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Differential immune activation following encapsulation of immunostimulatory CpG oligodeoxynucleotide in nanoliposomes

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ABSTRACT

The immunogenicity of a vaccine formulation is closely related to the effective internalization by the innate immune cells that provide prolonged and simultaneous delivery of antigen and adjuvant to relevant antigen presenting cells. Endosome associated TLR9 recognizes microbial unmethylated CpG DNA. Clinical applications of TLR9 ligands are significantly hampered due to their pre-mature *in vivo* digestion and rapid clearance. Liposome encapsulation is a powerful tool to increase *in vivo* stability as well as enhancing internalization of its cargo to relevant immune cells. The present study established that encapsulating CpG motifs in different liposomes having different physicochemical properties altered not only encapsulation efficiency, but also the release and delivery rates that ultimately impacted *in vitro* and *ex-vivo* cytokine production rates and types. Moreover, different liposomes encapsulating CpG ODN significantly increased Th1-biased cytokines and chemokines gene transcripts Additional studies demonstrated that co-stimulatory and surface marker molecules significantly upregulated upon liposome/CpG injection. Finally, co-encapsulating model antigen ovalbumin with CpG ODN adjuvant in nanoliposomes profoundly augmented Th1 and cell mediated anti-Ova specific immune response. Collectively, this work established an unappreciated immunoregulatory property of nanoliposomes mediating immunity against protein antigen and could be harnessed to design more effective therapeutic vaccines or stand alone immunoprotective agents targeting infectious diseases, as well as cancer or allergy.

1. Introduction

Innate immune cells respond to diverse components of microbial pathogens. These molecular patterns collectively are known as the pathogen associated molecular patterns (PAMPs). Host cell expressed pattern recognition receptors (PRRs) [1] recognizes PAMPs and generally initiates a signaling cascade leading to orchestrated innate immune activation [2]. The most studied PRR family is the Toll-like receptors (TLRs) [3,4], which are expressed by various cells of tissues such as spleen, lung, muscle, intestines and blood cells [5,6]. Recognition of diverse array of microbial components by different TLRs separates self antigen from non-self antigens [7] and it is the link between innate and adaptive immunity [8,9]. Microbial nucleic acids are recognized by endosomal Toll-like receptors on immune system cells of the vertebrates. Bacterial DNA containing CpG motifs and dsRNA, are recognized by TLR9 [10,11] and TLR3 [12] respectively in both murine and human immune cells whereas ssRNA are recognized by murine TLR7 and human TLR8 [13]. Certain TLRs induce a Th1 dominant immune response, yet others may drive a Th2-biased immune activation through MyD88 or TRIF pathways [14]. CpG ODNs with different sequences can also yield differential immune response [15,16]. CpG-B (or K-ODN) triggers monocytes and B cells to proliferate and secrete IL6 and IgM [15]. Moreover it induces plasmacytoid dendritic cells (pDC) to mature and also secrete TNFα by proceeding signaling from late endosome through MyD88 and IRF5. Whereas, CpG-A (or D-ODNs) induces pDCs to secrete IFNα [17] by signaling from early endosome through MyD88 and IRF7 [18] and it induces NK cells to produce IFNγ [15].

Nucleic acid based TLR ligands are promising vaccine adjuvants, anti-allergens, immunoprotective and anticancer agents [19]. However their clinical applications are significantly hampered due to their pre-mature *in vivo* digestion by endonucleases and rapid clearance via serum protein adsorption leading to limited *in vivo*

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stability and activity [20]. This problem can be overcome by encapsulating nucleic acid based TLR ligands into a depot carrier, whereas provide an increased *in vivo* stability as well as more pronounced targeting/internalization to relevant innate immune cells [21–23]. Liposomal encapsulation is an efficient tool that can improve stability, reduce pre-mature clearance and increase therapeutic efficiency of its cargo by prolonging the duration in circulation and thus improve bioavailability to relevant cells.

Liposomes are synthetically made phospholipids vesicle bilayers. By mixing various types of phospholipids with different molar ratios, it is possible to generate different liposomes possessing different physicochemical characteristics ranging from lamellarity to size or net surface charge [23] as well as PEG mediated hydrated surface. In this study, two different classes of TLR9 ligands (K and D-type CpG motifs) were encapsulated within five different liposomes possessing different surface charge and modification. The characteristic of liposome-ODN mediated differential innate immune activation was evaluated. Moreover, potential of these liposomes co-encapsulating antigen and adjuvant as vaccine carriers were also assessed.

2. Materials and methods

2.1. Reagents

All cell culture media components were from Hyclone (USA). Cytokine ELISA reagents such as recombinant cytokines, monoclonal unlabeled and biotinylated antibodies against IL6 and IFNγ, streptavidin-alkaline phosphatase (SA–AKP) and *p*-nitrophenyl phosphate disodium salt substrate (PNPP) were purchased from Thermo Scientific or Endogen Pierce (USA). Immunoglobulin ELISA reagents; goat anti-mouse IgG, IgG1, IgG2a, IgG2b monoclonal antibodies conjugated with alkaline phosphatase (AP) were from Southern Biotech (USA). Injectable endotoxin-free OVA was obtained from Pierce (USA). DNase/RNase free water was obtained from Hyclone (USA). TRI Reagent (Trizol) for RNA isolation was from Invitrogen (USA). DyNAmo™ cDNA Synthesis kit, DyNAzyme™ II PCR Master Mix for PCR was obtained from Finnzymes (Finland). 10–150 bp DNA ladder was from Fermentas, and 100–1000 bp DNA ladder was from Jena Bioscience.

L-α-Phosphatidylcholine (PC) was purchased from Sigma Aldrich (USA). Cholesterol (Chol), 3β-[N-(N′,N′-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Chol), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (Ammonium Salt) (PEG-PE), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) were all from Avanti Polar Lipids (USA). Heidolph Laborota Collegiate Rotary Evaporator (Germany), Vibra Cell cup type sonicator (Sonics and Materials Co., USA) and Maxi Dry Lyo, Heto-Holten (Denmark) freeze dryer were used during liposome preparation.

2.2. TLR ligands

TLR ligands for stimulation assays were as follows and supplied from several vendors: peptidoglycan (PGN) isolated from *B.subtilis*; (Fluka, Switzerland), lipopolysaccharide (LPS) (isolated from *E.coli*; Sigma, USA), and different classes of CpG motif expressing ODN and control GpC ODNs (please see Table 1 for sequence and size details) were synthesized by Alpha DNA (Montreal, Canada), and was kindly provided by Dr. Dennis M. Klinman (NCI/NIH, USA). All ODNs were free of endotoxin and protein. Bases shown in capital letter have phosphorothioate linkage and those in lower case have phosphodiester linkages. CpG or flip (GpC) motifs are underlined.

2.3. Maintenance of animals

Adult male or female BALB/c or C57/BL6 mice (6–10 weeks old) were used for *in vivo* experiments as well as generating primary spleen cells for *in vitro* assays. The animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled ambient conditions (22 ± 2 °C) regulated with 12 h light and 12 h dark cycles. They were provided with unlimited access of food and water. All experimental procedures have been approved by the animal ethical committee of Bilkent University (Bil-AEC, Protocol # 07/0029).

Table 1
Names, sizes and sequences of the CpG ODNs used in stimulation assays.

ODN Name and Size	Sequence
1555 (15mer) K-type	GCTAGACGTTAGCGT
K23 (12mer) K-type	TCGAGCGTTCTC
D35 (20mer) D-type	GGtgcatcgatgcaggggGG
D3CG (20mer) D-type	GGtcgatcgatcgaggggGG
1612 (15mer) Control ODN K-type	GCTAGAGCTTAGGCT
I-127 (20mer) Control ODN D-type	GGtgcatgcatgcaggggGG

2.4. Liposome preparation

Cholesterol and various phospholipids (Avanti Polar Lipids, Alabaster, AL) were combined in different ratios as shown in Table 2. Lipids were prepared in chloroform as stock solutions of 10 mg/ml and were stored at −40 °C until use. The liposome preparation method was reported earlier [23].

Briefly, different ratios of phospholipid mixtures in chloroform were evaporated in a round bottom flask using a rotary evaporator at 37 °C for 45–60 min. The solvent free lipid film was purged with argon or nitrogen to eliminate residual chloroform and replace oxygen, thereby preventing lipid peroxidation. To generate empty multilamellar vesicles (MLVs), sterile glass beads and 1 ml of PBS was added to each 20 μmol total dry lipid film. The MLV solution was then taken into sterile glass vial and sonicated 5–8 times for 30 s intervals at 4 °C using a cup sonicator. The generated small unilamellar vesicles (SUVs) were then mixed with 1 mg/ml ODN solution, and promptly frozen in liquid nitrogen and then freeze dried overnight. At this stage, the lipid/ODN powder is formed.

ODN encapsulation within the liposomes was achieved during controlled-rehydration step[20]. DNase/RNase free dH_2O (1:10 ratio of original solution volume before freezing) was added onto dehydrated ODN/liposome powder and vigorously vortexed for 15 s every 5 min for 30 min at room temperature. At the end of 30 min, PBS (1:10 original SUV/ODN solution volume) was added and gently mixed for 10 min. The liposome solution was completed to one ml by adding PBS and a final liposome concentration of 20 μM lipid/mg DNA is adjusted. The size of the final liposome preparation was reduced to <150 nm by extrusion through polycarbonate filters. Liposome formulations were stored at 4 °C until use.

2.5. Stimulation assays

BALB/c mice (3 months old) were sacrificed and their spleens were extracted. Single-cell suspensions from spleens were prepared in RPMI 1640 supplemented with 5% FCS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.11 mg/ml sodium pyruvate and 0.5 mM 2-ME. 4×10^5 cells were layered in each well of 96 well microtiter plates. Cells were stimulated with defined concentrations of ODNs and liposome formulations in triplicate. Final cell concentration at each well was adjusted to 2×10^6 cells/ml. The cells were cultured at 37 °C in a 5% CO_2 incubator for 36 h unless otherwise stated. After incubation supernatant were collected and stored at −20 °C for further use.

2.6. ELISA

The 96-well microtiter plates (Nunc, Denmark) were coated with 10 μg/ml of Abs that are specific for mouse IFNγ and IL6. The plates were blocked with PBS-5% BSA. Supernatants from cultured cells were added. Following the steps of incubating with biotin-labeled anti-cytokine Ab and phosphate-conjugated avidin and PnPP substrate, the cytokine content were quantitated according to standard curves generated by using known amount of recombinant mouse cytokines.

2.7. Semi-quantitative RT-PCR

Total RNA from 1×10^7 of stimulated single-cell suspension of splenocytes were extracted by Trizol extraction method. Synthesis of single-stranded DNA from mRNA was performed by cDNA synthesis kit (Finnzymes cDNA synthesis kit, Finland) according to the manufacturer's protocol. PCR was performed using 12.5 μl of master mix (Finnzymes PCR Master Mix, Finland) 1 μg cDNA, 10 pmol/μl sense primer, 10 pmol/μl antisense primer and 9.5 μl of ddH_2O. PCR conditions for β-actin, CD40 and IL18; 94 °C for 30s, 55 °C for 30s, 72 °C for 30s, 34 cycles and final extension at

Table 2
Lipid composition and molar ratios used to generate different liposome types.

Liposome Type	Liposome Composition (molar ratio)
Neutral	PC:Chol (1:1)
Anionic	PC:DOPE:PS (1:0.5:0.25)
Cationic	DC-Chol:PC:DOPE (4:6:0.06)
Stealth	Chol:DOPE:PEG-PE (4:6:0.06)
Sterically Stabilized Cationic Liposomes (SSCL)	DC-Chol:DOPE:PEG-PE (4:6:0.06)

PC, phosphatidylcholine; Chol, cholesterol; DOPE, dioleylphosphatidylethanolamine; PS, phosphatidylserine; DC-Chol, dimethylaminoethanecarbamol-cholesterol; PEG-PE, polyethylene glycol phosphatididyl ethanolamine.

Table 3
Primer sets and expected amplification products used in the RT-PCR assay.

Gene	Product Size	Sense primer	Antisense primer
mβactin	450-bp	GTATGCCTCGGTCGTACCA	CTTCTGCATCCTGTCAGCAA
mIFNα	92-bp	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG
mCD40	91-bp	GTCATCTGTGGTTTAAAGTCCCG	AGAGAAACACCCCGAAAATGG
mIL18	384-bp	GATCAAAGTGCCAGTGAACC	ACAAACCCTCCCCACCTAAC

72 °C for 5 min, for IFNα; initial denaturation at 94 °C for 2 min, 94 °C for 30s, 64.3 °C for 30s, 72 °C for 1 min, 40 cycles and final extension at 72 °C for 10 min. Primers used in the RT-PCR assay is presented in Table 3.

2.8. Cell viability assay

1×10^4 to 2.5×10^4 spleen cells were stimulated by 0.1 μM of free CpG ODN, liposomes alone and CpG ODN encapsulating liposomes in 96-well plates. After 48 h of incubation the assay was performed according to the protocol of Cell Counting Kit-8 (Dojindo-Japan).

2.9. Cell surface marker staining and analysis by FACS

For FACS analysis, original cell stock (2×10^6 cells) was transferred to 15 ml falcon tube. Total volume was completed to 1 ml with specific ODNs in 500 μl 5% FBS supplemented oligo medium. Final oligo concentration was 1 μM, unless otherwise stated. Incubation periods were 6–72 h for FACS analysis depending on the marker to be examined. Falcon tubes were left in tilted position with loosened caps to allow airflow during the incubation period in CO_2 incubator.

Cells were centrifuged at 1500 rpm for 7 min at the end of the incubation period. Supernatant was sucked. The protocol was slightly modified from earlier studies [15,17]. Briefly, pellet was disturbed by using a pin rack holder. If cells were to be stained and analyzed later, cells were fixed in 50 μl fixation medium (Caltag, Austria) and transferred to 1.5 ml eppendorf tubes. Cells were incubated in dark at room temperature for 15 min 1 ml PBS-BSA-Na azide was added into each tube to wash cells. Cells were spun at 2000 rpm for 5 min. Supernatant was discarded and the washing step was repeated. At the end of the second washing step, PBS-BSA-Na azide was discarded and cells were incubated in fresh 50 μl PBS-BSA-Na azide containing 3 μl of FITC-associated monoclonal antibody against CD86 (BD Pharmingen). If cells were to be stained and analyzed immediately, fixation was not required. The cells can be stained in 50 μl PBS-BSA-Na azide containing 2–6 μl of fluorochrome-associated cell surface marker. The remaining steps are similar but all steps were performed on ice if cells were not fixed. Cells were washed twice, resuspended in 500 μl PBS-BSA-Na azide, transferred to FACS tubes and analyzed in FACS Calibur (BD, USA).

2.10. Immunization protocol with specific ODNs and OVA

Adult male C57/BL6 mice (5/group) were injected intraperitoneally (ip) with 15 μg of D35, D35 encapsulated anionic liposomes or control ODN and 7.5 μg of OVA. Fourteen days later, booster injection was performed ip with the same ODN and OVA formulations. Animals were tail bled one day before each injection. Blood was incubated (to obtain mouse sera) at 37 °C for 1.5 h, the clot was discarded and then the remaining part was spun at 13200 rpm for 1 min. The serum was collected and stored at −20 °C for further use. Animals were sacrificed on day twenty-eight and their spleens were removed. Half of the spleen was used to obtain single-cell splenocyte suspension and were incubated to compare IFNγ secretion following *in vitro* 7.5 μg of OVA stimulation.

Table 4
The CpG ODN encapsulation efficiency (i.e. loading), and shelf-life stability of various liposome formulations.

Type	K-Type ODN Loading (%)[a]	D-Type ODN Loading (%)	Percent ODN Release [b] (%)	
			1mo	3mo
Neutral	67.4 ± 5.6	74.9 ± 3.3	4.9 ± 3.3	13.3 ± 3.7
Anionic	71.5 ± 4.2	89.5 ± 4.6	9.5 ± 4.6	21.5 ± 7.9
Cationic	93.1 ± 6.7	84.1 ± 2.4	3.1 ± 2.4	7.3 ± 4.7
Stealth	78.9 ± 2.0	76.7 ± 3.8	6.9 ± 3.8	14.9 ± 3.6
SSCL	85.4 ± 10.2	86.7 ± 3.5	5.4 ± 3.5	9.0 ± 3.7

All ODN measurements were done using 1555 (K-Type) and D35 (D-Type) CpG ODNs. Triplicate runs from at least three independent liposome preparations were recorded. Data represents Ave ± SEM.

[a] OD260 nm from supernatants were used to calculate loading efficiency following liposome centrifugation.

[b] Liposome shelf-life stability following 1 or 3 months of storage @ 4 °C was measured after liposome pellet was separated by centrifugation. The ODN content was calculated from supernatants by OD readings.

2.11. Statistical analysis

Statistical analysis was performed in SigmaSTAT 3.5 software. Student's *t*-test was used to evaluate the statistical differences between untreated groups, (or control-ODNs treated groups) with ODN-treated groups.

3. Results

3.1. Stability and release properties of nanoliposomes

Initial optimization studies aimed to establish the ODN loading efficiencies as well as *in vitro* release behavior and estimation of shelf-life stability when the formulations are kept at 4 °C following reconstitution. As seen in Table 4, all liposome formulations gave over 60% of ODN encapsulation upon reconstitution. Consistent with previous findings [23], the highest ODN loading was with SSCL liposome (Mean ± SEM: 85.4 ± 11.2%) and the lowest was with Neutral liposome (67.4 ± 5.6%, and Table 4). Interestingly a significant difference between the loading efficiencies of K-ODN was observed when cationic lipids were included in the liposome preparation. For K-ODN the *in vitro* release (at 37 °C with mild shaking) into PBS was followed for eight days (Fig. 1). Anionic liposome released 40% of its cargo whereas, SSCL type of liposome released only 14% at the end of 8 d period (Fig. 1). The shelf-life stability (at 4 °C) was followed for three months. It was observed that nearly all formulations had substantially good shelf-life. While Anionic liposome retained nearly 80% of its cargo at 4 °C, Cationic liposome retained over 92% of its cargo (Mean ± SEM: 21.5 ± 7.9 vs 7.3 ± 4.7) (Table 4). Collectively, these results implicate that current liposome formulations are an efficient depot system to store and deliver ODNs. The developed entrapment method of ODN within liposomes is mild and suitable for labile molecule encapsulations. To establish that liposome encapsulating ODNs retained their activity *in vitro* stimulation assays were conducted.

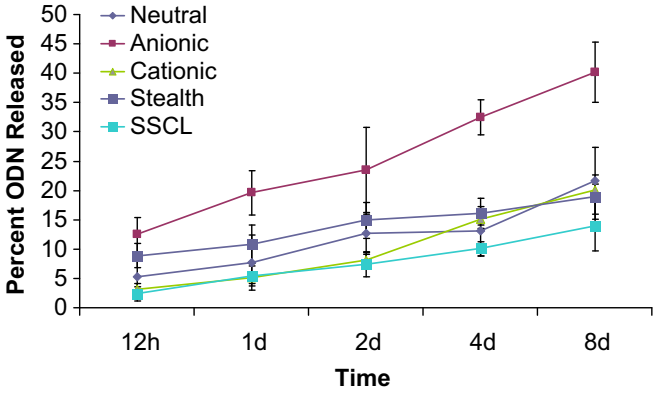

Fig. 1. Time dependent cumulative ODN release from different liposome formulations. Release of K-ODN @ 37 °C into PBS (while mild shaking) was followed by OD readings from supernatants after obtaining the liposome pellet.

3.2. In vitro activities of liposomal formulations

To compare the activity of K-ODN (1555, or K23) and D-ODN (D35, and D35-3CG) alone or when they are encapsulated into five different liposome types, spleen cells were stimulated with various doses of free or liposome encapsulating CpG ODNs. IFNγ and IL6 production were checked from cell supernatants by ELISA. While free 1555 ODN is active at 0.3 μM concentration (it yielded 35.6 ± 11.3 ng/ml, IL6 and 628 ± 18 pg/ml, IFNγ) when used with cationic and SSCL liposomes, it induced significantly high IFNγ than the free form (approximately 35 ± 5 and 25 ± 4 fold more respectively, Fig. 2a, $p > 0.001$). This augmented activity is solely dependent on the encapsulated CpG ODN, since neither the free liposome itself nor the liposomes encapsulating the control ODNs were active (Fig. 2d). IL6 response is also high for 1555 encapsulating SSCL liposome (3 fold more IL6 compared to free ODN, $p > 0.01$). However, in neutral, stealth or anionic liposomes 1555 CpG ODN activity was lost (Fig. 2a).

Alternative design of K-ODN, K23, was also used for stimulation. Of note the most distinguishable features of K23 from 1555 are, K23 is shorter (12mer vs 15mer) and it contains two CpG motifs in its sequence, whereas 1555 is 15-mer and has only one CpG motif. To

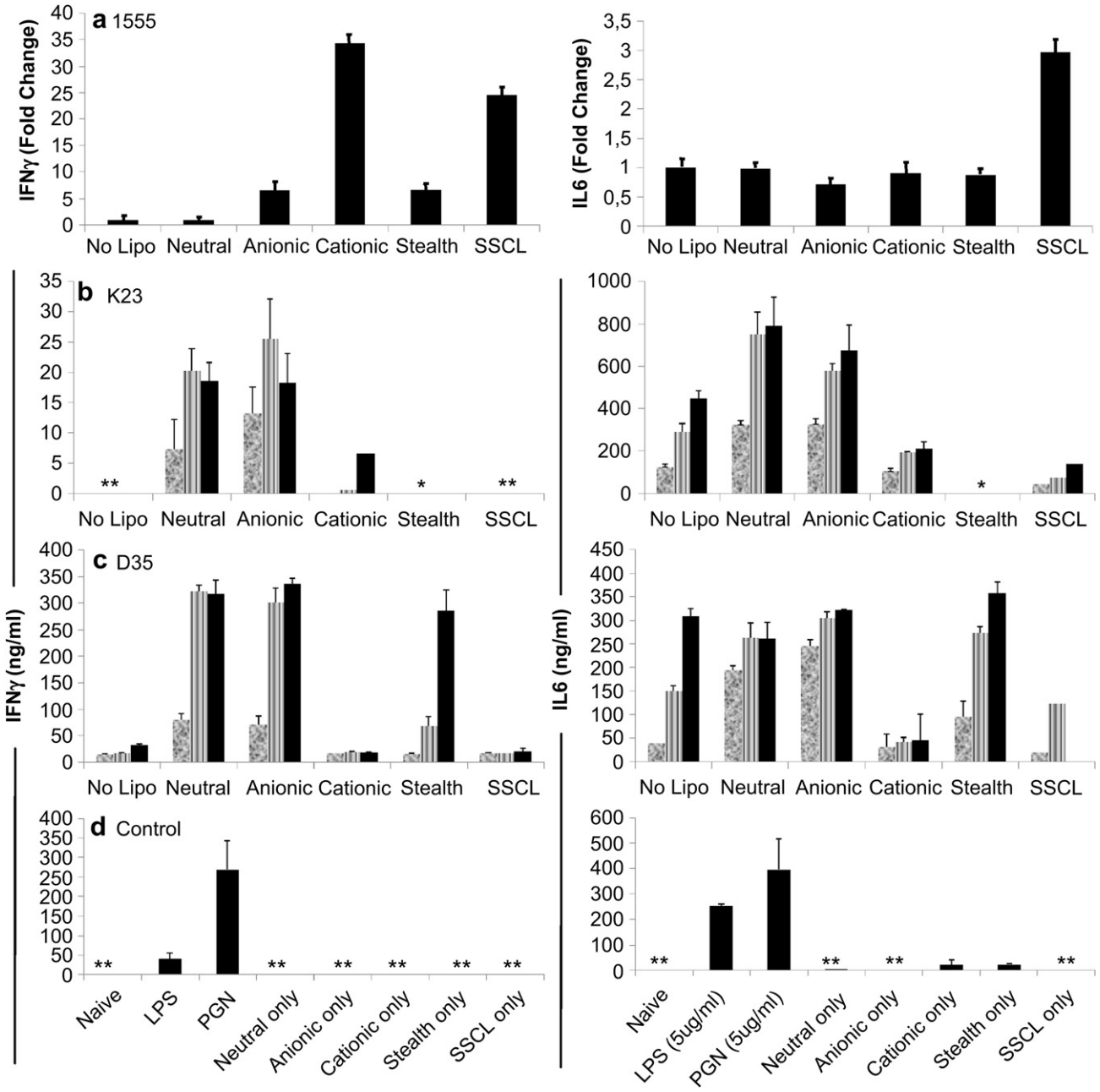

Fig. 2. IFNγ and IL6 production by splenocytes following stimulation with CpG ODN 1555. Splenocytes was stimulated with 0.3 μM of 1555 either in solution or within neutral, anionic, cationic, stealth or SSCL liposomes. Fold change of IFNγ and IL6 levels in cell supernatants was calculated according to stimulation with No Lipo (i.e. only 1555; stimulation index was based on 1555 mediated induction levels) for IFNγ, 628 ± 18 pg/ml, and for IL6, 35.6 ± 11.3 ng/ml. IFNγ and IL6), (a). Splenocytes were stimulated with 0.3 μM ▨, 1 μM ◧ and 3 μM ■ of K23 (b) and D35 (c) either free (No Lipo) or within neutral, anionic, cationic, stealth or SSCL liposomes. (d) Lipopolysacharide (LPS) (5 μg/ml), peptidoglycan (PGN) (5 μg/ml) and various liposomes without CpG ODNs were used as positive and negative controls. Cell supernatants were collected at 36 h of incubation IFNγ and IL6 levels were detected by ELISA. Data are representative of five independent experiments run in triplicates. * not determined, **; not detectable.

our surprise, following stimulation with K23-liposome formulations, the cytokine production levels (IFNγ and IL6) from spleen cells, resembled more to D-Type ODN-liposome stimulations rather than 1555-liposome formulation activities. At the lowest dose of K23 (0.3 μM) neutral and anionic liposomes gave 3 fold more IL6 (Fig. 2b). Although free K23 could not stimulate any detectable IFNγ secretion at that concentration, K23 ODN in neutral or anionic liposomes induced approximately 5 and 10 fold more IFNγ secretion ($p > 0.01$), respectively. Considering the lowest stimulation dose, while 1555 induces the strongest IFNγ and IL6 secretion when they are encapsulated into SSCL liposome, K23 (Fig. 2b) and D35 (Fig. 2c) yielded robust cytokine production either with anionic or neutral liposomes but not with SSCL. As the dose of ODNs increased, the IFNγ secretion increased correspondingly (Fig. 2b and c). Stimulation with D-ODN encapsulating various liposomes yielded cytokine responses unparallel to ODN1555 type. IFNγ and IL6 production from mouse splenocytes were significantly increased when D-ODN was encapsulated in neutral, anionic or stealth liposomes but not in cationic or SSCL liposomes (Fig. 2c). These results strongly support the view of differential immune activation by different liposomes. To determine whether loss of IFNγ is due to a shift in the cytokine milieu towards a Th2-biased immune response, IL4 levels in cell supernatants were also checked, (IL4 suppresses IFNγ production [24]). IL4 was not detected in any of liposome/ODN-treated cell supernatants (data not shown).

3.3. Cytokine expression by liposomal formulations

We have speculated that positively charged liposomes could alter subcellular location of D-ODN and thus prevent signaling to initiate from early endosome through MyD88 and IRF7. If this is the case, therefore, IFNα transcript levels should be lower upon stimulation with cationic and SSCL liposomes compared to neutral, anionic or stealth liposomes (liposome types lacking positively charged lipid). Total RNA of spleen cells were purified 1h or 8h after stimulations, and transcript levels of IFNα, in addition to IL18 and CD40 was checked by RT-PCR. As shown in Fig. 3, positively charged liposomes (Cationic and SSCL liposomes) encapsulating D35 (or D-3CG ODN) gave either similar or lower levels of mRNA transcripts for the tested cytokines and surface marker molecule CD40 to that of free D35. The transcript levels of spleen cells stimulated with neutral, anionic and stealth liposomes encapsulating D35 surpassed the performance of free or control D-ODN encapsulating liposomes (Fig. 3). As expected, CpG ODN 1555 (or K23 ODN) did not induce production of IFNα message. These results strongly suggested that when positively charged liposomes are internalized by immune cells they possibly either interfere or modify the fate of D-ODN subcellular localization and alter IFNα secretion effect. Neutral or negatively charged liposome formulations improved TLR9/D-ODN interaction and induced more pronounced IFNα transcipt. Of note, no significant difference was detected either in CD40 or in IL18 gene message levels following free or liposomal CpG ODN stimulations (i.e. 1 or 8 h).

3.4. Liposomal CpG ODN mediated proliferation

Cell viability and proliferation assays were performed to reveal the cytotoxic or proliferative effect of stimulation with free CpG ODN, and their liposome encapsulated forms on spleen cells. There was no adverse effect on cell viability when the liposome encapsulating ODN ligands were used to treat the spleen cells (data not shown). Table 5 summarizes proliferative index upon treatment with different liposome formulations. As expected all ODN sequences in their free forms induced very strong cell proliferation. Neutral and stealth liposomes were neither significantly contributing nor inhibiting the proliferative potential of the ligands. Cationic, anionic and SSCL liposome types, however, reduced the degree of ligand dependent cell division.

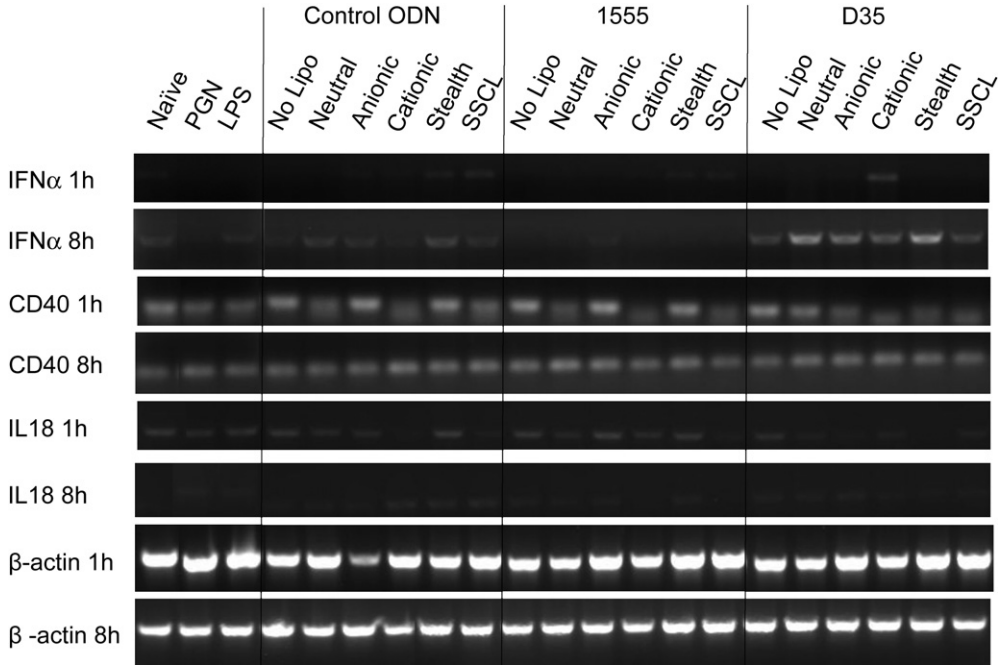

Fig. 3. IFNα, CD40 and IL18 transcript levels of spleen cells upon K-ODN and D-ODN stimulation. 10×10^6 spleen cells were stimulated with 1 μM control ODN (1612), K-type and D-type ODN alone (No Lipo) or with various combinations of liposomes (neutral, anionic, cationic, stealth, SSCL). After 1 h and 8 h of incubation, total RNA of splenocytes was isolated and RT-PCR was performed.

Table 5
Cell proliferation following treatment either with free or liposome encapsulating TLR ligands.

Types	No Ligand	Control ODN	1555	D-35
No Lipo	1.00 ± 0.09	1.38 ± 0.12	8.38 ± 1.47**	5.77 ± 0.65**
Neutral Lipo	1.55 ± 0.42	2.35 ± 0.92	5.55 ± 1.39**	4.30 ± 0.74*
Anionic Lipo	0.92 ± 0.34	1.56 ± 0.22	2.16 ± 0.82	3.81 ± 0.53*
Cationic Lipo	1.12 ± 0.45	1.65 ± 0.50	1.15 ± 0.10	1.89 ± 0.52
Stealth Lipo	1.34 ± 0.23	2.08 ± 0.54	3.88 ± 1.14*	2.48 ± 0.64*
SSCL Lipo	0.89 ± 0.44	1.29 ± 0.24	1.69 ± 0.44	1.21 ± 0.31

Stimulation index (SI) values are given. SI is calculated as the fold proliferation over untreated cells. Triplicate wells (Ave ± SEM) of two independent experiments are reported, * $0.05 < p$, ** $0.01 < p$ compared to untreated (no ligand) group.

3.5. APC activation by liposomal D-ODN

A naive T cell requires a second signal to proliferate and differentiate into effector T cells following engagement with an antigen. This second signal is known as co-stimulation. Co-stimulation is mediated by co-stimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86) expressed on mouse antigen presenting cells (APCs) including DCs, macrophages and B cells. The CD86 is expressed constitutively at low levels on APCs and its upregulation is initiated upon recognition either by a PAMP (i.e. through TLR triggering) or exposure to inflammatory cytokines such as IFNγ, IL12 or IL6. Of note, the CD80 upregulation requires longer exposure and may take days [25,26]. We investigated the activation ability (by anti-CD86 staining) of APCs by liposomes encapsulating only D-Type ODN ligand. This is due to the fact that, so far our findings indicated that the most promising vaccine adjuvant candidate is D-ODN encapsulated in negatively charged liposome. For this reason, these studies were only conducted with D-ODN formulations. Upon *in vitro* stimulation with five different liposome formulations, CD86 staining by FACS revealed that previous IL6 and IFNγ findings are in accordance with the co-stimulatory molecule upregulation (Fig. 4A and B). Similar to previous observations, the most potent formulation was anionic liposome encapsulating D-ODN (12.7 ± 1.2% CD86 positive DC population). The least effective treatment was again cationic and SSCL liposome types. Compared to free D-ODN, more than 4 fold CD86 upregulation was obtained with anionic liposomal D-ODN treatment.

The utmost aim of this study was to design an effective vaccine carrier co-encapsulating adjuvant and antigen of interest in a single depot package. As presented earlier, negatively charged liposomes encapsulating D-ODN's breadth of immune activity is more pronounced than other studied ODNs. Although *in vitro* studies are important, it does not warrant reproducible *in vivo* performance.

To reveal the *in vivo* activity of various liposome-D-ODN formulations, mice were ip injected and 4h post-injection, spleen cells were removed and *ex-vivo* cultured for an additional of 24h (no further external stimulation at this stage) and then stained with anti-CD86-FITC antibody to detect CD86 + cells. Knowing that free oligo is less stable than liposomal counterpart and also the internalization efficiencies of the free vs liposomal formulations vary at great extend [23,27,28], we intentionally injected 2.5 fold more of the free oligo (50 μg) during ip injections as opposed to 20 μg liposomal D-ODN. As presented in Fig. 4, compared to untreated animals, all tested free or liposomal D-ODN formulations induced significantly higher CD86 upregulation from DC. Among D35 group, similar what was seen during *in vitro* assays, anionic-D35 gave the most profound upregulation, (>3 fold more induction compared to free D-ODN group) CD86 upregulation was also stronger either

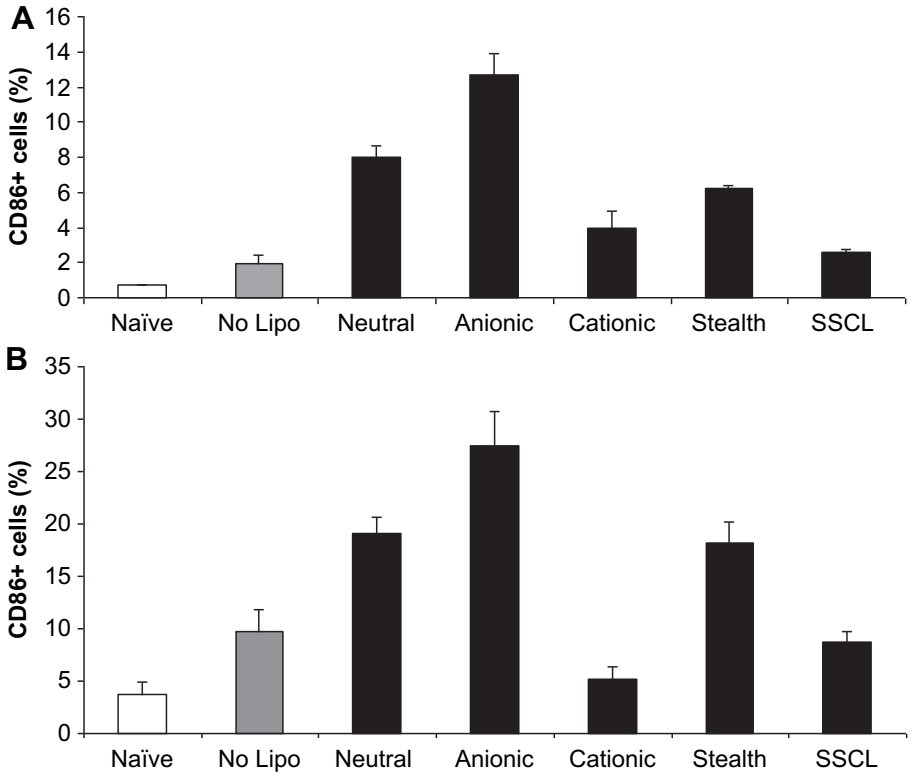

Fig. 4. FACS analyses of CD86 positive DC populations following (a) *in vitro* stimulation and (b) *in vivo* stimulation. (A) 1×10^7 splenocytes were stimulated with various liposome encapsulating or free 1 μM D35 and the percentage of CD86 expressing cells were determined by FACS. (B) Mice were immunized with 50 μg of free D35 or various liposome encapsulated D35. 4 h later mice were sacrificed and their spleens were extracted. 2×10^6 splenocytes were incubated for 24 h and then percentage of CD86 expressing cells were determined by FACS.

with neutral or stealth D-ODN encapsulating liposomes (19.1 ± 1.5 and 18.5 ± 2.0 respectively).

3.6. In vivo immunization studies

Following *in-vitro* and *ex-vivo* analyses, we next tested these D-ODN-liposome formulations in immunization studies. For this, a model protein antigen (Ovalbumin) was either mixed with free D-ODN or co-encapsulated form in anionic liposomes were prepared. Initial experiments revealed that encapsulating CpG and mixing with free Ag, or encapsulating Ova in liposomes and mixing with free D-ODN prior to injections had no significant contribution to anti-Ova specific Ab titers (data not shown). Furthermore, two different populations of liposomes (i.e. liposomal D-ODN and Ova loaded liposomes) and mixing just before immunization also did not significantly contribute anti-Ova Ab titers (data not shown).

As presented in Table 6, naive mice were divided into 5 different treatment groups. Before primary injection background un-immunized mouse sera was obtained. The next day (at $d = 0$) mice were i.p immunized with the indicated groups. On $d = 13$, all animals were tail bled and sera was saved to study primary anti-OVA Ig responses by ELISA. The next day, booster injections were performed. On $d = 28$ mice were first bled and then sacrificed, and spleens were frozen down for PCR study. The collected mouse primary and secondary sera were subjected to total IgG, anti-OVA IgG1, IgG2a and IgG2b ELISA following serial titrations (Table 6). It is clearly demonstrated that present vaccine formulation (Gr#5), that is, ovalbumin and D-ODN as the adjuvant, co-encapsulated in a single liposome vesicle (anionic liposomes) induced >150 fold more IgG over free Ag + CpG mixed group (i.e. Gr# 3), and 14 fold more from Ag + Control ODN (Gr#4). In vaccine trials, in addition to total IgG which is an indication of the generated humoral immunity, it is very important to mount cell mediated immune response, in which one can predict by analyzing the level of IgG2a titers. As seen in (Table 6a), over 30 fold more IgG2a from anionic liposomes co-encapsulating Ova and D-ODN (i.e. Gr#5) was detected compared to Gr#3 (428 ± 50 vs 14 ± 7, respectively).

Booster injection is the cardinal phase of any vaccination protocol, since it induces both effector T and memory B cells against injected antigen. As seen in Table 6b, a very potent anti-Ova humoral immunity is initiated at the end of 4 weeks (Gr#5, Table 6b). A very strong improvement in total IgG (that corresponded to >22 fold more IgG) was achieved when liposomal formulation is used instead of its free formulation (Gr#5 and Gr#3 titers are 28670 are 1300, respectively). Moreover, IgG1 anti-OVA titers were also significantly higher with liposomal formulation (more than 26 fold rose Gr#5 vs Gr#3). Of note, IgG2a level with Gr#5 was >13 fold more compared to Gr#3 animals. To further investigate the ability of the liposome formulation to mount cell mediated immune response, IFNγ production of the immunized animals were analyzed from the recovered spleen cell supernatants following *in vitro* Ova stimulation in culture for 36 h (Fig. 5). Data revealed that over 7 fold more IFNγ secretion was detected from Gr#5 animals to that of Gr#3 immunized mice. These findings collectively demonstrated that delivery of D-ODN and Ova antigen encapsulated in anionic liposome induced very strong humoral and cell mediated immunity than free D-ODN plus Ova formulation (Table 6 and Fig. 5).

4. Discussion

This work investigated the immune activity of liposomes harboring different classes of CpG ODNs, an important immune adjuvant intended for clinical use. In the present study, the shelf life of the generated liposomes showed quite promising stability and retention profiles up to three months (Table 4), duration much longer than any conventional vaccination scheme. Moreover, we demonstrated that K- and D-type ODN mediated cytokine secretion is differentially controlled by the liposome surface charge (Fig. 2). This data is in agreement with previously reported finding [23]. Interestingly, alternative design of K-ODN, K23, behaved differently in liposomes optimal for 1555 sequence (Fig. 2b). The vesicle lamellarity or the vesicle fluidity of the liposome formulations does not seem to influence the resultant immune activation induced by CpG ODNs, since the generated vesicles are kept at fixed molar ratio for all preparations and following liposome reconstitution their sizes were reduced to less than 150 nm via extrusion.

We have revealed that neutral, stealth and anionic liposomes encapsulating D-type ODNs boost IFNγ secretion compared to free form (Fig. 2c). Therefore we can conclude that neutral or anionic liposomes encapsulating D-ODN can promote substantial amount of cytokine secretion even at very low doses. Depot effect and prevention of pre-mature clearance in addition to sparing ODN from nuclease attack are important features provided by liposome encapsulation.

We have suspected that positively charged liposomes prevent ODN to be sequestered in early endosome where initiation of D-ODN mediated signaling cascade is required [15–18] and is

Table 6
Primary and secondary anti-Ova IgG subclass responses of mice immunized with free or liposome encapsulating Ag and CpG ODN.

A. Primary IgG Levels

Gr.#	Treatment Groups	IgG	IgG1	IgG2a	IgG2b
1	Untreated	7 ± 4	7 ± 4	7 ± 4	7 ± 4
2	Control ODN + OVA	7 ± 4	7 ± 4	7 ± 2	7 ± 1
3	D35 + OVA	28 ± 14	14 ± 7	14 ± 7	7 ± 2
4	Anionic Lipo(Control ODN + OVA)	300 ± 150	35 ± 10	7 ± 2	7 ± 1
5	Anionic Lipo(D35 + OVA)	4250 ± 1000*	112 ± 12*	428 ± 50	7 ± 1

B. Secondary IgG Levels.

Gr.#	Treatment Groups	IgG	IgG1	IgG2a	IgG2b
1	Untreated	7 ± 1	7 ± 2	7 ± 2	7 ± 4
2	Control ODN + OVA	7 ± 2	7 ± 4	7 ± 2	7 ± 1
3	D35 + OVA	1300 ± 450	828 ± 245	112 ± 12	112 ± 12
4	Anionic Lipo(Control ODN + OVA)	5168 ± 2590*	4355 ± 1430*	448 ± 20*	112 ± 20
5	Anionic Lipo(D35 + OVA)	28672 ± 5050[a]**	21672 ± 7150[a]**	1500 ± 250[a]*	28 ± 4

Female Balb/c mice (6–8 wks old, 5/group) was immunized with OVA (5 μg/ml) plus control or CpG ODN (15 μg/ml) (free or liposome encapsulated forms) at day 1 and bleed on $d = 13$ before booster injection. Two weeks post-booster injection mice were first tail bled and then sacrificed. Sera was titrated and assayed for anti-OVA IgG subclasses and reported as 1/T for primary and secondary antibody response. Results (mean titer ± SD) are combination of two independent experiments, *0.01 < p (represent the statistiacal significance of Gr#3 vs Gr#4 or Gr#5 paired *t*-test analysis comparison), ** 0.0001 < p (Gr#3 vs Gr#4 or Gr#5 paired *t*-test analysis comparison).

[a] 0.001 < p (Gr#4 vs Gr#5 paired *t*-test analysis comparison).

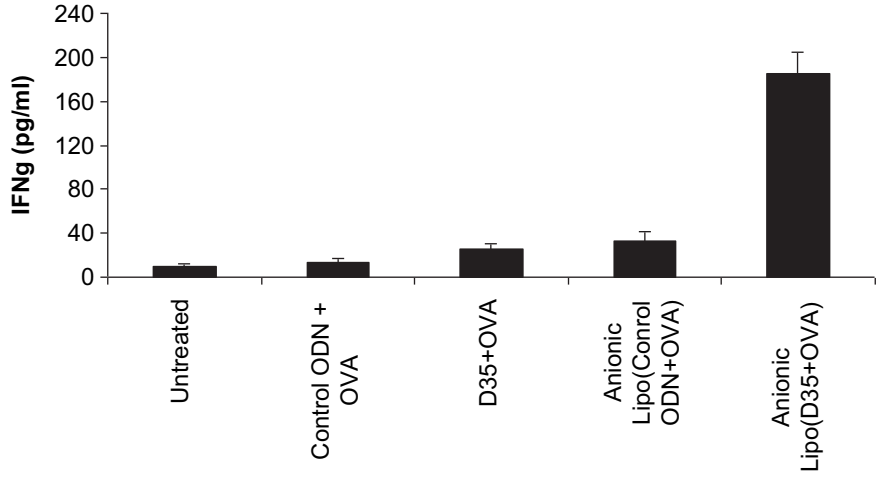

Fig. 5. Female Balb/c mice (6–8 wks old, 5/group) was immunized with OVA (5 μg/ml) plus control or CpG ODN (15 μg/ml) (free or liposome encapsulated forms), two weeks post-booster injection mice were sacrificed, spleen cells (5×10^6/ml) from each animal were recovered and cultured in the presence of 7.5 μg OVA for 36h. Supernatants were collected and IFNγ ELISA was studied to detect recall antigen response. Results are reported as IFNγ (pg/ml) (mean ± SD) for each group. Paired *t*-test analysis comparison between Gr#3 and Gr#5 is $P > 0.001$.

necessary for the production of IFNα transcript. We observed that D35 encapsulating cationic and SSCL liposomes failed to induce IFNα message as much as free D35 (following 8 h of treatment). However, neutral, anionic and stealth liposomes induced significantly high IFNα transcript (Fig. 3). Therefore, this data implied that positively charged liposomes somehow interfere with D-ODN/TLR9 interaction in the early endosome.

CD40 is a receptor expressed on macrophages and important in activation of infected macrophages with intracellular pathogens by CD4 T cells expressing CD40 ligand. We demonstrated that free D35 also induces CD40 expression (Fig. 3). Therefore we can say that K-ODN and D-ODN encapsulation into any of the liposomes do not augment its expression compared to free form, however, positively charged liposomes decreased the amount of CD40 transcript. IL18 is considered as IFNγ-inducing factor. Together with IL12, IL18 induces high amount of IFNγ expression [29]. It is an important cytokine in innate [30,31] and adaptive [32] immune regulation. Except the free form, liposomal forms of D35 do not induce IL18 expression. We can conclude that IFNγ induction by either liposomal D-ODN or K-ODN is not maintained by increased levels of IL18 production.

We observed that upon control ODN encapsulating liposome treatment considerable levels of IFNα, CD40 and IL18 was induced by immune cells. These stimulations are TLR independent since the control ODN does not contain any active CpG motif. However, it is of great importance to evaluate the activity of control ODN that is internalized into phagocytic cells within liposomes and whether it initiates a signaling pathways through recognition by cytosolic DNA sensors such as DAI [33] or AIM2 [34] and leads to these transcript upregulations.

In vaccine induced immunity an important step is to strongly activate T cells and for this co-stimulatory molecule upregulation on APCs is critical [35–37]. In this study, unexpectedly, anionic liposome encapsulating D-ODN consistently upregulated CD86 both upon *in vitro* and *in vivo* stimulation (Fig. 4). This effect was very encouraging since raising cell mediated immunity is as important as mounting a humoral response for the success of any immunization.

Immunization with liposome encapsulating antigen and CpG ODN compared to their free forms gave significantly high amount of IgG titers in mice. This heightened anti-ova response is provided by simultaneous signals delivered by the formulation leading to efficient naive T cell activation. In a depot vesicle formulation both the antigen and CpG adjuvant are together and liposomes indeed mimics a natural course of a pathogenic insult.

In the literature, Joseph et al. demonstrated that when mice were vaccinated once with liposomes (DMPC:DMPG mole ratio of 9:1) co-encapsulating 25 μg CpG ODN and and 0.5 μg influenza virus subunit hemagglutinin and neuraminidase (HN) antigen, no detectable IgG1 titer and 550 IgG2a titer was observed at 21 days post-injection [38]. We have injected mice with anionic liposome co-encapsulating 7.5 μg ova and 15 μg D-ODN. Only 13 days later we have obtained 122 IgG1 titer and 428 IgG2a titer. At the end of 4 weeks these titers were much higher than reported amounts by Joseph *et al.* Although amount and type of CpG ODN as well as antigen types and amounts are different, one can speculate that the immune response initiated by D-ODN encapsulating anionic liposome formulation is more immunogenic yielding stronger Th1-biased immunity against tested ovalbumin.

We previously demonstrated that when mice immunized with SSCL co-encapsulating 10 μg 1555 and 2 μg OVA. Following booster injection, total IgG titer reached 19000, the present study showed that a 1.5 fold more IgG (a titer of 28672) with a different type of CpG ODN and liposome type could be attained. Collectively, the present *in vivo* experiments demonstrated that contrary to the established dogma that D-type ODN is not that immunogenic than K-type, our findings suggests that when encapsulated within a proper liposome, D-ODN can induce antigen specific immunity.

5. Conclusion

This study established that different liposomes induced differential innate immune activation otherwise not possible to obtain with two different classes of TLR9 ligands (i.e. K- and D-types). The information from this work demonstrated an unappreciated feature of liposome-mediated immune modulation and could be harnessed to design more effective therapeutic vaccines targeting several infectious diseases, cancer and asthma or allergy.

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Appendix

Figures with essential color discrimination. Fig. 1 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.10.054.

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