Synthetic methylated CpG ODNs are potent *in vivo* adjuvants when delivered in liposomal nanoparticles

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Abstract

Although it is well documented that the immunological activity of cytosine–guanine (CpG) motifs is abrogated by 5′ methylation of the cytosine residue, encapsulation within stabilized lipid nanoparticles endows these methylated cytosine–guanine- (mCpG-) containing oligonucleotides (ODNs) with potent immunostimulatory activity in murine animal models. Surprisingly, not only do liposomal nanoparticulate (LN) mCpG ODN possess immunostimulatory activity, their potency is found to be equivalent and often greater than the equivalent unmethylated form, as judged by a number of *ex vivo* innate and adaptive immune parameters and anti-tumor efficacy in murine models. Preliminary data indicate that both methylated and unmethylated CpG ODN act through a common receptor signaling pathway, specifically via toll-like receptor (TLR) 9, based on observations of up-regulated TLR9 expression, induction of nitric oxide and dependence on endosomal maturation. This is confirmed in *TLR9* knockout animals which show no immunostimulatory activity following treatment with LN-mCpG ODN. These data, therefore, indicate that the mCpG DNA is fully competent to interact with TLR9 to initiate potent immune responses. Furthermore, this work implicates an as yet unidentified mechanism upstream of TLR9 which regulates the relative activities of free methylated versus unmethylated CpG ODN that is effectively bypassed by particulate delivery of CpG ODN.

Introduction

Prokaryotic DNA containing unmethylated cytosine–guanine (CpG) motifs is a well-recognized immunomodulator that can induce potent immune responses capable of providing significant protective and therapeutic immunity against a number of malignant and infectious diseases (1, 2), properties that are shared with short, CpG-containing synthetic oligodeoxynucleotide (ODN) (3). CpG-containing DNA is a member of a group of molecules known as pathogen-associated molecular patterns (PAMPs), highly conserved molecular motifs associated with a wide range of pathogens, other members which include LPS and peptidylglycans/lipopeptidies/lipoproteins from Gram-negative and -positive bacterial cell walls, respectively, flagellin and single- and double-stranded viral RNA. Eukaryotic organisms have evolved pathogen recognition receptors expressed in APCs that function to specifically recognize these PAMPs. The best described of these are the

toll-like receptors (TLRs), of which 13 have been identified in mammals and 10 are expressed in humans (4). PAMP recognition acts as a 'danger signal' indicative of bacterial and viral invasion that triggers rapid and potent innate and adaptive immune responses (5) characterized by activation of cytolytic cells, secretion of cytokines, chemokines and bactericidal effectors such as nitric oxide (NO) and induction of antigen-specific cellular and humoral immunity (6–8). While most TLRs are localized on the surface of APCs, those specific for prokaryotic nucleic acids [TLR3 (7–9)] are largely localized to the endosomal compartment. Therefore, prerequisites for CpG DNA activity include internalization and endosomal trafficking (3, 9, 10).

Since eukaryotic cells are continually exposed to self-DNA during normal development, growth and maintenance, the ability to distinguish immunostimulatory prokaryotic from

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eukaryotic DNA is vital. Discrimination is generally attributed to a combination of factors including CpG suppression [20-fold lower frequency in mammalian compared with bacterial DNA (7,11–13)] and context [occurrence of eukaryotic CpG motifs within 'immunosuppressive' flanking sequences (5, 14)]. In addition, methylation of CpG DNA is reported to play a major role in determining the immunostimulatory activity of CpG motifs. Since methylation has been extensively described to inactivate CpG motifs (7, 9, 15), the unmethylated nature of prokaryotic viral and bacterial CpG DNA [versus eukaryotic DNA in which the >70% of cytosine residues are methylated (11, 16)] is considered to be an essential characteristic for immunostimulatory activity. Finally, it has been recently proposed that intracellular localization/sequestration of TLR9 itself regulates DNA immunostimulatory potential (17).

While it is generally accepted that methylated eukaryotic DNA is relatively inactive, more recent studies suggest that it should not be considered immunologically inert (18–20). The present study demonstrates that methylated cytosine–guanine (mCpG) ODN sequences are actually immunologically active and that encapsulation within liposomal nanoparticles (LNs) endows them with potent immunostimulatory activity. Surprisingly, this immune activity and the resultant anti-tumor efficacy in animal models are at least equal to that induced by the equivalent, encapsulated unmethylated form. Finally, abrogation of activity in *TLR9* knockout (KO) animals confirms that mCpG ODN exert their activity through the same TLR9 pathway as their unmethylated counterparts. In summary, these studies show that mCpG DNA is able to interact with TLR9 and initiate potent immune responses that mediate effective anti-tumor activity. Furthermore, these results implicate an as yet unidentified, upstream mechanism regulating the activity of free methylated versus unmethylated CpG ODN, which is effectively bypassed when ODNs are delivered in LNs.

Methods

Animals and cell lines

Six- to 8-week-old female C57BL/6 and ICR mice were obtained from Charles River Laboratories (Saint-Constant, PQ, Canada) or Harlan (Indianapolis, IN, USA) and quarantined for 3 weeks prior to use. *TLR9*-KO mice (21) were obtained from Oriental Biosciences Inc. and backcrossed to C57BL/6 mice over eight generations. 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. All cells were obtained from the American Type Culture Collection (Manassas, VA, USA). EL4 and E.G7- ovalbumin (OVA) thymoma (22) cells were cultured in complete media (CM) consisting of RPMI 1640 medium supplemented with penicillin G (100 U ml^{-1}), streptomycin sulpfate (100 μg ml^{-1}), β-mercaptoethanol and 10% heat inactivated fetal bovine serum (FBS). RAW264.7 cells (23) were cultured in DMEM supplemented with 10% FBS, L-glutamine, penicillin G and streptomycin sulphate. Tissue culture media and supplements were obtained from Invitrogen (Burlington, ON, Canada).

Preparation of liposomal ODN

Distearoylphosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and cholesterol from Sigma (St Louis, MO, USA). 1,2-Dioleyloxy-3-*N*,*N*-dimethylaminopropane and polyethylene glycol–dimyristol glycerol were provided by Tekmira Pharmaceuticals Corporation (Burnaby, BC, Canada). These studies evaluated the immunostimulatory activity of the phosphorothioate ODNs INX-6295 (5′-TAA**CG**TTGAGGGGCAT-3′), INX-5001 (5′-AA**CG**TT-3′), CpG-2006 (5′-T**CG**T**CG**TTTTGT**CG**TTTTGT**CG**TT-3′) and CpG-1826 (5′-TCCATGA**CG**TTCCTGA**CG**TT-3′) containing either an unmethylated or methylated cytosine nucleotide within the CpG motifs indicated in bold. All ODNs were synthesized by Trilink Biotechnologies (San Diego, CA, USA) and encapsulated into LN-CpG ODN using methodology previously described (24–26). Particle size was 100 ± 25 nm in diameter as determined by quasi-elastic light scattering using a NICOMP submicron particle sizer (model 370, Santa Barbara, CA, USA).

Analysis of innate and adaptive immunopotency

The ability to induce innate immune responses was evaluated after subcutaneous (s.c.) and intravenous (i.v.) administration of free and encapsulated CpG and mCpG ODN to ICR and *TLR9*-KO mice, respectively. We have previously shown that s.c. and i.v. administered LN-CpG ODN induce very similar, dose-dependent systemic immune responses which are virtually indistinguishable (26). Adaptive immune parameters were assessed in C57BL/6 mice immunized s.c. with 20 μg OVA adjuvantated with 100 μg of free or encapsulated CpG and mCpG ODN. The terms CpG ODN and mCpG ODN refer to studies with unmethylated and methylated forms of INX-6295 unless otherwise noted.

Immune cell activation

For cell activation analysis, mice were terminally anaesthetized with ketamine/xylazine (3.2%/0.8%, v/v), and spleens and lymph nodes were collected and processed to single cells. These were analyzed for stimulation (as judged by activation marker expression) of specific immune cell populations (as determined by phenotype analysis) by flow cytometry. Cell suspensions were labeled with combinations of FITC-conjugated anti-CD11c, H-2K^b, PE-conjugated anti-IL-12R, CD11b, CD69, CD86 and allophycocyanin conjugated anti-CD11c, DX5, CD86 antibodies and analyzed using either a FACSort or LSRII flow cytometer with Cell-Quest Pro v4.0.1 or FACSDiva v4.1 software, respectively (BD Biosciences, San Jose, CA, USA). For the latter, data were analyzed with FlowJo flow cytometry analysis software v7.2.2 (Ashland, OR, USA). All fluorescently labeled antibodies were obtained from BD Biosciences. Propidium iodide was used to exclude dead cells and 150 000 and 80 000 events were collected to analyze dendritic cells (DCs) or NK cells and macrophages, respectively.

Plasma cytokine levels

For plasma cytokines, mice were terminally anaesthetized as previously described and blood was collected via cardiac

puncture. Plasma was isolated by centrifugation and frozen at −20°C until assayed. Plasma concentrations of IL-6, IL-12, IFN-γ and monocyte-chemotactic protein (MCP)-1 were determined using a cytometric bead array kit (BD Biosciences), as per the manufacturer's instructions.

MHC tetramer assay

The frequency of OVA-specific CD8 T lymphocytes was determined by MHC tetramer assay using PE-coupled H-2K^b MHC tetramers containing the immunodominant peptide of OVA (SIINFEKL; Beckman Coulter, Immunomics, San Diego, CA, USA) 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. At least 250 000 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 antigen-specific manner was assessed in splenocytes after 5 days *in vitro* re-stimulation with mitomycin-treated E.G7-OVA cells and recombinant human IL-2. OVA-specific cytotoxicity was assessed using a standard 4 h ^{51}chromium (^{51}Cr) release assay in which splenocytes were mixed in various effector:target ratios with ^{51}Cr-loaded parental EL4 or OVA-transfected E.G7 cells. The cellular cytotoxicity was calculated on the basis of ^{51}Cr released to the supernatant using the formula: % lysis = [(experimental c.p.m. − spontaneous c.p.m.)/(maximal c.p.m. − spontaneous c.p.m.)] × 100, where c.p.m. represents counts per minute, maximal c.p.m. was achieved by complete lysis of ^{51}Cr-labeled targets in 10% Triton X 100, spontaneous CPM was determined by incubating labeled targets in media and antigen-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

The IFN-γ secretion assay (Miltenyi Biotec Inc., Auburn, CA, USA) was performed according to the manufacturer's instructions to enumerate CD8 T lymphocytes secreting IFN-γ in response to antigen stimulation. Briefly, splenocytes were re-stimulated with OVA-expressing APCs prior to incubation with a bispecific antibody designed to bind to activated T lymphocytes and capture secreted IFN-γ. The frequency and phenotype of cells actively secreting cytokines were determined by flow cytometry as described previously using fluorescently labeled anti-IFN-γ and phenotype antibodies.

Antigen-specific anti-tumor activity

The syngeneic E.G7-OVA C57BL/6 tumor model was used to determine the efficacy of encapsulated ODN as a vaccine adjuvant to induce antigen-specific anti-cancer immune responses. For this model, mice were immunized prophylactically q7 × 3 with OVA mixed with free and encapsulated, unmethylated and mCpG ODN. One week later, mice were challenged with E.G7-OVA tumor cells (2.5 × 10^6 cells) by s.c. hind flank injection and monitored for tumor growth for ~50 days post-tumor challenge. Tumor volumes were calculated using the formula (length × width^2)/2. Animals that cleared their tumors were re-challenged 3 weeks later (~6 weeks following initial tumor challenge) and tumor growth was monitored as described above for ~50 days.

Up-regulation of TLR9

To assess the effects of LN-mCpG ODN on TLR9 expression, RAW264.7 cells were incubated for 4 h with 10 μg ml^{-1} free or encapsulated, methylated or unmethylated CpG ODN, washed, harvested and then fixed and permeabilized using BD Cytofix/Cytoperm Plus kit (BD Biosciences) according to the manufacturer's instructions. Cells were then incubated with biotinylated mouse mAb to TLR9 (Hycult, Uden, The Netherlands) followed by incubation with streptavidin–PE (BD Biosciences) prior to analysis by flow cytometry. Mean fluorescence intensity of cells incubated with streptavidin–PE only was subtracted from the data.

Nitric oxide production

To assess relative NO production induced by LN-CpG and mCpG ODN, RAW264.7 cells were incubated for 12, 24 or 48 h with 10 μg ml^{-1} encapsulated methylated or unmethylated CpG ODN. Supernatants were harvested and concentrations of NO determined by Greiss reaction (Nitric Oxide Quantitation Kit, Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

Inhibition by chloroquine

To assess the effects of chloroquine, an inhibitor of endosomal maturation, on LN-mCpG ODN immunostimulation, bone marrow from ICR mice were differentiated to DCs by culture in CM supplemented with 300 ng each of IL-4 and granulocyte macrophage colony-stimulating factor for 7 days, typically resulting in >85% CD11c$^+$ cells. These cells were incubated with 10 μg ml^{-1} chloroquine prior to treatment with 10 μg ml^{-1} LN-mCpG ODN or LN-CpG ODN and then stained with combinations of antibodies against activation and phenotype markers prior to analysis by flow cytometry.

TLR9-KO studies

To confirm a role for TLR9 in LN-mCpG ODN immunostimulatory activity, C57BL/6 WT and TLR9-deficient (*TLR9*–/−) mice were injected i.v. with encapsulated CpG or mCpG ODN or the equivalent lipid dose of empty LNs. *TLR9*-KO animals were injected i.v. with LPS as a positive control. Animals were euthanized at 7, 24 and 48 h and lymph nodes, spleens and blood harvested to assess the effect on cell activation (24 h data shown) and plasma cytokine levels (7 h data shown) as described above.

Statistical analyses

All statistical analyses 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. Independent *t*-tests were used to assess significance between wild-type and *TLR9*-KO animals. Probability (*P*) values <0.05 were considered significant.

Results

mCpG ODN induces potent innate and adaptive immune responses when delivered in LNs

We have previously demonstrated that encapsulated CpG ODN exhibit enhanced immunopotency and anti-tumor efficacy compared with free CpG ODN (24). As expected, the enhanced activity of the encapsulated ODN was CpG dependent since inversion of the dinucleotide effectively abro-

gated activity (data not shown). Somewhat surprising, however, was the observation that LN-mCpG ODN was active. Based on these and other preliminary data, a series of *in vivo* studies were undertaken to evaluate the immunostimulatory activity and anti-tumor efficacy of encapsulated mCpG ODN.

To assess the effect of encapsulation on the immunopotency of mCpG ODN, activation of splenic and lymph node immune cells (Fig. 1) and plasma levels of T_h1 and T_h2 cytokines (IFN-γ and IL-6, IL-10, respectively) as well as MCP-1 (a macrophage chemokine) were assessed (Fig. 2) over 72 h following administration of either free or encapsulated unmethylated or mCpG ODN. The data from the 24 h time point are presented and are representative of at least 10 independent experiments. ANOVAs on these data revealed a statistically significant difference between treatment groups at the $P < 0.001$ level. Consistent with the previously reported literature (7, 9, 15), Bonferroni *t*-tests showed that free mCpG ODN is not immunoactive, inducing little or no
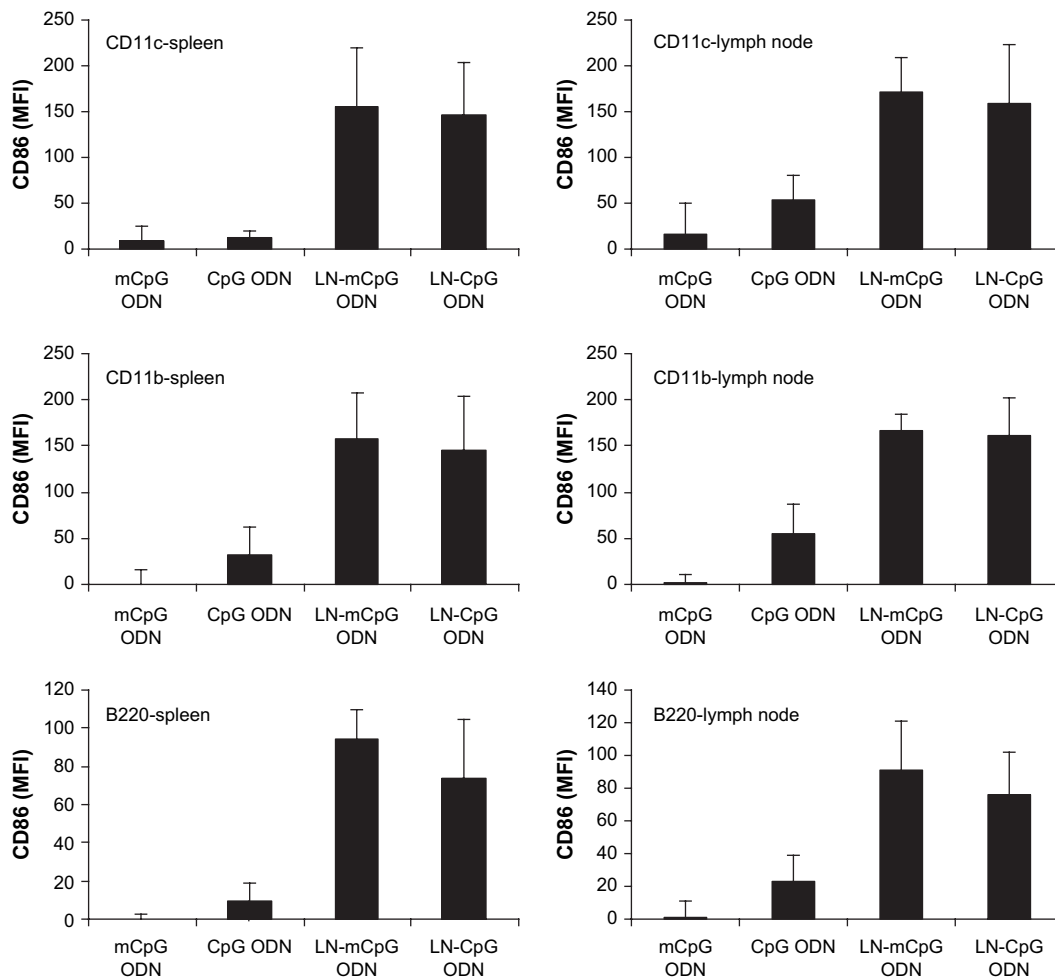

Fig. 1. mCpG ODN induces potent immune cell activation when encapsulated in LNs. Five milligrams per kilogram of free and encapsulated, unmethylated and mCpG ODN was administered s.c. to ICR mice (four animals per group). After 24 h, animals were euthanized and spleens and lymph nodes harvested and processed to single cell suspensions. Cells were analyzed for expression of CD86 cell surface activation markers [mean fluorescence intensity (MFI) ± SD] in conjunction with phenotype markers by flow cytometry as outlined in the Methods. MFI from control animals (PBS) was subtracted from the data. Data presented here is representative of at least 10 separate experiments.
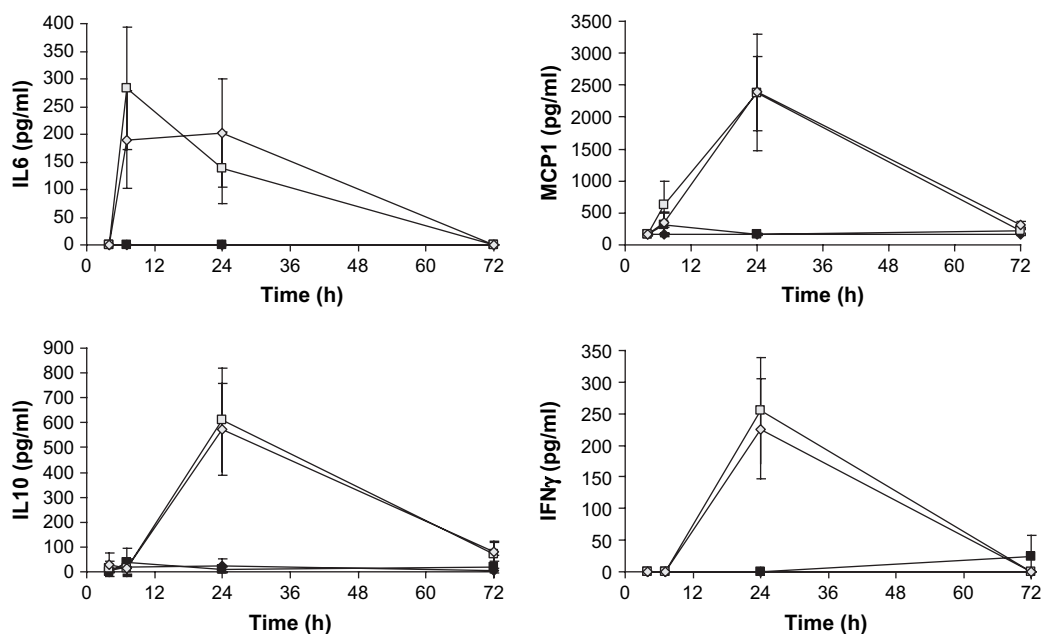
Fig. 2. Encapsulated mCpG ODN induces elevated plasma cytokine levels. Five milligrams per kilogram of free (closed symbols) or encapsulated (open symbols) unmethylated (squares) or methylated (circles) CpG ODN was administered s.c. to ICR mice (four animals per group). Blood was collected from animals by cardiac puncture and processed to collect plasma and cytokine levels (pg ml^{-1} ± SD) were determined by ELISA or cytometric bead array. Data presented here is representative of at least 10 separate experiments and each data point represents an average of four animals.

up-regulation of activation marker expression compared with control animals (CD86 expression shown) while free CpG ODN induces up-regulation of activation markers in CD11b$^+$ and c$^+$ cells in spleen and lymph node compartments compared with both control (HEPES-buffered saline (HBS) treated) and mCpG ODN-treated animals. As previously reported (27), encapsulation of CpG ODN dramatically enhances immunostimulatory activity, inducing in a statistically significant 4- to 6-fold ($P < 0.01$) and 3- to 4-fold ($P < 0.05$) increase in activation marker expression compared with free mCpG and CpG ODN, respectively. Statistically significant up-regulation of MHC class I and IL-12R on CD11c$^+$ cells and CD8 T lymphocytes, respectively, was also observed compared with free methylated and unmethylated CpG ODN (data not shown). Importantly, these data confirm preliminary observations that encapsulated CpG ODN regardless of methylation state induces significantly greater activation than free CpG ODN in all cell types examined with no significant difference detected between animals receiving encapsulated, CpG ODN and mCpG ODN. These data also reflect cytokine response (Fig. 2) where encapsulated CpG ODN, regardless of methylation state, is able to induce dramatic increases in plasma levels for all cytokines examined. While baseline levels of IL-6 were largely undetectable, animals treated with LN-CpG and mCpG ODN exhibited levels exceeding 200 pg ml^{-1} by 7–24 h following treatment. Similarly, IL-10 was enhanced ~40-fold, and IFN-γ and MCP-1 enhanced ~10–15-fold above control levels at 24 h post-administration. The kinetics and plasma cytokine levels were very similar for LN-CpG ODN and LN-mCpG ODN (Fig. 2). Dose–response studies showed that the ability

of LN-mCpG ODN to induce similar responses to LN-CpG ODN was consistent over a range of ODN concentrations (1–60 mg kg^{-1}), with no significant differences in either activation or cytokine data (data not shown). Furthermore, similar ability of encapsulation to endow mCpG ODN with immunostimulatory activity was also seen with natural, unmodified phosphodiester ODN (data not shown).

LN-mCpG ODN was also found to effectively adjuvanate adaptive, cell-mediated immune responses against the model antigen OVA as assessed quantitatively by MHC tetramer analysis (Fig. 3A) and functionally by IFN-γ secretion (Fig. 3B) and cytotoxic activity against OVA-expressing tumor cells (Fig. 3C). LN-mCpG ODN generated 9- to 10-fold more OVA-specific CD8 T lymphocytes than control animals (versus 2-fold enhancement with free CpG ODN). Interestingly, LN-mCpG ODN induced ~2.5-fold higher frequency of antigen-specific CD8 T lymphocytes compared with LN-CpG ODN (Fig. 3A), a trend that was observed for all encapsulated CpG/mCpG ODNs tested (Table I) including the hexameric sequence INX-5001, the primate optimized CpG-2006 and the murine optimized CpG-1826.

Functional assessment of antigen-specific CTLs using the IFN-γ secretion assay as an indicator of T$_h$1 response showed that immunization with encapsulated CpG ODN (unmethylated or methylated) induced 4- to 6-fold greater frequency of CD8$^+$ IFN-γ-producing cells compared with control or free CpG ODN-treated animals. Consistent with MHC tetramer results, LN-mCpG ODN appeared to be more immunopotent than the equivalent unmethylated ODN (0.37 versus 0.23%). In addition, antigen-specific cytolytic T lymphocyte responses following *in vitro* re-stimulation using
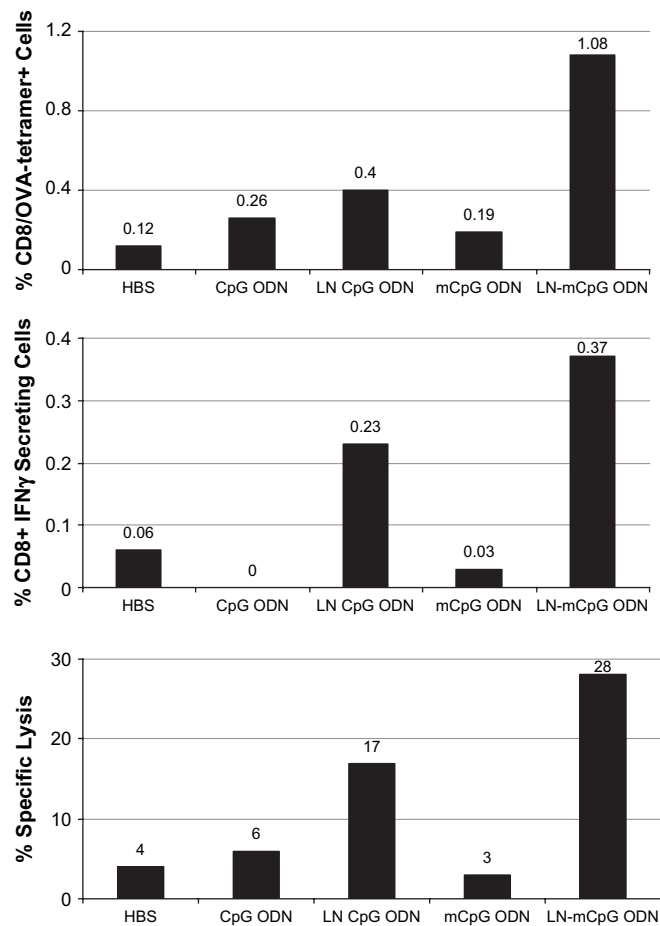
Fig. 3. LN mCpG ODN adjuvanates potent, antigen-specific adaptive cellular immune responses after s.c. immunization with OVA. C57BL/6 mice (four animals per group) were immunized s.c. with 20 μg OVA adjuvanated with 100 μg free or encapsulated CpG or mCpG ODN. (Panel A) Frequency of antigen-specific OVA-MHC tetramer-positive CD8^{+} T cells. Splenocytes were isolated from immunized mice, 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. (Panel B) Frequency of antigen-specific, IFN-γ-secreting CD8^{+} cells. Splenocytes were isolated from immunized animals and activated as described in the Methods. Briefly, IFN-γ-secreting ability of CD8^{+} cells was assessed after 8 h *in vitro* re-stimulation 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. (Panel C) Antigen-specific cytolytic activity of splenocytes against E.G7-OVA cells. Splenocytes were isolated as described in the Methods and used after 5 days *in vitro* re-stimulation 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.

a standard cytotoxicity assay show that immunization with encapsulated CpG and mCpG ODN resulted in a 4- and 7-fold increase, respectively, compared with control animals (Fig. 3C).

Table 1. Encapsulation in LNs confers immunostimulatory and anti-tumor activity on a variety of mCpG ODNs[a]

	% of CD8-SIINFEKL tetramer positive	Efficacy, day 21 tumor volume (mm^3)
Free 5001	0.17	>2000
LN 5001	0.62	781
Free 5001m	0.1	>2000
LN 5001m	0.98	419
Free 2006	0.18	1328
LN 2006	0.66	Nd
Free 2006m	0.26	>2000
LN 2006m	0.98	Nd
Free 1826	0.22	1000
LN 1826	0.24	128
Free 1826m	0.14	1563
LN 1826m	0.42	169

Nd, not detected.
[a]Frequency of MHC SIINFEKL tetramer-positive CD8^{+} T lymphocytes and anti-EG.7 tumor efficacy in animals immunized with OVA adjuvanated with free or lipid nanoparticulate, unmethylated and methylated forms of the CpG ODN 5001, 2006 and 1826 (see Methods).

Adaptive immune responses mediated by immunization with LN mCpG ODN mediates effective anti-tumor activity

To determine the therapeutic relevance of these results, the anti-tumor efficacy was evaluated in the well-defined, syngeneic EG.7—C57BL/6 thymoma tumor model in which animals immunized against OVA were challenged with OVA-expressing E.G7 tumor cells and tumor growth was monitored over 18 days (Fig. 4A). Results from these studies directly reflect the *ex vivo* cellular immune responses. Animals immunized with free mCpG ODN exhibited minimal inhibition of tumor growth compared with free CpG ODN (1700 versus 1050 mm^3, respectively, on day 15) while animals immunized with either LN-CpG or LN-mCpG ODN exhibited significant tumor growth inhibition within the 18 days after tumor implantation (400 and 125 mm^3, respectively). These data demonstrate the enhanced potency of unmethylated compared with mCpG ODN in free form and confirm the ability of liposomal encapsulation to either increase (as in the case of unmethylated) or endow (as in the case of methylated) CpG ODN immunostimulatory potency. To further evaluate and discriminate potency, the immunological memory in mice that had cleared the initial EG7 tumor challenge was assessed. Figure 4B shows tumor growth in mice that had cleared the initial tumor challenge and been re-challenged. These data show that mice initially immunized with OVA and LN-mCpG ODN maintained almost complete tumor rejection at day 26 post re-challenge compared with those initially immunized with OVA adjuvanated with LN-CpG ODN. As expected, both exhibited superior anti-tumor responses compared with naive animals. Taken together, these results demonstrate that encapsulation endows mCpG ODN that are essentially inactive in their free form with potent immunostimulatory activity. Particularly surprising is the fact that encapsulated mCpG ODN exhibits enhanced immune activity which is at least as potent as the equivalent unmethylated form. The ability of LN-mCpG ODN to induce protective immunity has also been
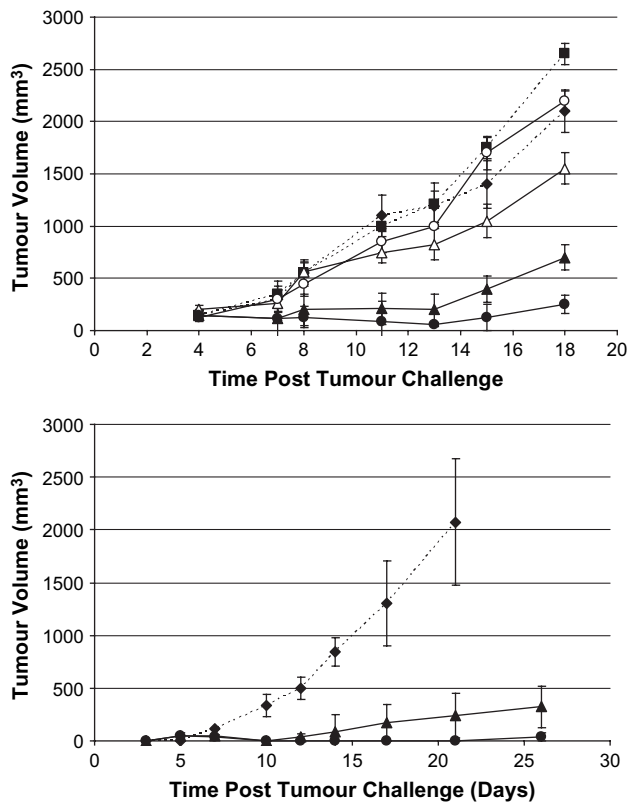
Fig. 4. LN mCpG ODN induces potent, antigen-specific anti-tumor activity following prophylactic immunization with OVA adjuvanated with free or encapsulated CpG ODN in a E.G7-OVA syngeneic tumor model. C57BL/6 mice were immunized prophylactically s.c. with OVA adjuvanated with saline (close diamonds), empty liposomes (closed squares) or 100 µg free and LN-CpG (open and closed, respectively) and mCpG ODN (triangles and circles, respectively). Mice were monitored for ~6–7 weeks and results for the initial 18–21 day period in which tumor data from control animals were available are shown here. (Panel A) 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 × W^2)/2. Each data point represents the mean and standard deviation of a group of five animals. (Panel B) A number of animals immunized with OVA adjuvanated with LN-CpG or mCpG ODN were able clear their initial tumor challenge. These mice were re-challenged s.c. with 2.5×10^6 E.G7-OVA cells and tumor growth was monitored as above.

demonstrated in more clinically relevant therapeutic cancer models as both a vaccine adjuvant to tumor-associated antigens (G. Chikh, L. Sekirov, S. G. Raney, P. R. Cullis and Y. K. Tam, in preparation) and an adjunct therapy to anti-cancer monoclonal antibodies (L. Sekirov, G. Chikh, S. Raney, M. Kazem, K. D. Wilson, S. D. de Jong, P. R. Cullis and Y. K. Tam, in preparation).

Importantly, these effects are not sequence or backbone modification specific and extend to other ODNs. Anti-tumor activity analysis of encapsulated synthetic CpG ODN INX-5001, CpG-2006 and CpG-1826 yield similar results (Table I). Similarly, encapsulated CpG and mCpG ODN with phosphodiester backbones demonstrate parallel activity, although comparison to their equivalent free forms, in this case, is not possible due to their labile nature (data not shown).

Encapsulated mCpG ODN induces its immunostimulatory activity through TLR9

Analysis of a number of parameters, previously reported in the literature to be implicated in TLR9-mediated responses, found that LN-mCpG ODN induced responses similar to the unmethylated CpG ODN, thus suggesting that both act through TLR9. While intracellular flow cytometry showed that TLR9 is up-regulated by free CpG ODN but not mCpG ODN (28), expression levels were significantly increased by treatment with both LN-CpG and LN-mCpG ODN (P < 0.01) (Fig. 5A). Similarly, NO levels were found to be induced by free unmethylated CpG ODN (29) as well as both LN-CpG and LN-mCpG ODN. Levels were significantly elevated at 24 and 48 h and 12, 24 and 48 h for LN-CpG and mCpG ODN, respectively (P < 0.05), compared with cells treated with saline (Fig. 5B) or free CpG ODN (data not shown) although no significant differences were observed between LN-CpG and LN-mCpG ODN. Finally, consistent with free CpG ODN, both LN-CpG and LN-mCpG ODN were inhibited by chloroquine (30–32) due to either inhibition of endosomal maturation (30) and/or competition for TLR9 binding (33, 34) (Fig. 5C).

To confirm the seminal role for TLR9 in mediating activity of LN-mCpG ODN, studies in *TLR9*-KO animals were undertaken. As expected, while wild-type animals significantly up-regulated CD69 and CD86 on a variety of splenic and lymph node (data not shown) immune cell populations, independent *t*-tests revealed that the administration of LN-mCpG ODN to *TLR9*-KO mice failed to induce any immune responsiveness (P < 0.01), with cells exhibiting only background expression levels (Fig. 6A). Similarly, evaluation of IFN-γ, IL-12, MCP-1 and IL-6 plasma levels indicated that while wild-type animals displayed rapid statistically significant (P < 0.001) increases in plasma cytokine/chemokine levels following treatment with LN-mCpG ODN, *TLR9*-KO animals failed to respond (P < 0.05, independent *t*-tests) (Fig. 6B). Bonferroni *t*-tests confirmed no significant differences between wild-type and KO animals receiving HBS or empty LNs.

Discussion

Encapsulation within LNs protects CpG ODN from degradation and avoids the disadvantages associated with protective backbone modification which can include toxicities following i.v. administration (35) and reduced immunopotency [G. Chikh, L. Sekirov, S. G. Raney and Y. K. Tam, unpublished data (27, 36)]. Furthermore, encapsulation also functions to improve pharmacokinetic [such as avoiding rapid elimination from circulation and degradation by serum proteases (27)] and biodistribution characteristics (enhancing delivery to target immune tissues and cells) provide a depot effect upon s.c. administration to prolong bioavailability (26, 37) and enhance cellular uptake. As a result, liposomal encapsulation has the potential to increase CpG ODN potency as we have previously shown with respect to a number of innate and adaptive immune parameters and anti-tumor activity (24, 26, 27). Consistent throughout many of these studies has been the use of ODN containing unmethylated CpG motifs in deference to the significant body of research indicating that methylation abrogates immunostimulatory activity (6, 7, 38, 39). However, we report here that when delivered within stabilized lipid
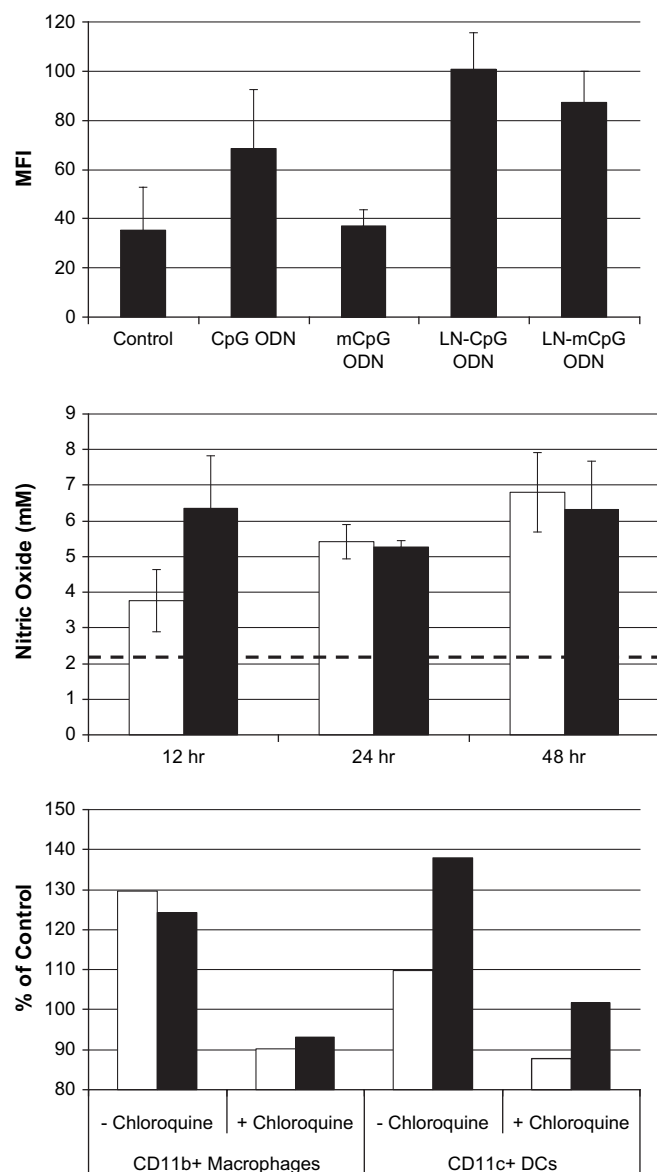
Fig. 5. LN, unmethylated and mCPG ODN induce similar responses. (Panel A) LN, mCpG ODN induces TLR9 up-regulation. One million RAW264.7 murine macrophage cells ml^{-1} were incubated with 10 μg ml^{-1} of free and LN-mCpG ODN and CpG ODN for 1.5 h. Cells were harvested, fixed and permeabilized and subjected to intracellular flow cytometry analysis for TLR9 expression as described in the Methods section. (Panel B) LN, mCpG ODN induces nitric oxide production. One million RAW264.7 murine macrophage cells ml^{-1} were incubated with 10 μg ml^{-1} of LN-CpG ODN (white bars) and LN-mCpG ODN (black bars) CpG ODN for 12, 24 and 48 h. Supernatants were harvested and assessed for NO by Greiss reaction as described in the Methods section. NO level in control cells (12 h after injected with saline) is indicated by dashed line. (Panel C) Immune activation by LN, mCPG ODN is inhibited by chloroquine. Bone marrow-derived DCs from ICR mice with IL-4 and granulocyte macrophage colony-stimulating factor were incubated with 10 μg ml^{-1} chloroquine prior to treatment with 10 μg ml^{-1} LN-CpG ODN (white bars) and LN-mCpG ODN (black bars). Cells were analyzed for activation of immune cell populations by flow cytometry as described in the Methods section, specifically CD86 expression by CD11c$^+$ DCs and CD11b$^+$ macrophages. Data are expressed as percentage of expression on cells from control animals.

nanoparticles, mCpG ODN are potent immunomodulators, exhibiting activity which is at least as potent as the equivalent unmethylated form based on a number of innate and adaptive immune parameters as well as anti-tumor efficacy in animal models.

CpG motifs are known to act through TLR9 to trigger potent innate immune responses and, to this point, it has been largely assumed that TLR9 specifically recognizes unmethylated CpG motifs. Conversely, CpG ODN bearing modifications such as methylation that deviate it from the 'conserved molecular motif' have been assumed to prevent recognition/binding by TLR9 and abrogate immunostimulatory activity. However, it is reported here that LN-mCpG ODN induce potent immune responses that are at least equal to the equivalent encapsulated unmethylated ODN. Insomuch as these observations represent a deviation from the generally accepted mechanism by which prokaryotic and eukaryotic DNA is distinguished, this conclusion was predicated on an assumption that LN-mCpG ODN mediates its effect through the TLR9 signaling pathway rather than a novel pathway as confirmed by studies of TLR9 expression, NO production, chloroquine inhibition and immune response in *TLR9*-KO animals.

While an extensive body of research exists to show that mCpG motifs exhibit little or no immune activity, there is limited data to demonstrate differential binding of methylated and unmethylated ODN to TLR9 or a causal relationship with immunopotency. Researchers have used surface plasmon resonance (Biacore) biosensor technology and NF-κB assays in *TLR9*-transfected cells (34, 40) to evaluate CpG–TLR9 interaction, and while these data support an effect of CpG methylation on TLR9 binding, the assay systems are highly artificial and the results are somewhat ambiguous. In fact, direct binding of CpG ODNs to TLR9 *in vivo/in vitro* has not yet been demonstrated, although confocal microscopy studies show that TLR9, MyD88 and CpG co-localize in late endosomes and require endosome maturation for signaling (10, 41).

The studies reported here extend preliminary observations first made by our group (42) and later confirmed by Yasuda *et al.* (43, 44) that mCpG ODN is, in fact, capable of initiating TLR9-mediated immune responses when delivered in lipid particles. However, while Yasuda *et al.* reported immunostimulation following mCpG ODN delivery in cationic lipid complexes, they observed responses that were significantly lower than observed for free CpG ODN. The authors hypothesized that inefficient natural uptake restricts endosomal accumulation of CpG ODN, allowing only ligands with high affinity (such as unmethylated CpG ODN) and not those with low affinity (such as mCpG ODN) to initiate immune responses. Therefore, consistent with the prevailing view of the role of methylation in regulating CpG ODN-TLR9 activity, Yasuda *et al.* postulated that delivery in lipid complexes achieves sufficiently high levels to overcome the low affinity of mCpG ODN for TLR9 and enable immunostimulatory activity.

In contrast to this conclusion, our results suggest that mCpG ODN is fully capable of interacting with and triggering TLR9 signaling at least as effectively as the equivalent unmethylated ODN, inducing responses that often exceed those found with equivalent amounts of either free or encapsulated unmethylated CpG ODN. Significantly, the ability of encapsulated mCpG ODN to induce equivalent responses
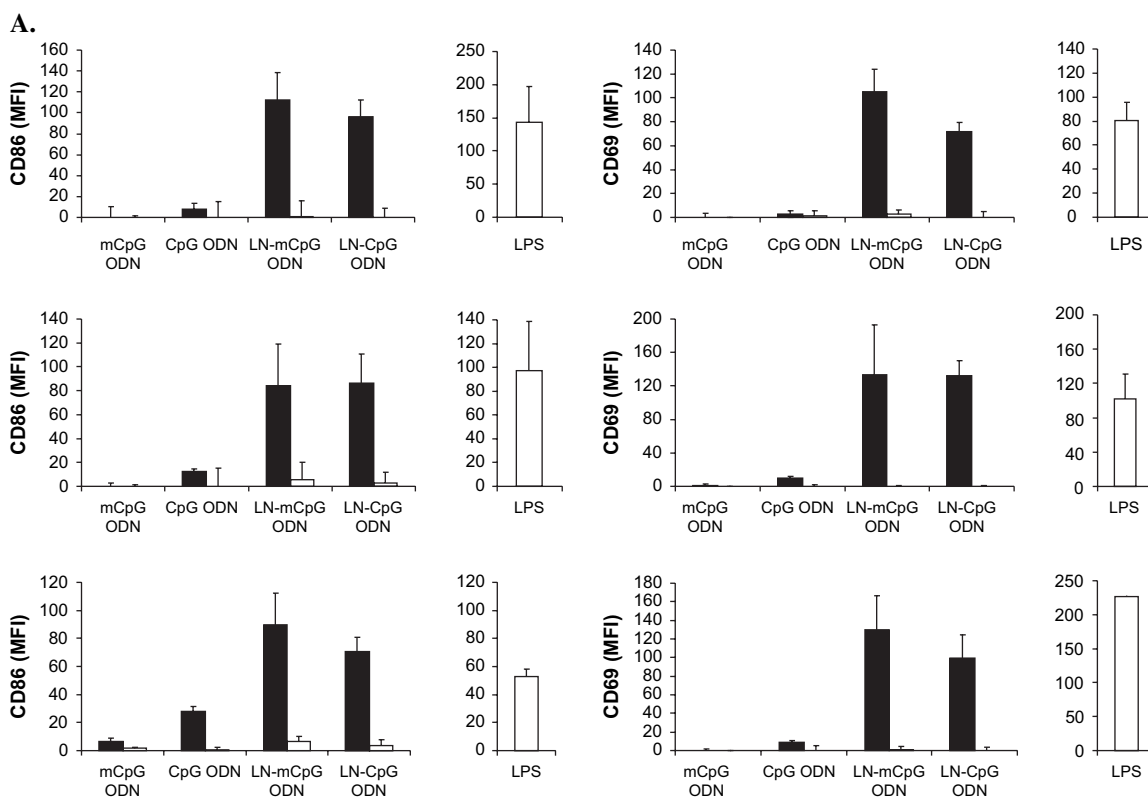
A.

Fig. 6. LN, mCpG ODN mediates immune cell activation through TLR9. Twenty milligrams per kilogram free or encapsulated, mCpG or CpG ODN was administered i.v. to wild-type and *TLR9*-KO animals (three animals per group). After 7, 24 and 48 h, animals were euthanized and spleens and blood harvested. The 24-h time point shown here. (Panel A) 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 Methods. Mean fluorescence intensity (MFI) of CD69 and CD86 expression on macrophage, DCs and B lymphocyte in wild-type control (black bars) are shown in comparison to TLR-KO (white bars) animals. *TLR9*-KO mice treated with LPS were used as a positive control and baseline. MFI from wild-type and *TLR9*-KO control animals have been subtracted from the data. (Panel B) Blood was collected from animals by cardiac puncture, processed to plasma and frozen at −20°C until analysis. Cytokine levels (pg ml^{-1} ±SD) in plasma isolated from wild-type animals (black bars) compared with *TLR9*-KO animals (white bars) determined by cytometric bead array as outlined in the Methods. *TLR9*-KO mice treated with LPS were used as a positive control and baseline cytokine levels observed in wild-type and *TLR9*-KO control animals have been subtracted.

to LN-CpG ODN was observed over a range of doses, further supporting the contention that immunostimulatory activity is not based simply on achieving a sufficient intracellular concentration of a low-affinity ligand. The basis for the discrepancy between the data reported here and previously reported work is unclear, but possibilities include potential toxicity (45, 46) or disruption of the endosome (and thus TLR9 signaling) by cationic complexes.

Results from our studies raise questions regarding mechanisms that regulate CpG DNA/TLR9 immunostimulatory activity. If TLR9 is in fact able to effectively bind and mediate immune responses to both unmethylated and methylated ODN, then what is the true mechanism responsible for differentiating between free CpG and mCpG ODN and how is it overcome by nanoparticulate delivery? Furthermore, if both unmethylated and methylated ODN mediate their activity through TLR9, what is the basis for the observation that encapsulated methylated ODNs are often more potent that the unmethylated counterpart, particularly for adaptive immune responses. Of relevance is a recent report proposing the immunostimulatory activity of natural, non-modified DNA via TLR9 is not actually dependent on the presence of CpG

motifs but rather is mediated by the deoxyribose backbone itself (47). Although these data remain to be confirmed by other groups, they call into question the role of CpG methylation in regulating the immunostimulatory activity of DNA and may indicate that while DNA methylation impacts upstream events controlling CpG ODN-TLR9 activity, it is not methylation of CpG motifs specifically, that may be relevant.

Ultimately, these data indicate that the mammalian immune system is not inherently inert to methylated eukaryotic DNA and implies that methylated DNA from apoptotic/necrotic self cells must be subjected to other regulatory mechanisms that promote self-tolerance in healthy individuals and that may be inactive in individuals prone to autoimmune diseases. This is consistent with reports demonstrating that genomic DNA released by necrotic cells can induce the maturation/activation of APCs (48). Based on these findings, we hypothesize that discrimination between methylated and unmethylated ODN occurs upstream of TLR9 and the lack of mCpG ODN immunological activity is due to an inability of mCpG ODN to access rather than bind the TLR9 receptor. This could be mediated by differences in uptake (i.e. discrimination by the plasma membrane-resident 'uptake' receptor) or trafficking (i.e. differential trafficking to
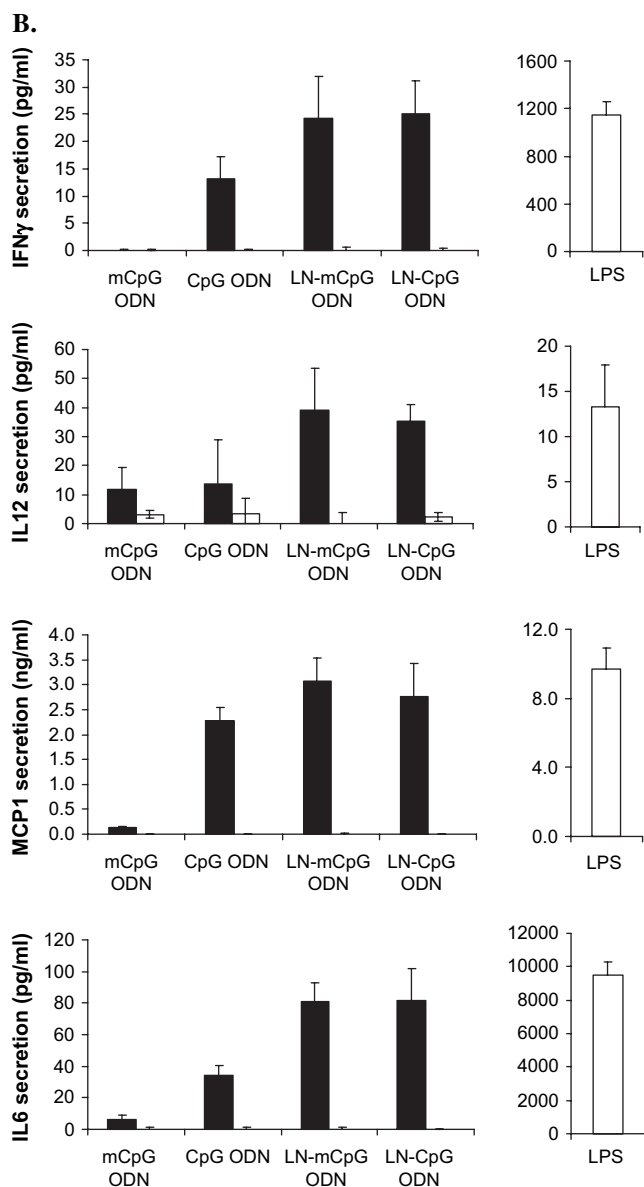
B.

Fig. 6. *Continued.*

appropriate intracellular compartments) that are overcome by encapsulation, thus providing mCpG ODN access to TLR9 by altering the uptake and/or trafficking pathways. Work is currently under way in our laboratory to elucidate the potential roles of mCpG ODN uptake and trafficking in regulating CpG ODN/TLR9-mediated immune responses.

In regards to the relative potency of encapsulated CpG versus mCpG ODN, since LN delivery is able to overcome a primary barrier to mCpG ODN activity (such as access to TLR9), it may be expected that previously unobserved aspects of the nature of CpG/mCpG-mediated immune response (such as potency) may manifest themselves. In this regard, the ability of both CpG and mCpG ODN to access the TLR9-containing compartment by delivery in LNs has revealed differences in immunostimulatory potency. Although the basis for this difference is unknown, one possibility is that mCpG may actually bind TLR9 more effectively *in vivo* than

unmethylated motifs since TLR9 has been reported to share homology with methyl-CpG-binding proteins and DNA methyl-transferases (34, 49), containing the motifs responsible for the methylated DNA-binding activity of these proteins.

While there have been sporadic observations of immunostimulatory activity from methylated DNA in the literature, these have generally agreed with the widely held notion that methylated DNA is poorly active. To the contrary, this study provides evidence for the ability of LN-mCpG motifs to act through TLR9 to mediate vigorous immune responses. It supports a major role for sequestration in regulating TLR9-CpG immune activity and implicates a mechanism by which relative immunostimulatory activity of methylated and unmethylated CpG is based on differential access to TLR9 rather than differential binding affinity. Therefore, this research raises several important questions as to the nature of the TLR9–CpG interaction, how this interaction is regulated and the implications on the resultant immune response. Elucidating this process is vital in understanding both the natural process of self-tolerance as well as designing effective prophylactic and therapeutic intervention strategies with nucleic acid-based drugs such as adjuvants, vaccines, genes and small interfering RNA.

Abbreviations

ANOVA	analysis of variance
APC	antigen-presenting cell
CM	complete media
CpG	cytosine–guanine
^{51}Cr	^{51}chromium
DC	dendritic cell
FBS	fetal bovine serum
i.v.	intravenous
HBS	HEPES-buffered saline
KO	knockout
LN	liposomal nanoparticle/nanoparticulate
mCpG	methylated cytosine–guanine
MCP-1	monocyte chemotactic protein-1
NO	nitric oxide
ODN	oligodeoxynucleotide
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
s.c	subcutaneous
TLR	toll-like receptor

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