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ORIGINAL ARTICLE

Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN

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Abstract Immunostimulatory oligodeoxynucleotides (ODN) containing cytosine-guanine (CpG) motifs are powerful stimulators of innate as well as adaptive immune responses, exerting their activity through triggering of the Toll-like receptor 9. We have previously shown that encapsulation in liposomal nanoparticles (LN) enhances the immunostimulatory activity of CpG ODN (LN-CpG ODN) (Mui et al. in J Pharmacol Exp Ther 298:1185, 2001). In this work we investigate the effect of encapsulation on the immunopotency of subcutaneously (s.c.) administered CpG ODN with regard to activation of innate immune cells as well as its ability to act as a vaccine adjuvant with tumor-associated antigens (TAAs) to induce antigen (Ag)-specific, adaptive responses and anti-tumor activity in murine models. It is shown that encapsulation specifically targets CpG ODN for uptake by immune cells. This may provide the basis, at least in part, for the significantly enhanced immunostimulatory activity of LN-CpG ODN, inducing potent innate (as judged by immune cell activation and plasma cytokine/chemokine levels) and adaptive, Ag-specific (as judged by MHC tetramer positive T lymphocytes, IFN-γ secretion and cytotoxicity) immune responses. Finally, in efficacy studies, it is shown that liposomal encapsulation enhances the ability of CpG ODN to adjuvanate adaptive immune responses against co-administered TAAs after s.c. immunization, inducing effective anti-tumor activity against both model and syngeneic tumor Ags in murine tumor models of thymoma and melanoma.

Keywords CpG · Liposome · Nanoparticle · Immunostimulatory · Anti-tumor · Vaccine

Abbreviations

APC	Antigen presenting cells
LN	Liposomal nanoparticles
CpG	Unmethylated CpG dinucleotides
ODNs	Oligodeoxynucleotides
TRP-2	Tyrosinase-related protein 2

Introduction

It is well established that bacterial DNA and synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs are capable of inducing potent immune responses. CpG-containing DNA directly activates antigen presenting cells (APCs) such as dendritic cells (DCs) and B lymphocytes, resulting in enhanced antigen (Ag) processing and presentation, upregulation of co-stimulatory molecules and secretion of immunomodulatory cytokines, including interleukin- (IL-) 6, 10, 12, 18 and interferon- (IFN-) α/β. This, in turn, activates innate immune effector cells such as natural killer (NK) cells, results in the production of additional immunoactive cytokines (including IFN-γ) and chemokines and promotes the development of adaptive responses, with induction of Ag-specific effector cells such as cytotoxic and helper T lymphocytes (CTLs and T_H cells, respectively) [6, 19, 25, 30, 35, 58, 64, 65].

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A hallmark of CpG ODN activity is its capacity to induce strong T_H1-biased responses [7, 8, 59, 60, 67].

Binding, internalization and trafficking of CpG ODN to the endosomal compartment by APCs are requisites for immunostimulatory activity [18, 30, 53], allowing recognition and engagement by Toll-like receptor 9 (TLR9), a pattern recognition receptor specific for CpG DNA [2, 20, 53, 56].

Its immunopotency and broad range of activity suggests that CpG ODN may be an effective immune modulator for the treatment and prophylaxis of a wide variety of diseases [3, 12, 21, 56]. In fact, CpG ODN are currently undergoing extensive clinical evaluation of their safety and tolerability, as well as their therapeutic potential as a single agent, adjunct therapy and vaccine adjuvant for treatment and prevention of malignant, infectious, allergic, inflammatory and autoimmune diseases [9, 10, 14, 29, 52]. Preliminary results from these studies are promising, showing that these compounds are well tolerated and mediate significant immune modulation [9, 24, 29, 54].

Despite encouraging preclinical and clinical data, the use of free CpG ODN still has several disadvantages. First, free ODN have unfavorable pharmacokinetics and lack specificity for target cells, with rapid and broad tissue disposition after systemic administration. Furthermore, free ODN exhibit poor cellular uptake characteristics. Finally, ODN with natural phosphodiester (PO) backbone linkages are extremely sensitive to nuclease degradation, rendering them inactive in their free form [1, 48, 66]. Therefore, the majority of the work with CpG ODN has employed ODN with nuclease-resistant phosphorothioate (PS) backbone modifications [9, 28, 30]. While effectively enhancing stability, use of free PS-modified ODN also has inherent disadvantages, foremost being non-sequence specific complement- and coagulation-related toxicities associated with PS-containing ODN [33].

To address these issues, we have previously reported on the development of a lipid-based nanoparticulate delivery system for ODN [22, 50] that allows efficient encapsulation within liposomes possessing optimal characteristics (i.e. neutral surface charge, small particle size) for effective intravenous (i.v.) delivery and targeting of ODN to immune effector cells such as macrophages and DCs. Furthermore, liposomal encapsulation enhances intracellular delivery compared to equivalent doses of free CpG ODN through endocytosis-mediated cellular uptake [34, 50]. Finally, encapsulation completely protects ODN from nuclease degradation thus allowing for the use of more immunopotent PO ODN [44]. As a result, the use of lipid-based nanoparticulate delivery systems enhances targeting to and uptake by immune cells as well as providing an alternative to chemical modification to increase the stability of CpG ODNs in the circulation [16–18, 34, 62]. A recent study demonstrates that these factors result in improved immunostimulatory activity following i.v. administration as demonstrated by plasma cytokine levels and immune cell activation [44].

While the benefits of encapsulation in nanoparticles are clear for i.v. administered CpG ODN, encapsulation may also improve the activity of locally delivered CpG ODN. This may be achieved through enhancing ODN uptake by phagocytic APCs, facilitating intra- and extra-cellular ODN trafficking, serving a source of drug (i.e. depot effect) and enabling the use of more immunopotent forms (i.e. PO) of CpG ODN. The ability of encapsulation to enhance activity of both locally and systemically administered CpG ODN drug is relevant for the development of liposomal nanoparticles (LN)-CpG ODN as a platform technology with broad immunostimulatory applications. In particular, as a vaccine adjuvant, subcutaneously (s.c.) administration is the route of choice for many prophylactic and therapeutic vaccines aimed at inducing adaptive immune responses, allowing access to a large number of APCs. Therefore, it is of interest to define and evaluate the capacity of liposome encapsulation to enhance the activity of CpG ODN following local delivery.

In this work we expand on previous i.v. data, focusing primarily on the capacity of LN-CpG ODN to serve as an effective vaccine adjuvant to mediate Ag-specific immune responses. Specifically, we investigate the immunopotency of s.c. administered LN-CpG ODN and its ability to induce adaptive cellular responses against co-administered Ags and effective anti-tumor activity in animal models of cancer. As hypothesized, LN-CpG ODN demonstrated enhanced delivery and uptake by APCs following s.c. administration and, consistent with previous observations following i.v. administration, enhanced immunopotency, mediating more vigorous innate immune responses compared to free ODN. These enhanced responses were also reflected by more potent induction of adaptive cellular responses by LN-CpG ODN and ultimately, enhanced anti-tumor activity as a cancer vaccine adjuvant in combination with tumor-associated antigens (TAAs). These results confirm that encapsulation within LN profoundly enhances the immunopotency of CpG ODN and demonstrate the effectiveness of LN-CpG ODN in promoting the induction of Ag-specific cellular immune responses, thus dramatically increasing anti-tumor activity in various animal models. These results strongly suggest that liposomal encapsulation is an effective strategy to optimize the activity of CpG

ODN and that LN-CpG ODN may be a suitable adjuvant for the development of effective vaccines for the treatment and prevention of cancer and other diseases.

Materials and methods

Animals and cell lines

Six- to eight-week-old female C57BL/6 and ICR mice were obtained from Charles River Laboratories (Saint-Constant, PQ, Canada) or Harlan (Indianapolis, IN). Mice were held in a pathogen-free environment and all procedures involving animals were performed in accordance with the guidelines established by the Canadian Council on Animal Care. Murine B16-F10 melanoma and EL4 and E.G7-OVA (EL4 transfected to express OVA [43]) thymoma cells were obtained from the American Type Culture Collection (Manassas, VA). Parental EL4 and transfected E.G7-OVA thymoma cells were cultured in complete medium (CM) consisting of RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with penicillin G (100 U/ml), streptomycin sulphate (100 μg/ml), 5×10^{-5} M β-mercaptoethanol and 10% fetal bovine serum. B16-F10 cells were cultured in CM supplemented with 0.1 mM nonessential amino acids and 1 mM sodium pyruvate, 0.3% glutamine and 50 μg/ml gentamicin.

ODN and preparation of LN

INX-6295 (5′-TAACGTTGAGGGGCAT-3′), a PS 16-mer ODN, was synthesized by Trilink BioTechnologies (San Diego, CA) for use in these studies. This ODN does not conform to any of the classes of CpG ODN previously described based on backbone chemistry, nucleotide sequence and immunostimulatory characteristics [26, 38]. INX-0167 contains a single CpG and a poly-G motif within a fully phosphorothioated ODN and when encapsulated, induces activation of wide variety of immune cells (unpublished data). INX-6295 was encapsulated in lipid nanoparticles containing an ionizable aminolipid using an ethanol dialysis procedure, as previously described [49]. Briefly, lipid combinations consisting of the bilayer forming lipids distearoylphosphatidylcholine, or palmitoyloleoylphosphatidylcholine and cholesterol (Avanti Polar Lipids, Alabaster, AL), the ionizeable lipid 1,2-dioleyloxy-3-*N*,*N*-dimethylaminopropane (for efficient ODN encapsulation) and the steric barrier lipid polyethylene glycol (PEG)-dimyristol glyceol or PEG-ceramide C_{14} (to prevent vesicle aggregation during formation) at a molar ratio of 25/45/20/10 were solubilized in ethanol and added to 50 mM citrate buffer containing approximately 3.33 mg/ml of ODN to give a final ethanol concentration of 40%. The ODN/lipid mixture was passed twice through stacked 200 nm + 100 nm polycarbonate membranes (Whatman Nuclepore, Clifton, NJ) using a thermobarrel extruder (Lipex Biomembranes, Vancouver, BC, Canada) to produce a homogeneous population of vesicles approximately 100 nm in diameter. The resulting vesicles were dialyzed first against citrate followed by HEPES-buffered saline (HBS) at pH 7.5. Unencapsulated ODN was subsequently removed by anion exchange chromatography on DEAE-Sepharose CL-6B columns equilibrated in HBS. As previously described, this process results in discrete vesicles completely encapsulating the ODN within an aqueous interior which are distinctly different from lipid complexes [39, 49]. Oligonucleotide concentrations were determined by UV spectroscopy (260 nm) on a Beckman DU 640 spectrophotometer (Beckman Coulter, San Diego, CA) following solubilization of the samples in chloroform/methanol at a volume ratio of 1:2.1:1 chloroform/methanol/aqueous phase (sample/HBS). Lipid concentrations were determined using an inorganic phosphorus assay after separation of the lipids from the oligonucleotides by a Bligh and Dyer extraction [4]. The ODN-to-lipid ratio was typically 0.10 to 0.13 (w/w). Particle size, as determined by quasi-elastic light scattering using a NICOMP submicron particle sizer (Model 370, Santa Barbara, CA), was approximately 100 ± 25 nm.

Cell uptake analysis

The effect of encapsulation on the uptake of CpG ODN by murine immune cell populations following s.c. administration was assessed in ICR mice injected with 5 mg/kg free or encapsulated 5′-carboxyfluorescein (FAM)-labeled INX-6295 PS ODN (Trilink Biotechnologies). For uptake analysis, mice were anaesthetized with ketamine/xylazine (3.2%/0.8%, v/v) 1, 4, 7 and 24 h post administration, and spleens and lymph nodes were collected and processed to single cell suspensions by passage through a sterile 100 μm nylon mesh (Becton Dickenson, Franklin Lakes, NJ). Splenocytes were depleted of red blood cells by ammonium chloride lysis. Cells were analyzed for ODN uptake (as judged by intensity of the fluorescently labeled ODN on a per cell basis) by specific immune cell populations (as determined by phenotype analysis; cell suspensions were stained with phycoerythrin [PE]-conjugated or allophycocyanin [APC]-conjugated anti-CD11b, anti-CD11c, anti-CD8 and anti-DX5

phenotype antibodies) using a 4-colour FACSort flow cytometer and CellQuest Pro software (BD Biosciences, San Jose, CA). All fluorescently labeled antibodies were obtained from BD Biosciences. Propidium iodide was used to exclude dead cells and 150,000 and 20,000 events were collected to analyze DCs or NK cells, macrophages, B cells, CD4 and CD8 T lymphocytes, respectively.

Ex-vivo analysis of immune parameters

Plasma cytokine analysis

The effect of encapsulation on the potency of ODN-mediated activation of murine immune cell populations was evaluated after s.c. administration of free and encapsulated ODN to ICR mice. For plasma cytokine levels, mice were anaesthetized as previously described and blood was collected via cardiac puncture into Vacutainer tubes containing EDTA (Becton Dickinson). Plasma was isolated by centrifugation and frozen at −20°C until assayed. Plasma concentrations of IL-6, IL-10, MCP-1 and IFN-γ were determined using commercially available ELISA or cytometric bead array (both from BD Biosciences) kits, as per the manufacturer's instructions.

Cell activation analysis

For cellular assays, peripheral blood, spleens and lymph nodes were collected after treatment and single cell suspensions were generated from the organs as described above. Blood and splenocytes were depleted of red blood cells by ammonium chloride lysis and analyzed for immune stimulation as judged by activation marker expression. Cell suspensions were stained with fluorescein isothyocianate (FITC)- and APC-labeled phenotype antibodies (anti-CD11b and anti-CD11c, anti-CD8, anti-DX5, respectively) in combination with PE-conjugated antibodies directed against the activation markers CD69 or CD86. Cell activation analyzes were performed by flow cytometry as described above.

Assessment of Ag-specific CD8 T lymphocytes

The ability of free and encapsulated ODN to induce Ag-specific CD8 T lymphocytes was assessed in C57BL/6 mice immunized s.c. with hen egg albumin (ovalbumin or OVA) mixed with saline, free ODN or LN-CpG ODN using MHC tetramer, chromium release cytotoxicity, and IFN-γ cytokine secretion assays.

MHC tetramer assay

After immunization, the frequency of OVA-specific CD8 T lymphocytes was determined using an MHC-tetramer assay. Briefly 5×10^6 spleen cells were incubated with PE-coupled H-2D MHC tetramers containing the immunodominant peptide of OVA (SIINFEKL; Beckman-Coulter, Immunomics, San Diego, CA) and FITC-labeled anti-mouse-CD8 and PE-cyanin-labeled anti-TCRβ phenotype antibodies (BD Biosciences) prior to analysis on a flow cytometer as previously described. One hundred and fifty thousand events were collected to analyze the frequency of OVA-specific CD8 T lymphocytes in immunized animals.

Cytotoxicity assay

The ability of cells from immunized animals to lyse target cells in an Ag-specific manner was assessed in splenocytes, either immediately following completion of the immunization regimen with OVA or after in vitro Ag-restimulation. For the latter, E.G7-OVA cells were treated with mitomycin C (50 μg/ml) and combined with splenocytes from immunized animals for 5 days with the addition of human recombinant IL-2 (100 IU/ml; BD Biosciences). Ovalbumin-specific cytotoxicity was assessed using a standard 4 h ^{51}chromium (^{51}Cr) release assay. Briefly, splenocytes were mixed in various effector:target ratios with ^{51}Cr-loaded parental EL4 or OVA-transfected E.G7 cells and the percentage cellular cytotoxicity was calculated on the basis of ^{51}Cr released to the supernatent using the formula: % lysis = [(experimental CPM − spontaneous CPM)/(maximal CPM − spontaneous CPM)] × 100, where maximal cpm was achieved by complete lysis of ^{51}Cr-labeled targets in 10% Triton X 100, spontaneous CPM was determined by incubating labeled targets in CM and Ag-specific killing was determined by comparison of cytotoxicity of ^{51}Cr-labeled OVA-expressing and non-expressing E.G7 and EL4 cells, respectively.

Cytokine secretion assay

Interferon-γ secreting CD8 T lymphocytes were detected using the IFN-γ secretion assay (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's instructions. This assay is designed to quantify Ag-specific CD8 T lymphocytes by enumerating the number of CD8 T lymphocytes that secrete IFN-γ in response to Ag stimulation. Briefly, splenocytes were restimulated with OVA-expressing APCs prior to incubation with a bispecific antibody designed to bind to activated T lymphocytes via the CD25 activation marker and

capture secreted IFN-γ. The frequency and phenotype of cells that responded to OVA-stimulation by actively secreting cytokines were determined by flow cytometry as described previously using a fluorescently labeled anti-IFN-γ antibody in combination with previously described fluorescently labeled phenotype antibodies.

Tumor challenge efficacy studies

Antigen-specific cancer vaccine

The xenogeneic E.G7-OVA and syngeneic B16 C57BL/6 tumor models were used to determine the efficacy of encapsulated ODN as a vaccine adjuvant to induce Ag-specific anti-cancer immune responses. For the EG7-OVA model, mice were immunized prophylactically with OVA mixed with 100 μg free or encapsulated INX-6295 ODN adjuvant weekly for 2 or 3 weeks. One week following the last vaccination, mice were injected s.c. with 2.5×10^6 E.G7-OVA tumor cells into the left flank and monitored for tumor growth. Tumor size was assessed every second day using digital calipers (Mitutoyo, Mississauga, ON, Canada). Tumor volumes were calculated using the standard formula for ellipsoid tumor volumes, (length × width^2)/2. For the syngeneic B16-Tyrosinase-related protein 2 (TRP-2) model, mice were immunized prophylactically with TRP-2 mixed with 100 μg free or encapsulated INX-6295 ODN adjuvant weekly for 2 or 3 weeks. Two days following the final vaccination, mice were injected i.v. with 1.0×10^5 B16 cells. Mice were euthanized 18 days later, lungs were excised and metastases were enumerated using a stereomicroscope.

Statistical analyzes

All statistical analyzes were performed using SPSS Ver 14.0. Initially, a one-way analysis of variance (ANOVA) was used to statistically evaluate the differences between treatment groups. In the case of statistically significant results, the differences between treatment groups were assessed through the use of Bonferroni adjusted t tests, a post hoc test which controls for error rate. Probability (P) values less than 0.05 were considered significant.

Results

Encapsulation in LN enhances uptake of CpG ODN by immune effector cells in the lymph nodes and spleen after subcutaneous administration

Preliminary pharmacokinetic and biodistribution studies following s.c. administration using radiolabeled lipids and CpG ODN to determine the fate of administered LN-CpG ODN, indicates preferential accumulation in local draining lymph nodes on a per gram basis, as well as secondary accumulation patterns consistent with i.v. delivery, particularly in the spleen and liver, (manuscript in preparation) following s.c. administration. Based on these results, studies were undertaken to characterize the cellular uptake of s.c. administered free and LN-CpG ODN in the draining lymph nodes and spleen using fluorescently-labeled ODN. Results from these studies demonstrate a two to ninefold enhancement of uptake for encapsulated ODN by phagocytic APCs including macrophages and DCs (as judged by expression of CD11b and CD11c ± CD11b, respectively; Fig. 1) compared to free ODN in the lymph nodes over a 24 h period. Similarly, enhanced uptake for LN-CpG ODN is also observed in spleen following s.c. administration albeit at a much lower relative level than that observed in lymph nodes, with splenic uptake of free ODN being similar to control levels.

Encapsulation of CpG ODN in LN enhances immune cell activation

To assess the effect of encapsulation on the immunopotency of CpG ODN, the expression of CD69, an early activation marker of leukocytes (T and B lymphocytes and NK cells), macrophages and neutrophils [47] and CD86, a co-stimulatory molecule expressed primarily by activated APCs such as monocytes/macrophage and DCs [31, 40, 41] was monitored. Similarly, the production of T_H1 and T_H2 cytokines (IFN-γ and IL-6, IL-10, respectively) as well as MCP-1 (a macrophage chemokine) was also assessed. These data were collected over a 72 h time period following administration of either free or encapsulated CpG ODN to ICR mice. Data presented here are representative of at least three independent experiments.

Immune cell activation is observed in response to s.c. treatment with both free and encapsulated CpG ODN, with upregulation of CD69 and CD86 expression on spleen and lymph node (data not shown) cell populations that peaks at 24 h post injection. Based on CD69 expression, free CpG ODN induces a 44% increase in the number of activated CD11b+ cells, a fourfold increase in activated CD11c+ cells and a twofold increase in activated DX5+ cells above control levels. No appreciable effect is observed in CD8+ cells (Fig. 2). In contrast, the administration of encapsulated CpG ODN leads to a greater than fivefold, 12-fold, fourfold and a 12-fold increase in activated CD11b, CD11c, DX5 and CD8+ cells, respectively, compared to cells from

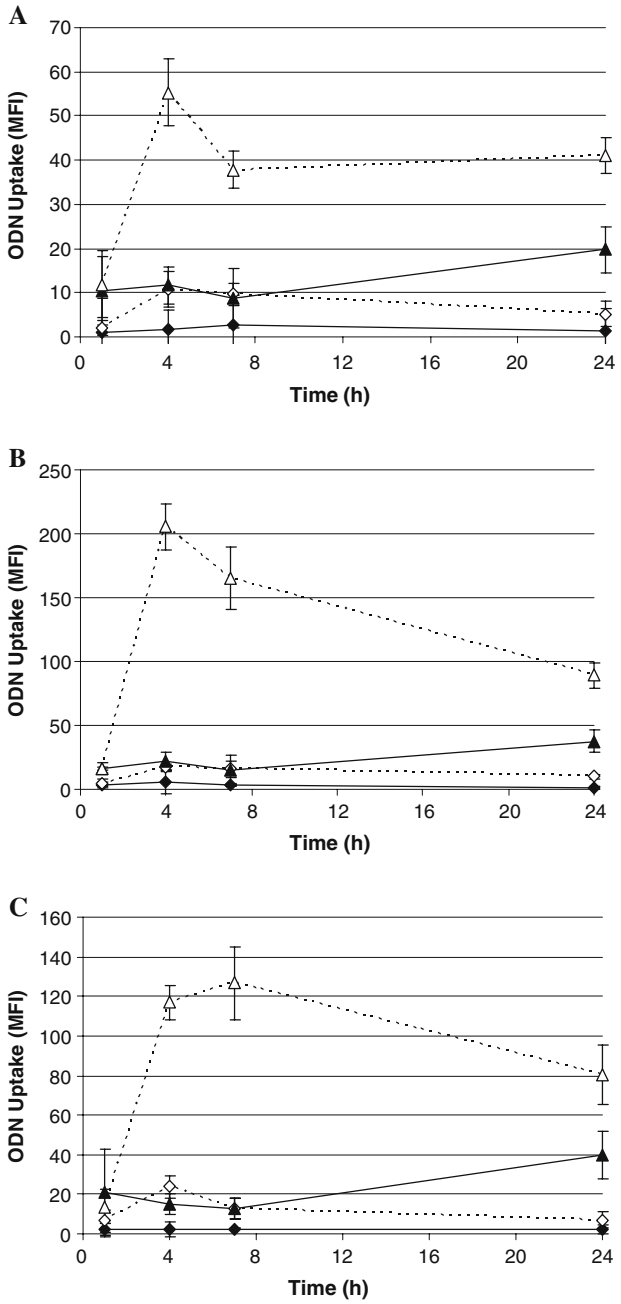

Fig. 1 Uptake of free and liposome nanoparticulate formulations of CpG ODN by immune cells in spleen and lymph nodes following s.c. administration. Five mg/kg of free (denoted by *closed symbols*) or encapsulated (denoted by *open symbols*) fluorescently-labeled (5′-FAM) INX-6295 CpG ODN was administered s.c to mice (four animals/group). After 1, 4, 7 and 24 h, animals were euthanized and spleens (denoted by *diamonds*) and lymph nodes (denoted by *triangles*) were harvested and processed to single cells. Samples were analyzed for uptake of the ODN (as judged by mean fluorescence intensity or MFI ± SD) by specific cell types [as judged by expression of the phenotype markers CD11b (*Panel A*), CDllc (*Panel B*) and CD11b/CD11c (*Panel C*)] by flow cytometry as outlined in the "Materials and methods". Background fluorescence levels of 17.5–18.3 were subtracted from the data. Data presented here is representative of two separate experiments

HBS-treated control animals. This corresponds to a fourfold increase in activated CD11b+ cells, a threefold increase in activated CD11c+ cells, a twofold increase in activated DX5+ cells, and a 12-fold increase in activated CD8+ cells above levels seen after administration of free CpG ODN. Similarly, the expression of the co-stimulatory molecule CD86 is upregulated approximately twofold in both CD11b+ and CD11c+ cells in animals treated with free CpG ODN but upto sixfold in those treated with LN-CpG ODN. An ANOVA was performed on each of the six groups of data shown in Fig. 2. The results from each analysis showed that there is a statistically significant difference between the treatment groups at the $P < 0.001$ level. Further analysis using a Bonferroni t test showed that the upregulation of both CD69 and CD86 was significantly increased ($P < 0.005$) in mice treated with LN-CpG ODN compared with both the control and mice treated with free CpG-ODN.

The relative immunopotency of encapsulated CpG ODN compared to free is also reflected in plasma cytokine levels. Mice injected with free CpG ODN show modest increases in cytokine levels over 72 h (Fig. 3) while LN-CpG ODN is able to exert a dramatic effect. The plasma concentration of IL-6 at the 7 h peak concentration is 240-fold above baseline while both IL-10 and MCP-1 expression is enhanced 14-fold at the 24 h peak time point. The concentration of IFN-γ is also greatly enhanced by encapsulation, showing a 250-fold enhancement in plasma levels above that of free CpG ODN.

As expected, these data are similar to those results previously reported after i.v. administration of LN-CpG ODN demonstrating dramatic immunostimulation as judged by plasma cytokines and cell activation marker levels compared to control animals and those treated with equivalent doses of free CpG ODN [44]. Furthermore, results with other cytokines (e.g. IL-12) and activation markers (CD16 and IL-12 receptor) show similar trends (data not shown).

In summary, encapsulation dramatically enhances the immunogenicity of CpG ODN following s.c. administration compared to free CpG ODN, resulting in enhanced expression of cell activation markers CD69 and CD86 and elevated plasma cytokine/chemokine levels. No appreciable immunostimulatory activity, based on immune cell activation or plasma cytokines were detected in animals treated with empty liposomes (unpublished data).

Encapsulation of CpG ODN in LN enhances the generation of Ag-specific immune responses

To assess the effect of encapsulation on the development of adaptive responses, studies with OVA were

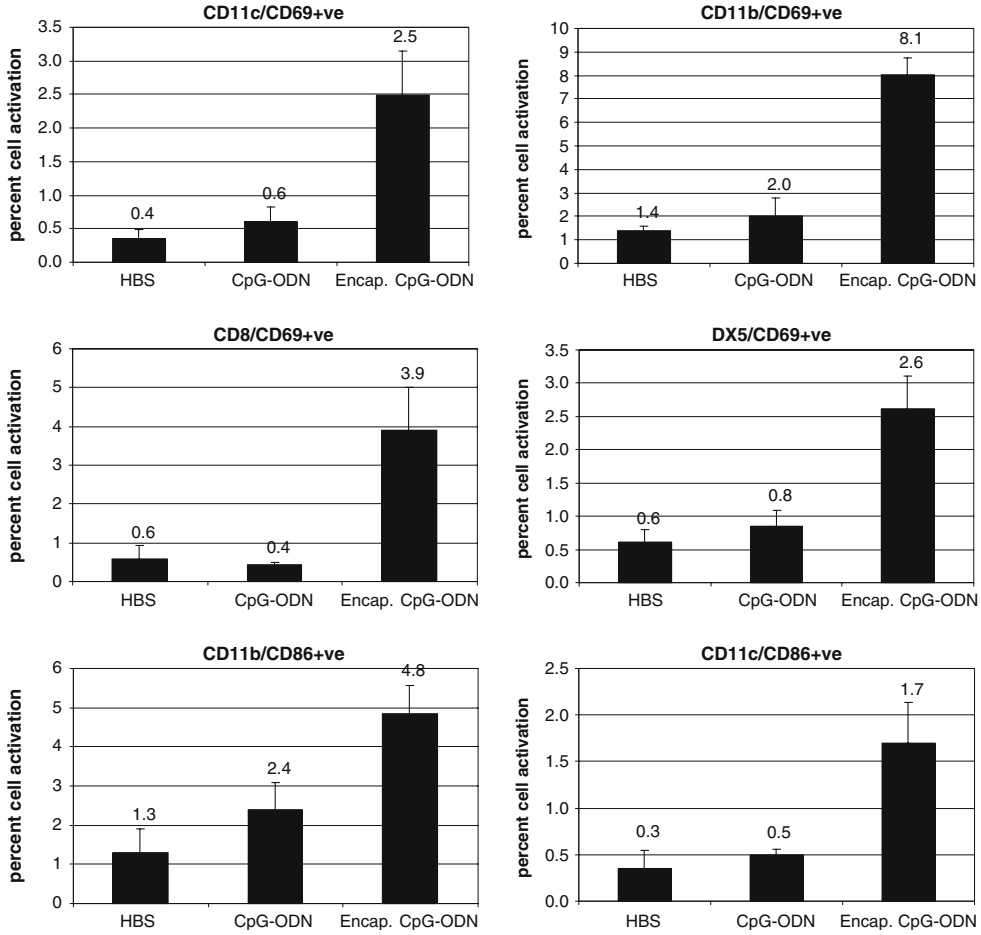

Fig. 2 Comparison of immune cell activation following s.c. treatment with free and encapsulated CpG ODN. Five mg/kg of free or encapsulated CpG ODN was administered s.c. to ICR mice (four animals/group). After 24 h, animals were euthanized and spleens harvested. Splenocytes were analyzed for expression of the CD69 and CD86 cell surface activation markers (% of total cell population ± SD) in conjunction with phenotype markers by flow cytometry as outlined in the "Materials and methods". Data presented here is representative of three separate experiments

undertaken. Ovalbumin is widely studied as a model Ag and thus its antigenic determinants have been mapped and reagents and models are available to monitor both humoral and cell-mediated immune responses. In our studies, OVA was employed as an artificial TAA to evaluate the ability of LN-CpG ODN to act as an adjuvant in the generation of tumor-specific cell-mediated immune responses. In vitro immune parameters included quantitative and functional assessments of OVA-specific cytotoxic T lymphocytes (CD8+) using an MHC tetramer assay and cytotoxicity and cytokine secretion assays, respectively.

The ability of encapsulated CpG ODN to generate OVA-specific CD8 T lymphocytes was assessed using a standard MHC tetramer assay on splenocytes from C57BL/6 mice immunized with OVA adjuvanated with free or encapsulated CpG ODN. The use of tetramers is a quantitative method of determining the frequency of Ag-specific CTL that is not dependent upon limiting dilution or in vitro culture methods. Using this assay, the percentage of CD8/TCR-β OVA-tetramer positive cells is enhanced 20- and 5-fold following treatment with encapsulated and free CpG ODN, respectively, compared with control animals (Fig. 4). Further analysis revealed statistically significantly greater frequency of Ag-specific OVA-MHC tetramer positive CD8 cells following immunization with OVA adjuvanated with LN-CpG OVA compared with free CpG ODN plus OVA [$t(7) = -2.157$, $P < 0.05$].

In addition to a quantitative assessment, the relative ability of liposome-encapsulated versus free CpG ODN to generate Ag-specific CTLs was also assessed functionally. One such functional assay used the secretion of IFN-γ (as an indicator of T_H1 response) by splenocytes of C57BL/6 mice to monitor the number of T lymphocytes in immunized animals capable of responding to Ag-specific stimulation. While a 2.5-fold increase in the percentage of CD8+ IFN-γ producing cells is observed

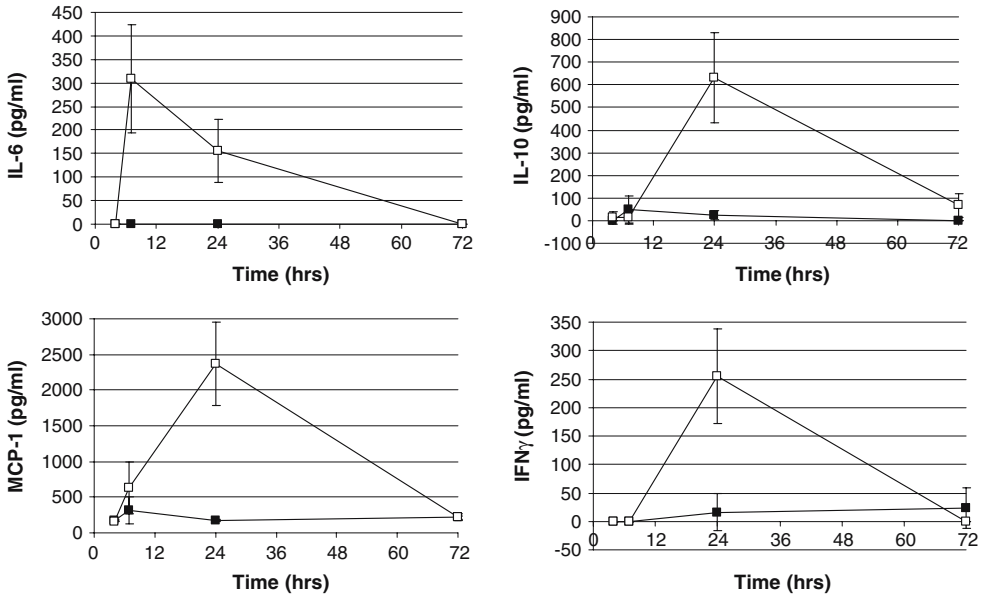

Fig. 3 Comparison of plasma cytokine induction following s.c. treatment with free and encapsulated CpG ODN. Five mg/kg of free or encapsulated CpG ODN was administered s.c. to ICR mice (four animals/group). Blood was collected from animals by cardiac puncture, processed to collect plasma and cytokine levels (pg/ml ± SE) were determined by ELISA or cytometric bead array. Data presented here is representative of three separate experiments and each *data point* represents an average of four animals

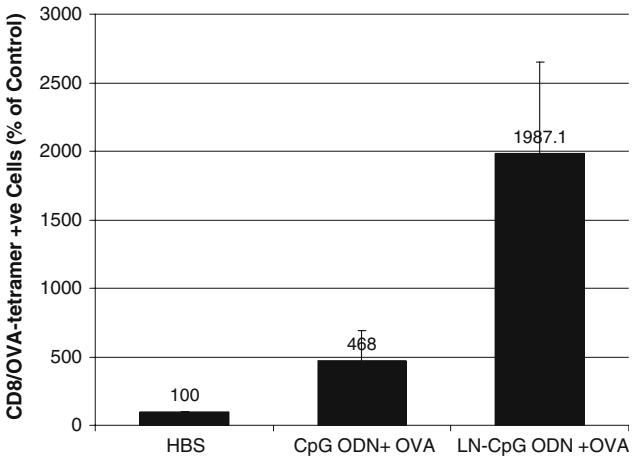

Fig. 4 Frequency of antigen-specific OVA-MHC tetramer+ CD8+ cells following s.c. immunization with OVA adjuvanated with free or encapsulated CpG ODN. C57BL/6 mice (four animals/group) were immunized s.c. with OVA adjuvanated with 100 μg free or encapsulated CpG ODN. Splenocytes were isolated, incubated with an OVA-specific PE-labeled H-2D-SIINFEKL MHC tetramer in conjunction with fluorescently labeled anti- CD8 and TCRβ antibodies and analyzed by flow cytometry to quantitate the frequency of OVA-specific CD8 T lymphocytes in animals following immunization. These data are derived from five separate studies and expressed as a percentage of control levels ± SD

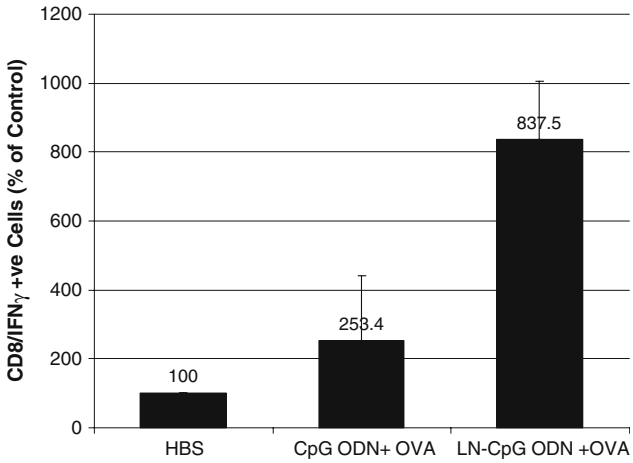

Fig. 5 Frequency of antigen-specific, IFN-γ secreting CD8+ cells following s.c. immunization with OVA adjuvanated with free or encapsulated CpG ODN. C57BL/6 mice (four animals/group) were immunized s.c. with OVA adjuvanated with 100 μg free or encapsulated CpG ODN. Splenocytes were isolated and activated as described in the "Materials and methods". Briefly, IFN-γ secreting ability of CD8+ cells was assessed after 8 h in IVR with OVA expressing E.G7 cells as determined by cytokine secretion assay. These data are derived from five separate studies and expressed as a percentage of control levels ± SD

in mice treated with free CpG ODN compared to control mice (Fig. 5), a further significant increase in the percentage of IFN-γ-secreting CD8+ cells is obtained when mice are immunized with OVA adjuvanated with LN-CpG ODN [$t(7) = -4.584$, $P < 0.05$].

As an additional functional assessment, the induction of Ag-specific CTL responses was assessed in a standard chromium release cytotoxicity assay. The relative ability of splenocytes, isolated from animals immunized with OVA and encapsulated or free CpG ODN, to lyse OVA-expressing E.G7 target cells in an Ag-specific manner was assessed immediately after

isolation or following 5 days of in vitro restimulation (IVR) with OVA-expressing APCs. In both primary and IVR evaluation of CTL activity, effector cells from control animals exhibit only minimal levels of target cell lysis while immunization with OVA and free CpG ODN results in a twofold and a fourfold increase, respectively (Fig. 6). However, significantly higher CTL responses in both the primary and the IVR assays are observed in mice that received OVA and LN-CpG ODN compared to mice vaccinated with free CpG ODN ($P < 0.005$—Bonferroni adjusted t tests) and control animals. No Ag-specific responses, as judged by either the quantitative or functional assays were detected following immunization with empty liposomes (unpublished data).

Anti-tumor efficacy of encapsulated ODN as a vaccine adjuvant in xenogeneic and syngeneic tumor models

Using the parental and OVA-expressing EL4 and E.G7 tumor cell lines, we assessed the relative ability of free and encapsulated CpG ODN to induce anti-OVA immunity and effective anti-tumor efficacy. Specifically, C57BL/6 mice were immunized prophylactically with OVA combined with free or LN-CpG ODN, challenged s.c. with E.G7 tumor cells and monitored for effect on tumor growth. As a standardized control, these data were compared to animals immunized with OVA adjuvanated with complete Freund's adjuvant (CFA), a potent, widely used, research adjuvant against which the majority of new adjuvants are measured. Immunization with OVA alone or in combination with free CpG ODN results in only minor anti-tumor activity, with animals exhibiting vigorous tumor growth similar to that observed in control animals and those immunized with CFA alone (Fig. 7). Alternatively, OVA adjuvanated with CFA induces a moderate anti-tumor response while mice immunized prophylactically with OVA and LN-CpG ODN show pronounced responses, exhibiting complete tumor regression that persists prior to eventual tumor regrowth. Interestingly, the use of encapsulated CpG ODN as an adjuvant also results in less variability in response compared with CFA, most likely due to difficulties in obtaining a homogeneous Ag-adjuvant emulsion with CFA for immunizations. Furthermore, animals treated with CFA also develop local inflammatory responses and granuloma formation unlike those treated with LN-CpG ODN.

However, while LN-CpG ODN elicits potent anti-tumor activity in the E.G7-OVA tumor model, it has been noted that OVA, being a xenogeneic Ag, is highly immunogenic. Therefore induction of effective anti-OVA immune responses would be expected to be relatively easy compared to true, syngeneic TAAs that are characterized by low immunogenicity and host

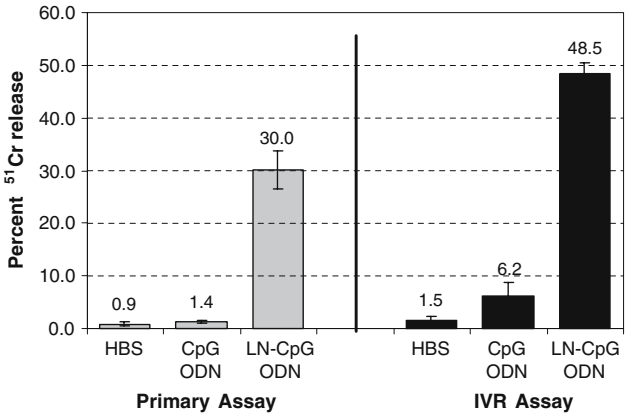

Fig. 6 Antigen-specific cytolytic activity of splenocytes against E.G7-OVA cells following s.c. immunization with OVA adjuvanated with free or encapsulated CpG ODN. C57BL/6 mice (four animals/group) were immunized s.c. with OVA adjuvanated with 100 μg free or encapsulated CpG ODN. Splenocytes were isolated as described in the "Materials and methods" and either used immediately (primary) or after 5 days IVR as effector cells in a standard ^{51}Cr release assay. The percentage of chromium released from radiolabeled E.G7 (for specific lysis) and EL-4 (for non-specific lyis) targets after 4 h co-incubation with isolated splenocytes was used to calculate specific cytolytic activity ± SD. Effector cells and target cells were plated at a variety of ratios; the 100:1 effector-target ratio is shown above. Data presented here is representative of five separate studies

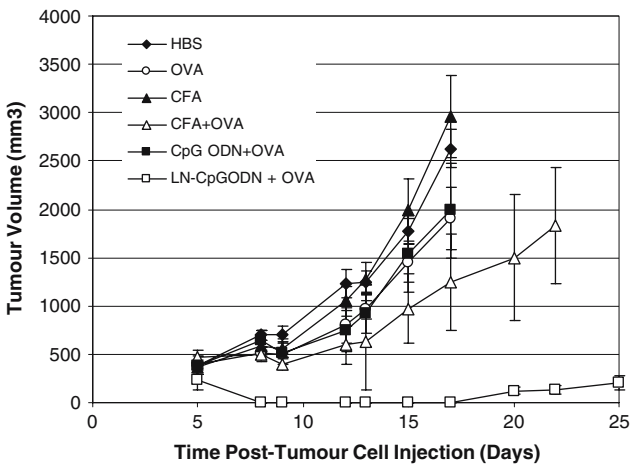

Fig. 7 Antigen-specific anti-tumor activity following prophylactic s.c. immunization with OVA adjuvanated with free or encapsulated CpG ODN in a E.G7-OVA Xenogeneic Tumour Model. C57BL/6 mice were immunized prophylactically s.c. with OVA adjuvanated with 100 μg free or encapsulated CpG ODN. One week following the last vaccination, mice were challenged s.c. with 2.5×10^6 E.G7-OVA cells and tumor growth was monitored. Tumor volume was calculated using the formula $V = (L \times W^2)/2$. Each *data point* represents the mean and standard deviation of a group of five animals

tolerance. To address this concern, the ability of free and encapsulated CpG ODN to induce immune responses against TRP-2 was assessed in a B16 pulmonary metastasis model to establish whether free or encapsulated CpG ODN provides sufficient immunological stimulus to generate immune responses and therapeutic activity against self Ags. TRP-2 is a tissue specific syngeneic Ag, expressed on melanoma, melanocytes and the retina and anti-B16 CTLs have been shown to recognize the immunodominant MHC class I (H-2K^b) epitope of TRP-2 ($TRP_{181–188}$; [5]). Since TRP-2 has been identified as a potential TAA against which to target CTL responses for cancer therapy in humans [57], the murine TRP-2-B16 model provides a system that mimics human melanoma without the introduction of a xenogenic Ag. C57BL/6 mice were immunized prophylactically with the immunodominant peptide $TRP_{181–188}$ co-administered with free or encapsulated CpG ODN, challenged i.v. with B16 cells and after 18 days, euthanized, lungs collected and lung metastases enumerated. Animals immunized with the TRP-2 peptide alone show no anti-tumor effect, with similar numbers of lung metastases (mean = 82) compared to untreated animals (data not shown). Compared with untreated animals, there is a non-significant but suggestive decrease in the total number of lung metastases in mice treated with TRP-2 peptide adjuvanated with CFA (mean = 46) and free CpG ODN (mean = 35; Fig. 8). However, animals immunized with $TRP_{181–188}$ adjuvanated with encapsulated CpG ODN have a significant reduction in lung metastases (mean = 8) compared to control or CFA-adjuvanted animals (Bonferroni adjusted t tests; vs. HBS: $t(7) = 4.244$, $P < 0.05$; and vs. CFA: $t(7) = 4.965$, $P < 0.01$). A suggestive but non-significant reduction in lung metastases was observed also between animals immunized with TRP-2 peptide adjuvanated with free versus encapsulated CpG ODN. No anti-tumor activity was observed for empty liposomes in either of these models (unpublished data).

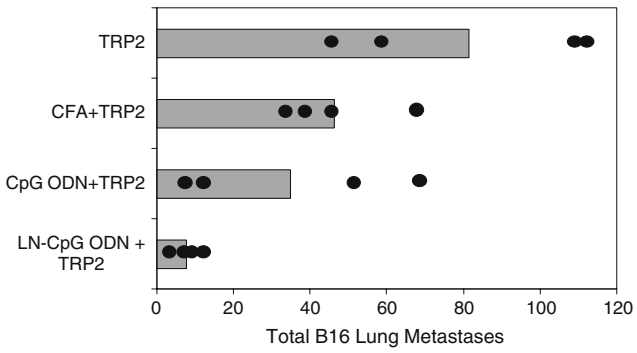

Fig. 8 Antigen-specific anti-tumor activity following prophylactic s.c. immunization with TRP-2 adjuvanated with free or encapsulated CpG ODN in a B16 Syngeneic Tumor Model C57BL/6 mice were immunized prophylactically s.c. with TRP-2 adjuvanated with 100 μg free or encapsulated CpG ODN or complete Freund's adjuvant. Two days following final immunization, mice were injected intravenous with 1×10^5 B16 cells. Animals were euthanized on day 18, lungs were excised and metastases were enumerated using a stereomicroscope. The *bar* represents the mean number of lung metastases per group ($n = 4$); *circles* represent individual animals

Discussion

Broadly active biological response modifiers that target the innate as well as the adaptive arms of the immune system and enhance cell-mediated immunity are continuously being explored for their clinical potential in the treatment of cancer [63]. It has been clearly established that bacterial DNA or synthetic ODN containing CpG motifs have potent immunostimulatory properties through their interaction with TLR9 [23, 27, 51]. CpG ODN-mediated stimulation of APCs induce the secretion of immunomodulatory cytokines and chemokines and promote the upregulation of costimulatory molecules which, in turn, activate NK and other innate immune cells. This results in elaboration of secondary cytokines such as IFN-γ and facilitates the priming and expansion of T lymphocytes, ultimately giving rise to Ag-specific effector T lymphocyte populations. This ability to elicit potent innate responses and directly and indirectly induce adaptive, cellular immunity supports the concept of CpG ODN as an effective cancer therapeutic with a variety of potential applications, including as a bioresponse modifier and vaccine adjuvant [11].

While free CpG ODN mediate potent immunostimulatory effects, its limited delivery to target cells or tissues, poor cellular uptake, rapid degradation (for the native PO form) and toxic side-effects represent limitations for its clinical use [45]. As an alternative strategy, it has been well demonstrated that encapsulation in LN has the capacity to dramatically alter the pharmacokinetic and biodistribution characteristics of a drug. In immunotherapeutic applications, encapsulation of immunomodulatory compounds results in targeted delivery to immune cells [46]. For antisense ODNs, encapsulation in LN has been shown to significantly enhance the plasma and tissue levels and improve anti-tumor effects in human melanoma and laryngeal squamous carcinoma in vivo [15, 16, 32].

Although all micro and nano-particulate carrier systems preferentially accumulate in macrophages and professional APCs following systemic administration, traditional complexes consisting of cationic lipids and

CpG ODN are not suitable for systemic delivery in humans as they are rapidly eliminated from the blood due to their large (micron) size and positive charge. The charge associated with complexes can also give rise to non-specific immunological activity as a result of complement activation via the alternative pathway, resulting in damage to the liver and other tissues [33]. However, the nanoparticulate lipid-based delivery system utilized in this study is characterized by low surface charge and small particle size, resulting in reduced clearance rates, relatively long circulation lifetimes and minimal toxicity [22, 50]. These nanoparticulate systems protect the ODNs from nuclease degradation and promotes uptake of relatively large amounts of intact ODN by target immune cells. We show in these studies that s.c. delivered LN-CpG ODN is preferentially delivered to and taken up by target APCs, providing, at least in part, the basis for the enhanced immunopotency of encapsulated CpG ODN.

Using this formulation, we have previously demonstrated the enhanced immunopotency of LN-CpG ODN compared to free ODN following i.v. administration [44], consistent with other observations of immune stimulation and anti-tumor activity following systemic administration of lipid-DNA complexes for gene transfer applications [13, 61] and report here that a parallel response is induced following s.c. administration. Expression of activation markers such as CD69, which is low on resting immune cells such as NK and T lymphocytes, and minimal in mice injected with free CpG ODN is dramatically upregulated on immune cell populations following s.c. (and i.v., as previously reported [44]) administration of LN-CpG ODN. These results are supported by similar observations of plasma cytokine levels in which s.c. (and i.v. [44]) administered LN-CpG ODN dramatically enhances levels of a number of cytokines and chemokines compared to equivalent doses of free ODN, both in terms of the magnitude and duration of cytokine expression [44].

Furthermore, this enhanced immunopotency is also reflected in the capacity of LN-CpG ODN to induce more vigorous adaptive immune responses. It has been well documented that CpG ODN have properties that make them ideal immune adjuvants for cancer vaccines [37, 42, 55, 63], promoting the generation of T_H1-biased, Ag-specific immune responses against co-administered peptide and protein tumor Ags including vigorous cell-mediated responses. Since priming and expansion of tumor-specific T lymphocytes is considered to be an essential component of an effective anti-tumor immune response [54], it was of particular interest to assess the capacity of liposome encapsulation to enhance CpG ODN induced Ag-specific adaptive cellular responses. In these studies, the generation of high frequencies of functional Ag-specific CTLs, as assessed using MHC tetramer, IFN-γ secretion and cytotoxicity assays, directly supports the characterization of LN-CpG ODN as a highly effective adjuvant that is able to promote generation of TAA-specific immune responses. While very low levels of Ag-specific T lymphocytes are detected in splenocytes of control mice and animals immunized with TAAs adjuvanated with free CpG ODN, a concomitant increase in MHC-tetramer positive cells, IFN-γ secretion and cytolytic activity is detected in the splenocytes of mice immunized with TAAs co-injected with LN-CpG ODN, indicating an induction of functional Ag-specific CD8+ T lymphocytes. Thus, both quantitative and functional assays demonstrate that encapsulation results in a dramatic increase in the ability of CpG ODN to support development of Ag-specific cytotoxic T lymphocytes compared with free CpG ODN. Furthermore, although not described here, LN-CpG ODN was also found to be effective in inducing humoral immune responses, resulting in elevated plasma levels of Ag-specific immunoglobulins which, upon analysis of IgG1 and IgG2a isotypes, indicated either a slight Th1-biased or a balanced Th1/Th2 immune response (unpublished results).

While evaluation of ex vivo immune parameters provides valuable insight into mechanisms of action and allows quantitative and functional comparisons of the immunostimulatory capacity of free and encapsulated CpG ODN, evaluation of anti-tumor efficacy provides a more relevant assessment of the ultimate potential of liposomal CpG ODN as a cancer therapeutic. Towards this end, the relative ability of LN-CpG ODN to provide anti-tumor activity was evaluated in a number of animal models. As a vaccine adjuvant, LN-CpG ODN is able to support the generation of protective immunity against tumor challenge, promoting significantly more effective tumor immunoprophylaxis than free CpG ODN as evidenced by the enhanced inhibition of tumor growth in animals compared to free CpG ODN or CFA, a potent, commonly used research adjuvant. Importantly, this therapeutic strategy is able to not only provide effective protection against model Ags, but is capable of breaking tolerance to self Ags, an aspect that is vital for the development of clinically relevant therapies. Encapsulated CpG ODN is sufficiently potent to induce immune responses against poorly immunogenic syngeneic Ags, providing effective anti-tumor activity against even aggressive tumors expressing only very low levels of MHC class I Ag [36].

In summary, the immunological potency and therapeutic efficacy of immunostimulatory CpG ODN is greatly enhanced by encapsulation in LN. In the work

described here, we confirm and further define the previous observations of enhanced immunopotency of LN-CpG ODN compared to free CpG ODN. In view of the interrelated nature of immune regulation, it should perhaps not be surprising that this enhanced capacity of LN-CpG ODN to induce innate responses following i.v. administration would also be reflected in enhanced innate and adaptive immune responses following s.c. administration. Importantly, we also extend these observations, clearly demonstrating the potential of encapsulated CpG ODN to induce potent and effective in vivo anti-tumor activity. As an immune adjuvant to support vigorous adaptive cellular immune responses, encapsulated CpG ODN mediates effective anti-cancer activity, acting to reduce tumor burden and enhance survival. Thus, encapsulation of CpG ODN within LN offers an attractive strategy for significantly enhancing the activity of free CpG ODN and improving its therapeutic activity in the treatment of cancer.

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