD45R for B-lymphocytes) with fluorescently-labelled antibodies (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions and analysed on a LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) for SPLP uptake by specific immune cell populations. Dead cells were excluded with propidium iodide (Invitrogen Molecular Probes) and data were acquired using FACSDiva software and analysed using FloJo software V (BD Biosciences).

SPLP-mediated transfection of APC in vitro

RAW264.7 cells were plated at a concentration of 4×10^4 cells/ml. Splenocytes isolated from BALB/c mice were prepared as single cell suspensions as described previously and were plated at a density of 5×10^6 cells/ml. Cells were treated with either PBS (control) or some combination of LN CpG-ODN (3 µg/ml, added 4 h after plating for 12 h at $37^\circ\text{C} + 5\%$ CO₂) and SPLP (luc) at 4 µg/ml for the indicated time points. RAW264.7 cells and splenocytes were harvested manually and washed with PBS. For appropriate studies, CD11b+ cells were isolated from whole splenocyte populations by immunomagnetic sorting with CD11b microbeads (Miltenyi Biotech, Auburn, CA, USA). For each treatment, 5.0×10^7 splenocytes were magnetically-labelled and sorted. All cell populations were lysed with 0.25 ml of Reporter Lysis Buffer (Promega, Madison, WI, USA) followed by a freeze-thaw cycle. Luciferase assays were performed using the Promega Luciferase Assay reagent kit (Promega), according to the manufacturer's instructions, in a Centro LB 960 microplate luminometer (Berthold Technologies, Oak Ridge, TN, USA). A standard curve was generated from purified firefly luciferase (Roche, Montreal, QC, Canada) and used to calibrate luminescence readings and luciferase activity was normalized against cell protein concentration determined using the Micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA).

SPLP-mediated transfection of APC in vivo

BALB/c mice were seeded with 1×10^5 CT26 tumour cells in 100 µl of PBS by subcutaneous hind flank injection. Tumours were allowed to reach an approximate volume of 200 mm³ [volume = (length \times width²)/2] prior to i.v. administration of 5 mg/kg of SPLP (β gal) alone or followed 24 h later by LN CpG-ODN (10 mg/kg) via the lateral tail vein. For the 24-h time-point, LN CpG-ODN was administered 12 h after the addition of SPLP. Animals were sacrificed 24, 48, 72 and 96 h after SPLP administration, spleens were harvested and single cell suspensions were prepared as previously described. β gal activity was detected using the fluorogenic substrate 5-chloromethylfluorescein di-b-d-galactopyranoside (CMFDG) and the DetectaGene Green lacZ Detection Kit as per manufacturer's instructions (Invitrogen Molecular Probes). Briefly, 2×10^6 splenocytes were incubated in media containing 200 µM chloroquine for 30 min at 37°C , 5% CO₂. Cells were loaded

with CMFDG using the Influx Reagent and incubated for 3 h at 37 °C, 5% CO₂, following which the reaction was terminated by the addition of the competitive inhibitor phenylethyl β-D-thiogalactopyranoside at 1 mM. Verapamil was included in all solutions at a final concentration of 100 μM to block the efflux of the fluorescent product. Cells were analysed immediately on a flow cytometer as previously described using propidium iodide to exclude dead cells.

SPLP-mediated induction of adaptive immune responses

Vaccination

Eight- to 10-week-old BALB/c mice were injected i.v. by lateral tail vein injection with SPLP (βgal) or SPLP (luc) corresponding to a dose of 5 mg/kg once a week for 3 weeks. Mice receiving both SPLP and LN CpG-ODN received LN CpG-ODN, at a dose of 10 mg/kg, 24 h following the administration of SPLP. One week following final vaccination, mice were euthanized as described above and blood was collected via cardiac puncture in Vacutainer tubes containing EDTA (BD Biosciences). Serum was isolated and stored at -70 °C until assayed. Spleens were harvested and processed as described above.

Serum immunoglobulin analysis

Pooled serum samples were analysed in quadruplicate for anti-βgal antibodies by an enzyme-linked immunosorbent assay (ELISA). In brief, plates (Nunc-Immuno MaxiSorp; Nalge Nunc, Denmark) were coated overnight at 4 °C with 10 μg/ml βgal protein (Calbiochem, San Diego, CA, USA) in a solution containing 0.1 M sodium carbonate, pH 9.5. After blocking with 10% FBS in PBS (pH 7.4) for 1 h at room temperature (RT), plates were incubated for a further 2 h at RT with the serially diluted experimental mouse serum and then washed with PBS + 0.05% Tween 20. Mouse immunoglobulin (Ig)G1 and IgG2a antibodies were detected by biotin-conjugated rabbit anti-mouse isotype-specific antibodies (Rockland, Gilbertsville, PA, USA) and avidin-horse radish peroxidase (BD Biosciences)/TMB substrate (BD Biosciences) at OD 492 nm corrected for absorbance at 520 nm. Antibody titre was defined as the highest serum dilution giving an absorbance twice that of serum from non-immune control animals.

Determination of cytotoxic T-lymphocyte (CTL) activity by ⁵¹chromium release assays

One week following the final vaccination, CTL responses were assessed *ex vivo* or following 5 days of *in vitro* restimulation using a standard ⁵¹chromium (⁵¹Cr) release assay. For *in vitro* restimulation, splenocytes from immunized animals were co-cultured with control splenocytes pulsed with 0.2 μM of the βgal-immunodominant peptide TPHPARIGL (Sigma-Genosys, Oakville, ON, Canada) and treated with mitomycin C (50 μg/ml), at a ratio of

10 : 1 for 5 days with the addition of human recombinant interleukin (IL)-2 (100 IU/ml; BD Biosciences). βgal-specific cytotoxicity was assessed using a standard 5-h ⁵¹Cr release assay where splenocytes were mixed in effector to target (E : T) ratios of 5 : 1, 25 : 1, 50 : 1, 100 : 1 with ⁵¹Cr-loaded parental CT26 or βgal-expressing CT26.CL25 cells. The percentage of cellular cytotoxicity was calculated on the basis of ⁵¹Cr released to the supernatant using the formula: [sample counts per min (cpm) – spontaneous (cpm)]/(maximum cpm) × 100, where maximal cpm was achieved by complete lysis of ⁵¹Cr-labelled targets in 10% TritonX-100, spontaneous cpm was determined by incubating labelled targets in media and antigen-specific killing was determined by comparison of cytotoxicity of ⁵¹Cr-labelled βgal expressing and non-expressing CT26.CL25 and CT26 cells, respectively.

Interferon (IFN)-γ cytokine secretion assay

CD8+ T lymphocytes capable of responding to antigen-specific stimulation were detected using the IFN-γ secretion assay (Miltenyi Biotec Inc., Auburn, CA, USA) according to the manufacturer's instructions. Briefly, splenocytes from immunized mice were re-stimulated with 10 μg/ml of the peptide TPHPARIGL for 5 h prior to incubation with a bi-specific antibody designed to bind to activated T-cells via the CD25 activation marker and capture secreted IFN-γ. The frequency and phenotype of cells responding to βgal stimulation were determined by flow cytometry as described previously using a fluorescently-labelled anti-IFN-γ antibody in combination with the previously described fluorescently-labelled phenotype antibodies.

Statistical analysis

All statistical analyses were performed using SPSS, version 14.0 (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance was used to evaluate the differences between treatment groups. In the case of statistically significant results, the differences between treatment groups were assessed using Tukey's HSD test where *p* < 0.05 was considered statistically significant.

Results

APC uptake SPLP *ex vivo*

Studies employing Di-I-labelled SPLP demonstrate that *ex vivo*, SPLP is taken up by splenic CD11b+, CD11c+ and B220/CD45R+ APC when present at concentrations typically used in transfection experiments (Figure 1). The maximum percentage of cells positive for uptake was reached between 4 and 8 h for all three cell-types and was greatest for CD11b+ cells (37%), followed by CD11c+ cells (30%) and B220/CD45R+ cells (21%). On a per cell basis as judged by mean fluorescence intensity

(MFI), CD11b+ and CD11c+ cells showed a similar level of uptake, whereas uptake was approximately two-fold lower in B220/CD45R+ cells at 8 h. Uptake by CD8+ and CD4+ cells was only visible at 8 h where 3.0% and 1.3% of cells were positive for uptake, respectively. Similar uptake levels were also observed in cells treated with LN CpG-ODN 12 h prior to the addition of SPLP. Nonspecific binding was less than 2% in all instances as determined by incubation at 4 °C (data not shown).

SPLP transfect the cultured macrophage cell line RAW264.7, whole splenocyte populations and CD11b+ cells *ex vivo*

Investigation of the ability of SPLP (luc) to transfect the cultured macrophage cell line RAW264.7 and cultured splenocytes *ex vivo*, demonstrates that SPLP (luc) is capable of transfecting both, albeit at low levels, and that similar levels of gene expression are obtained when cells are pretreated with LN CpG-ODN. Detectable levels of luciferase expression were observed in RAW264.7 cells 48 and 72 h after incubation with SPLP in both the presence and absence of LN CpG-ODN (Figure 2A)

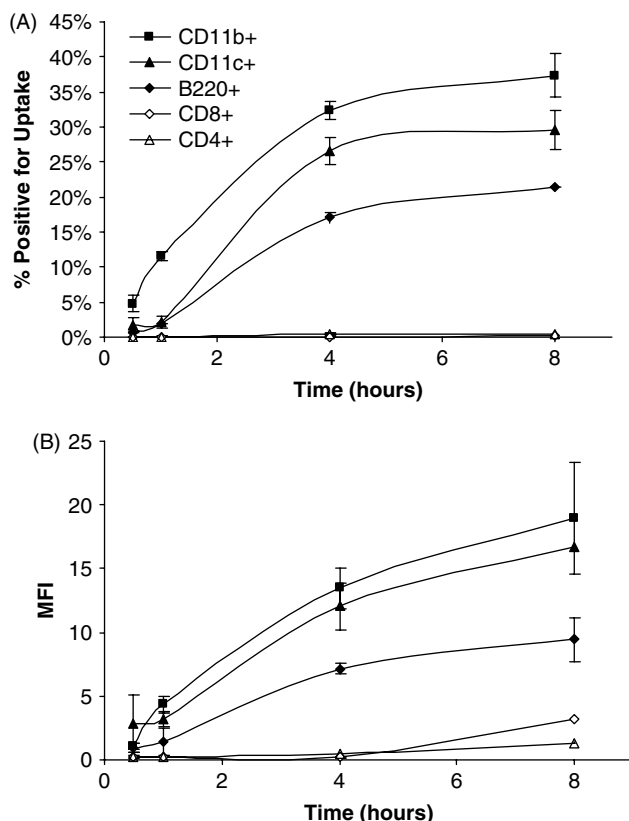


Figure 1. SPLP is preferentially taken up by CD11b+, CD11c+ and B220/CD45R+ cells *ex vivo*. Splenocytes isolated from BALB/c mice were treated with Di-I labeled SPLP (4.0 μ g/ml) *ex vivo*. (A) Percent uptake and (B) MFI, as determined by flow cytometry, was calculated by subtracting nonspecific binding for each cell-type at 4 °C from uptake at 37 °C. Each data point represents $n = 3$ (\pm SD)

and similarly in cultured splenocytes 36 and 48 h after incubation with SPLP in both the presence and absence of LN CpG-ODN (Figure 2B). Similar expression levels were observed when cells were treated with LN CpG-ODN 12 h after the addition of SPLP (data not shown).

Immunomagnetic sorting was used to specifically demonstrate SPLP-mediated transfection of CD11b+ cells in whole splenocyte populations isolated from BALB/c mice. A two- to three-fold increase in luciferase expression per mg of protein was observed at 48 h in CD11b+ enriched populations over that observed for unseparated or CD11b depleted populations indicating that CD11b+

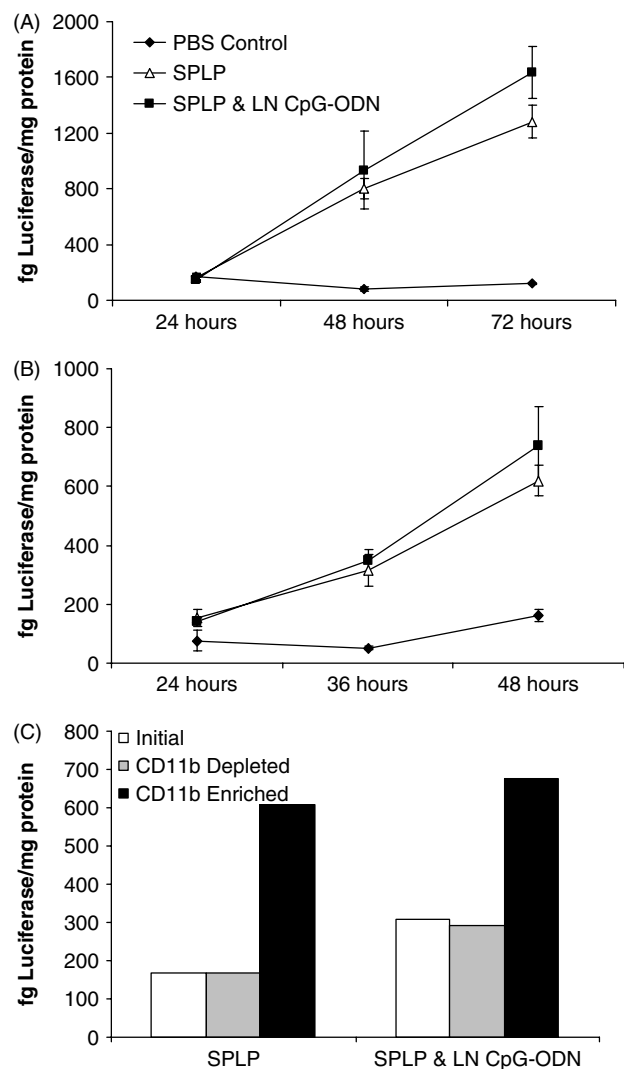


Figure 2. SPLP transfects the cultured macrophage cell line RAW264.7, whole splenocyte populations and primary macrophage *ex vivo* both in the presence and absence of LN CpG-ODN. (A) RAW264.7 cells or (B, C) splenocytes were treated with SPLP (luc) (4.0 μ g/ml), SPLP (luc) (4.0 μ g/ml) and LN CpG-ODN (3.0 μ g/ml) or (\blacklozenge) PBS. Wells receiving LN CpG-ODN were treated 12 h prior to SPLP addition. Luciferase expression was normalized to protein concentration, error bars represent $n = 4$ (\pm SD) (A, B). (C) Forty-eight hours after treatment with SPLP, splenocyte populations were immunomagnetically sorted to obtain CD11b+ enriched and CD11b+ depleted populations prior to the quantitation of luciferase expression; data represent the findings from two independent experiments

cells are transfected by SPLP *ex vivo* (Figure 2C). On average, immunomagnetic sorting resulted in a 12–20-fold increase in the percentage of CD11b+ cells over that of the depleted population or unsorted population.

SPLP are taken up by APC in the spleen following intravenous administration

Using Di-I-labelled SPLP, we were able to demonstrate the *in vivo* uptake of SPLP by CD11b+, CD11c+ and B220/CD45R+ cells in the spleen within 1 h of i.v. administration. The percentage of cells positive for uptake peaked 1 h following administration for CD11b+ cells (66%) and CD11c+ cells (51%) and, whereas B220/CD45R+ cells showed a similar level of cells positive for uptake (58%), this cell-type displayed a different uptake profile, exhibiting maximum uptake 4–12 h after administration (Figure 3A). Although a similar percentage of B220/CD45R+ cells were found to be positive for uptake compared to CD11b+ and CD11c+ cells, they were found to have an eight- to ten-fold lower level of uptake on a per cell basis based on MFI compared to CD11b+ and CD11c+ cells (Figure 3B). It should be noted that measures to quench cell surface fluorescence were not taken as we have previously observed using confocal microscopy that following the *in vivo* administration of fluorescently-labelled LN virtually all of the fluorescence detected in antigen-presenting cells (including CD11b+, CD11c+ and B220/CD45R+ cells) is inside the cell (data not shown).

SPLP transfect CD11b+ and CD11c+ cells in the spleen following intravenous administration

The ability of SPLP (β gal) to transfect APC in the spleen 24, 48, 72 and 96 h following i.v. administration was demonstrated by flow cytometry using the fluorogenic substrate CMFDG in conjunction with fluorescently-labelled phenotype markers. β gal activity was observed in CD11b+ cells 48–96 h after SPLP administration both in mice receiving SPLP (β gal) alone, or SPLP (β gal) followed 24 h later by LN CpG-ODN (Figure 4). Peak levels of cells positive for gene expression were observed 72 h following transfection with SPLP (β gal) alone, or in combination with LN CpG-ODN. Although the percentage of cells positive for gene expression at 72 h in the presence of LN CpG-ODN was similar to that observed when mice were treated with SPLP (β gal) alone (9.4% and 12.6%, respectively), treatment with LN CpG-ODN appeared to alter expression kinetics as the level of expression on a per cell basis peaked 72 h after administration of SPLP (β gal) in the presence of LN CpG-ODN and 96 h after the administration of SPLP alone (MFI of 166 and 168, respectively). Low, but detectable levels of expression were observed in CD11c+ cells at 72 and 96 h; however, a distinct population of B220/CD45R+ cells demonstrating

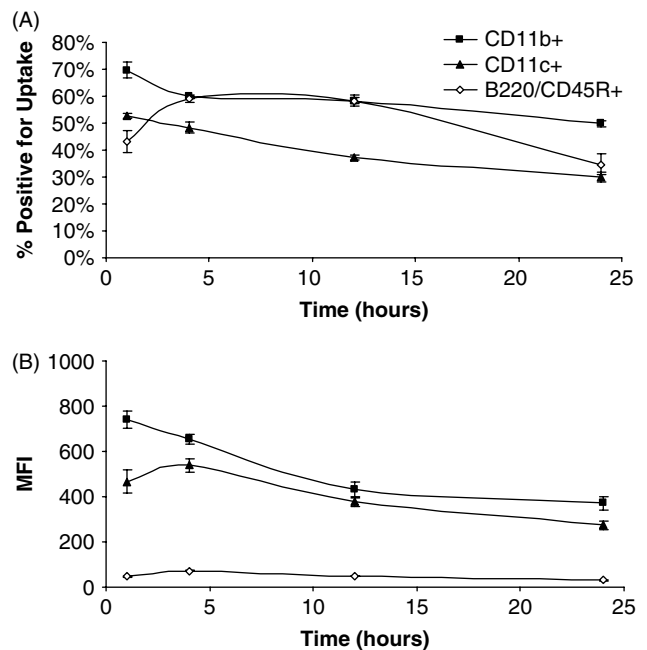


Figure 3. Uptake of SPLP by CD11b+, CD11c+ and B220/CD45R+ cells in the spleen following intravenous administration. Uptake of Di-I labelled SPLP by CD11b+, CD11c+ and B220/CD45R+ cells in the spleen following i.v. administration to BALB/c mice at a plasmid dose of 5 mg/kg. Results are expressed as the percent of cells positive for uptake (A) and MFI (B), as determined by flow cytometry. Each data point represents $n = 4$ (\pm SEM)

β gal expression was not observed (data not shown). Mock transfection with SPLP (luc) alone or in combination with LN CpG-ODN resulted in background levels of fluorescence at 48 h which were similar to that observed for the PBS control (data not shown).

The increase in the population of CD11b+ cells observed in the spleen following administration of SPLP in the presence or absence of LN CpG-ODN is thought to stem from a combination of the influx of CD11b+ cells, in response to the presence of strong immunostimulatory agents, and their proliferation, as SPLP and LN CpG-ODN have been demonstrated to promote both *in vivo* (unpublished results, Kaley D. Wilson and Susan D. de Jong).

Vaccination with SPLP followed 24 h later by LN CpG-ODN primes the generation of transgene-specific humoral and cellular immune responses

To evaluate the ability of SPLP to act as a vaccine and prime the generation of adaptive antigen-specific humoral and cellular immune responses animals were immunized with SPLP (β gal) or the mock vaccine SPLP (luc) alone or followed 24 h later by LN CpG-ODN once a week for 3 weeks. β gal-specific immune responses were assessed one week following the final vaccination. With respect to the generation of antigen-specific humoral

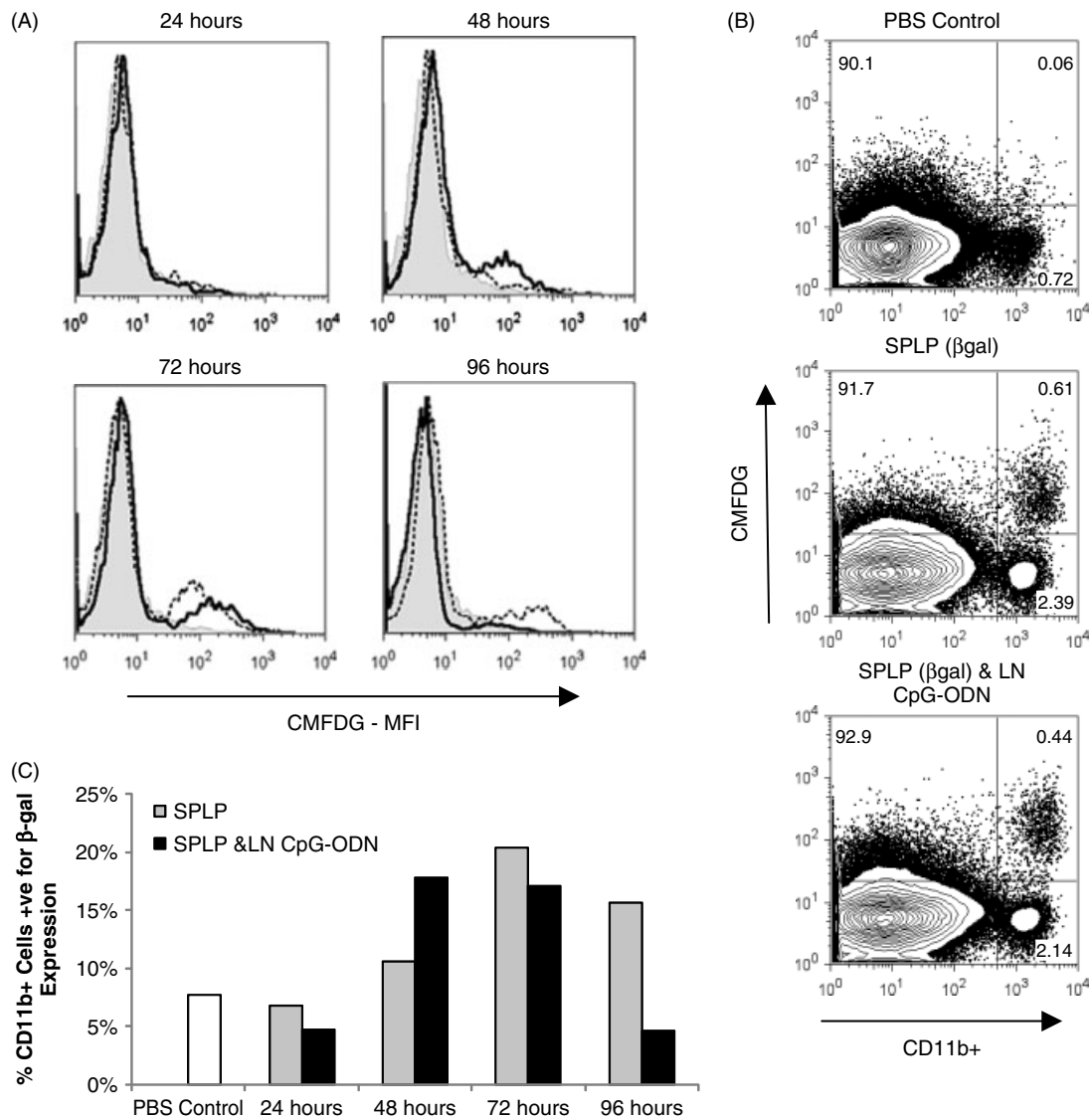


Figure 4. SPLP-mediated transfection of CD11b⁺ cells in the spleen following the intravenous administration of SPLP (β gal) alone or followed 24 h later by LN CpG-ODN. Transfection of CD11b⁺ cells in the spleen of BALB/c mice bearing CT26 tumours as determined 24, 48, 72 and 96 h following the i.v. administration of PBS (shaded curve), SPLP (5 mg/kg) encoding β gal alone (dashed line: grey) or followed 24 h later by LN CpG-ODN (solid line: black) (10 mg/kg). Mice receiving LN CpG-ODN were treated 24 h after the administration of SPLP, except for the 24-h timepoint where mice received LN CpG-ODN 12 hours after SPLP. β gal expression was detected using the fluorogenic substrate CMFDG in conjunction with fluorescently-labelled phenotype markers and flow cytometry. Control mice receiving PBS showed similar background as mice receiving SPLP (luc) in the presence or absence of LN CpG-ODN. (A) Histogram representation of data obtained from three mice pooled; (B) dot plot representation for CD11b⁺ cells at the 72-h timepoint; (C) percent CD11b⁺ cells positive for β gal expression as determined by flow cytometry

immune responses, immunization with SPLP (β gal) followed 24 h later by LN CpG-ODN resulted in the generation of β gal specific IgG1 and IgG2a responses, with a titre of 100, whereas minimal responses (titre ≤ 1) were observed following vaccination with SPLP (β gal) alone or the mock vaccine (Figure 5).

The ability of vaccination with SPLP (β gal) to promote the generation of β gal-specific CTLs was assessed functionally by quantitating the number of CD8⁺ T-cells capable of responding to antigen-specific stimulation by secreting IFN- γ (a Th1 response indicator) in a cytokine secretion assay. Vaccination with SPLP (β gal) followed 24 h later by LN CpG-ODN resulted in a six-fold increase in the percentage of CD8⁺ cells producing

IFN- γ following antigenic stimulation with a β gal-derived peptide (Figure 6), whereas no such increase in the frequency of CD8⁺/IFN- γ T-cells was observed following antigen exposure in splenocytes isolated from control or mock vaccinated mice.

Additional functional assessment of the induction of antigen-specific CTL responses following vaccination was determined in a standard ⁵¹Cr release cytotoxicity assay where the relative ability of splenocytes, isolated from immunized animals, to lyse β gal-expressing CT26.CL25 target cells in an antigen-specific manner was assessed immediately after isolation or following 5 days of *in vitro* restimulation with peptide-pulsed APC. In both primary and secondary assays performed following

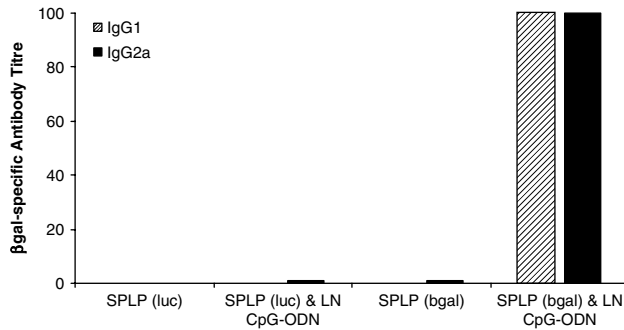


Figure 5. Vaccination with SPLP (β gal) followed 24 h later by LN CpG-ODN primes the generation of a β gal specific humoral immune response. β gal specific IgG1 and IgG2a serum antibody responses in BALB/c mice vaccinated with SPLP (β gal) or SPLP (luc) (5 mg/kg) alone or followed 24 h later by LN CpG-ODN (10 mg/kg) once a week for 3 weeks. Each bar represents the antibody titre of plasma isolated from five mice pooled as assessed by ELISA 1 week following the final vaccination. Antibody titre was defined as the highest serum dilution giving an absorbance twice that of serum from non-immune control animals at 492 nm. Data represent findings from two independent experiments

in vitro restimulation, an increase in antigen-specific CTL responses were observed in mice vaccinated with SPLP (β gal) followed 24 h later by LN CpG-ODN. Specifically, at an E:T ratio of 100:1, significantly higher CTL activity was observed in splenocytes isolated from mice vaccinated with SPLP (β gal) and LN CpG-ODN (16.8%) compared to that observed for control animals vaccinated with PBS (2.5%) or with the mock vaccine (1.3%) ($p < 0.05$) for which only minimal levels of target cell lysis (less than 4%) were observed at all E:T ratios investigated (Figure 7).

Discussion

Although the use of pDNA for immunization purposes is an attractive approach for immunotherapy, the relatively low levels of gene expression and weak immune responses attained following immunization with naked pDNA constitute significant hurdles to clinical application. A number of approaches have been taken to enhance pDNA delivery to APC in the hopes of improving immunogenicity and enhancing vaccine activity, but sub-optimal responses and/or serious safety and toxicity issues remain [17,19,33]. In the present study, we show that SPLP, which have previously been used as a nontoxic systemic delivery system for gene therapy, can function as a systemically administered genetic vaccine. Specifically, we demonstrate first that SPLP are taken up by and effectively transfect APC of the spleen compartment both *ex vivo* and *in vivo* following i.v. administration, and second that the SPLP-mediated transfection of APC, when combined with a strong immunostimulatory signal such as that provided by the adjuvant LN CpG-ODN, can promote the priming of transgene-specific humoral and cellular immune responses.

It has been conclusively demonstrated that bone marrow-derived APC play a key role in the induction of the immune response following vaccination with pDNA [34–36], making the efficient delivery of plasmid to APC a primary goal for DNA vaccines. As it is well established that APC avidly accumulate lipid particulate delivery systems *ex vivo* and *in vivo* [14–16], it was of considerable interest to investigate the ability of SPLP to passively target to, be taken up by, and transfect APC. We demonstrate that SPLP is taken up by approximately 21–38% of B220/CD45R+, CD11c+ and CD11b+ cells *ex vivo*, when present at the same concentrations as those typically used for *in vitro* transfection. Furthermore, SPLP demonstrates the ability to transfect both the cultured macrophage cell line RAW264.7 and primary splenocytes *ex vivo*, albeit at low levels, and enhanced luciferase expression per mg of protein is observed in populations enriched for CD11b+ cells, indicating that SPLP is able to transfect this cell-type.

Although numerous attempts have been made to design vectors which both protect pDNA from degradation and target its delivery to APC, few studies have directly characterized the uptake of nonviral vectors by APC and their subsequent transfection *in vivo*. Using fluorescently-labelled SPLP it is demonstrated here that SPLP are rapidly taken up by CD11b+, CD11c+ and B220/CD45R+ cell populations in the spleen within 1 h of i.v. administration. These results are in agreement with previous findings demonstrating the efficient uptake of physically similar LN CpG-ODN by APC following systemic administration [29]. Furthermore, using a very sensitive flow cytometry-based assay for the detection of β gal activity [37], we are able to quantitatively characterize the transfection of CD11b+ cells in the spleen following i.v. administration of SPLP encoding β gal. Although detectable levels of β gal expression were evident in a high percentage of CD11b+ (12.6%) cells from 48 to 96 h following administration, the transfection of CD11c+ cells was considerably lower and more transient. Although transgene expression by macrophage and dendritic cell (DC) populations following genetic vaccination has been previously assessed using detection methods that include the expression of a fluorescent product (i.e. GFP) in combination with fluorescent microscopy, expression levels reported are typically very low (i.e. 50–100 antigen positive DCs per inguinal lymph node) and it is difficult to directly compare these findings with our results because they are typically assessed following intramuscular or gene-gun administration of naked pDNA [38–40]. Our observations are in agreement, however, with those observed by Garg *et al.* [41] who, using a Cre/loxP recombination strategy that results in β gal expression by transfected cells, observed that approximately 12% of the purified CD11c+ DCs from draining lymph nodes of gene gun immunized mice were β gal positive 60 h after immunization. These authors did not detect transfected macrophage following vaccination, a discrepancy that is most likely due to differences in the method and route of delivery.

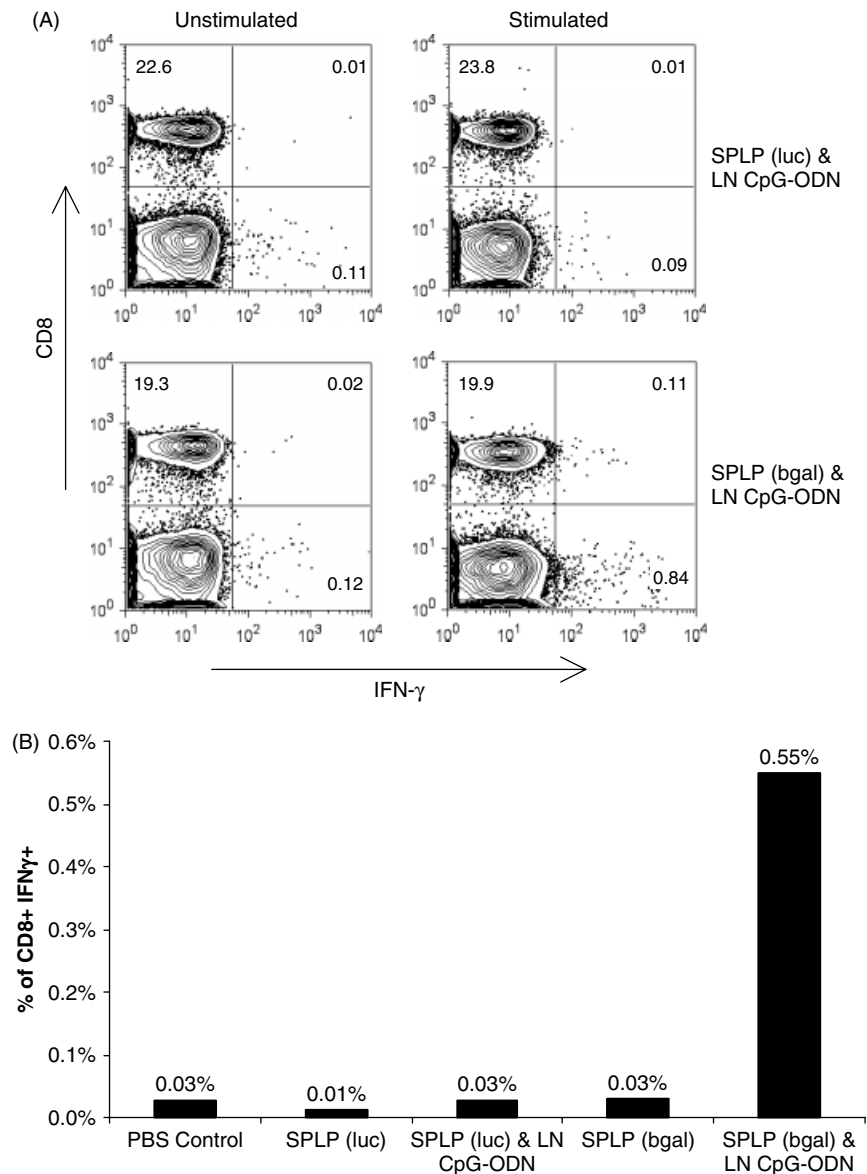


Figure 6. Vaccination with SPLP (β gal) followed 24 h later by LN CpG-ODN increases the frequency of antigen-specific IFN- γ secreting CD8+ T-cells. Frequency of IFN- γ + CD8+ T-cells in BALB/c mice vaccinated with SPLP (β gal) or SPLP (luc) (5 mg/kg) alone or followed 24 h later by LN CpG-ODN (10 mg/kg) once a week for 3 weeks. One week following the final vaccination the frequency of antigen-specific, IFN- γ secreting CD8+ was assessed by an IFN- γ secretion assay following stimulation for 5 h with the immunodominant peptide of β gal (TPHPARIGL). (A) Dot plot representation of splenocytes isolated from mock vaccinated mice [SPLP (luc) and LN CpG-ODN] or mice vaccinated with SPLP (β gal) and LN CpG-ODN, before and after peptide stimulation. (B) Bar graph representation of the percentage of CD8+ cells positive for IFN- γ secretion following peptide stimulation for all vaccination strategies. Data represent splenocytes isolated from five mice pooled and are representative of the findings from two independent experiments

It is proposed that the efficient uptake and transfection of APC in the spleen observed in the present study depends largely on our use of newly-formulated SPLP particles that have been modified compared to their traditional counterparts to allow for repeat administration [22]. The shorter circulation lifetime ($t_{1/2} = 1$ h) of the SPLP formulated in the present study with the shorter chain PEG-lipid PEG-DMG, as compared to the long circulation halftimes ($t_{1/2} = 7$ h) observed for traditional SPLP formulated with longer chain PEG-lipid, PEG-DSG, results in increased accumulation of the particle in the spleen [24], which is well suited to the role for SPLP as a genetic

vaccine carrier. The efficient transfection of CD11b+ and CD11c+ cells by SPLP *in vivo* may also rely in part on the fact that SPLP and LN CpG-ODN have been demonstrated to promote the proliferation of these cell-types *in vivo* within 24 h of systemic administration, as assessed by bromodeoxyuridine incorporation (unpublished results, Kaley D. Wilson and Susan D. de Jong) because transfection of cells by SPLP depends strongly on mitotic activity [42]. Importantly, co-administration of the adjuvant LN CpG-ODN did not down-regulate SPLP-mediated gene expression levels in either of the cell-types investigated. It is well documented that unmethylated

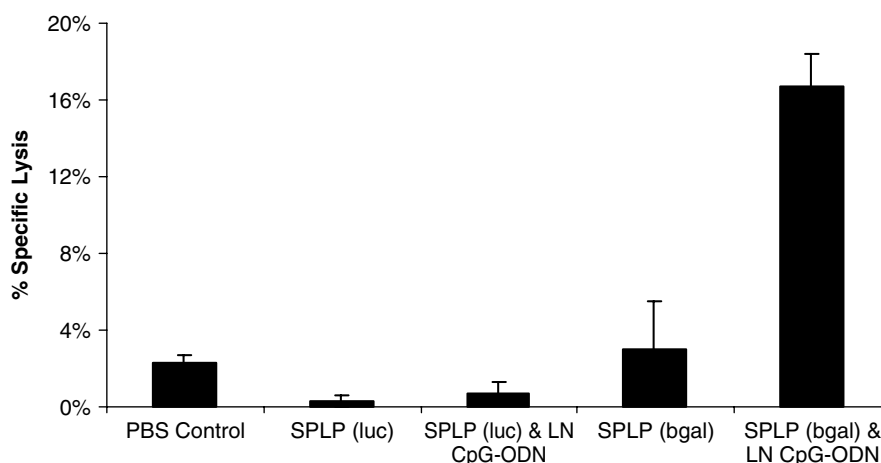


Figure 7. Vaccination with SPLP (β gal) followed 24 h later by LN CpG-ODN primes the generation of β gal specific CTLs. β gal specific cytolytic activity in BALB/c mice vaccinated with SPLP (β gal) or SPLP (luc) (5 mg/kg) alone or followed 24 h later by LN CpG-ODN (10 mg/kg) once a week for 3 weeks. One week following the final vaccination, splenocytes were restimulated for 5 days *in vitro* with β gal-peptide pulsed splenocytes and cytotoxicity was assessed in a standard ^{51}Cr release assay. Results are expressed as the percent specific lysis, calculated by subtracting the percentage of nonspecific lysis (CT26) from the percentage of specific lysis (CT26.CL25) after 5 h of co-incubation at an effector to target ratio of 100 : 1. Each data point represents the specific lysis from five mice pooled (\pm SD). Data represent the findings from two independent experiments

CpG motifs present in pDNA and synthetic ODN promote the production of cytokines, including IFN- γ and TNF- α , which can act to down-regulate gene expression from viral promoters such as cytomegalovirus promoter [43–45], particularly when administered systemically as cationic lipid-containing/DNA particles [30,46,47].

The strength and nature of immune responses primed by DNA vaccines are strongly influenced by their route and method of administration [9,12,48]. Although the efficacy of systemically administering genetic vaccines has been evaluated in several instances for both free or cationic lipid-complexed forms of pDNA [10,12,48,49], the instability of naked pDNA combined with the toxicity and rapid clearance often associated with cationic carriers has significantly reduced the viability of this approach. However, due to the advantage of direct access to the relatively large number of APC in the spleen, systemic administration of genetic vaccines still represents an attractive alternative approach to traditional routes of administration if the obstacle of poor delivery can be overcome. In the present study, we demonstrate the generation of functional antigen-specific cellular responses, as assessed by cytokine secretion and cytotoxicity, and humoral responses, as assessed by serum immunoglobulin titres, which directly support the ability of SPLP to act as a systemically administered genetic vaccine when combined with a strong immunostimulatory signal. Although very low levels of antigen-specific CTLs are detected in splenocytes of control or mock vaccinated mice, a concomitant increase in β gal-specific IFN- γ secretion and cytolytic activity is detected in the splenocytes of animals immunized with SPLP in conjunction with LN CpG-ODN. Not surprisingly, it should be noted that the systemic administration of SPLP (β gal) alone can, on some occasions, prime antigen-specific humoral and

cellular responses (data not shown); however, these responses are sporadic and consistently weaker than those observed in the presence of LN CpG-ODN. Our observations that LN CpG-ODN can act as an adjuvant for a DNA-based vaccine, enhancing the generation of antigen-specific humoral and cellular immune responses, are in agreement with several groups who have also demonstrated enhanced immune responses to a DNA vaccine following administration of CpG containing DNA [50–52]. Consistent with observations made by other groups [51,52], we have observed (unpublished results, Kaley D. Wilson, Susan D. de Jong and Mikameh Kazem) that the relative timing of administration of the DNA vaccine and CpG ODN adjuvant is critical, with delivery of the CpG-ODN after the vaccine resulting in an enhanced immunogenic boost.

In conclusion, our studies demonstrate that SPLP represents an effective candidate for the nonviral delivery of a systemic genetic vaccine when combined with additional immune stimulation. It is evident that SPLP are taken up by and effectively transfect CD11b+ and CD11c+ cells in the spleen compartment following systemic administration, and, in the presence of additional immune stimulation provided by LN CpG-ODN, induces the generation of detectable antigen-specific humoral and cellular responses. These data highlight the benefit of additional immune stimulation, particularly CpG-mediated, in enhancing the generation of immune responses to genetic vaccines. Although optimization of dosing and dosing schedules are required, our demonstration of the ability of SPLP combined with LN CpG-ODN to promote the priming of antigen-specific immune responses, while at the same time being well-tolerated following systemic administration, supports the potential of SPLP as a genetic vaccine.

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