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The effect of circulation lifetime and drug-to-lipid ratio of intravenously administered lipid nanoparticles on the biodistribution and immunostimulatory activity of encapsulated CpG-ODN

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Abstract

The encapsulation of conventional drugs in lipid nanoparticles (LNs) has been extensively utilized to enhance therapeutic activity by altering their pharmacokinetic (PK) and biodistribution (BD) properties. We have previously shown that the immunostimulatory activity of unmethylated cytidine-guanosine (CpG)-containing immunostimulatory oligodeoxynucleotides (ODN) is greatly enhanced when encapsulated in an LN (LN CpG-ODN). Here, we investigate the effect of circulation lifetime (determined by lipid composition) and drug-to-lipid (D/L) ratio of intravenously (i.v.) administered LN CpG-ODN on PK, BD, and cellular uptake and correlate these parameters with the immunostimulatory activity. Results from these studies show that despite significant differences in the circulation lifetime and the D/L ratio, the immune response is similar with respect to immune cell activation and cytolytic activity in the spleen and the blood compartments. Our findings indicate that the benefits of liposomal nanoparticles for the delivery of immunomodulatory drugs such as CpG-ODN are defined by a different paradigm than that for conventional drugs.

Keywords: *adjuvant, biodistribution, CpG-oligodeoxynucleotide, immunostimulatory, liposomal nanoparticle, pharmacokinetics*

1. Introduction

Over the last several decades, lipid nanoparticles (LNs) have been extensively utilized as delivery vehicles for systemically administered drugs, representing one of the most advanced drug delivery technologies to date with five liposomal drugs currently approved in the United States and numerous others in the late-stage clinical development. Traditionally, liposomes have been used as vehicles to alter the pharmacokinetic (PK) and biodistribution (BD) properties of systemically administered drugs such as conventional chemotherapeutics and antibiotics, with the aim of extending their circulation lifetimes and allowing for accumulation at the sites of disease. In support of this, a considerable body of data exists to demonstrate that the efficacy of i.v. administered LN-encapsulated drugs is enhanced with increased circulation lifetimes (Gregoriadis

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1995; Allen and Cullis 2004). This relationship is based on the fact that pathological conditions such as inflammation and solid tumors display increased vascular permeability, which provides the opportunity for nanoparticles to extravasate and accumulate at these sites of disease (Hashizume et al., 2000; Maeda et al., 2000). A second characteristic that significantly influences the efficacy of LN-encapsulated drugs is their D/L ratio. While intuitively it is assumed that LN with higher D/L ratios will allow for greater drug delivery and accumulation at the sites of disease, optimized D/L ratio has also demonstrated the potential to exert additional effects. Specifically, the D/L ratio has been reported to directly impact the rate of drug release from liposomes, a characteristic that can have a profound effect on drug activity (Johnston et al. 2006). With this in mind, the development of encapsulated drugs has traditionally included the design of LNs with extended circulation lifetimes and D/L ratios targeted toward maximal delivery and optimal timing of drug release.

Consistent with these characteristics, the LN system described in this work was initially developed for the optimized delivery of therapeutic antisense oligodeoxynucleotides (ODNs), protecting them from nuclease degradation and enhancing their delivery to disease sites following systemic administration (Klimuk et al. 2000; Semple et al. 2000). Recently, however, we have demonstrated that the immune responses to CpG-containing ODNs are greatly enhanced when encapsulated in LN (Mui et al. 2001; de Jong et al. 2007; Wilson et al. 2007). Bacterial DNA and synthetic ODN containing unmethylated CpG motifs are known to activate a wide array of immune effector cells through interaction with Toll-like receptor 9 (TLR9), which is localized in the endosomal compartment of antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages/monocytes, and B cells (Hemmi et al. 2000; Takeshita et al. 2001). The enhanced activity of LN CpG-ODN is evidenced by elevated plasma cytokines (interleukin-12, interferon-γ, interleukin-6, and macrophage chemoattractant protein-1), more potent humoral and cellular immune responses, as well as improved antitumor efficacy in animal models of cancer following intravenous or subcutaneous administration (Mui, Raney et al. 2001; de Jong, Chikh et al. 2007; Wilson, Raney et al. 2007).

It is clearly evident that encapsulation within an LN has the potential to enhance the activity of both 'conventional' (i.e. chemotherapeutics and antibiotics) and immunostimulatory (CpG-ODN) drugs. However, the rationale for the encapsulation of immunomodulatory drugs may be distinctly different than for other therapeutic agents. Optimal characteristics of liposomal delivery vehicles for conventional small-molecule drugs, such as reticular endothelial system (RES) evasion, long circulation lifetime, disease site accumulation, and high D/L ratio, are not necessarily ideal for CpG-ODN as their primary targets are the very cells (i.e. macrophages and DCs) that conventional liposomes are designed to avoid. Indeed, it is proposed that the enhanced activity of LN CpG-ODN is likely due, in part, to the tendency of LN delivery systems to naturally target to and be taken up by APCs of the RES, particularly in the liver and spleen (Juliano 1986). In addition, since unlike traditional chemotherapeutics CpG-ODN act through a receptor-mediated process (Hemmi, Takeuchi et al. 2000), it is likely that their activity is subject to a threshold/saturation effect. Lastly, due to their different physical and chemical nature, it is highly likely that the release of CpG-ODN from LN is controlled by factors that differ from those guiding the release of conventional drugs.

As immunotherapy assumes a greater role in therapeutic intervention for various diseases and lipid nanoparticles become increasingly utilized in applications of immune stimulation (Gregoriadis 1994; Chen and Huang 2005), it is vital that we characterize and understand the fundamental paradigms that influence the action and, ultimately, the effectiveness of LN for immunotherapeutics. The studies described here elucidate fundamental aspects of this paradigm, and serve to inform the design of LN, particularly for the delivery of CpG-ODN. Our data indicate that as with conventional drugs, LN delivery can enhance the activity of immunostimulatory drugs. However, unlike conventional drugs, the increased activity is not dependent on increased circulation lifetime or D/L ratio.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), and cholesterol from Sigma (St Louis, MO, USA). 1,2-Dioleyloxy-3-*N*,*N*-dimethylaminopropane (DODMA) and polyethylene glycol-dimyristol glycerol (PEG-DMG) were provided by Tekmira Pharmaceuticals Corporation (Burnaby, BC, Canada). INX-6295 and INX-6303, 16-mer phosphodiester, or phosphorothioate ODNs (5′-TAACG-TTGAGGGGCAT-3′) containing an unmethylated or methylated cytosine residue in the CpG motifs, respectively, were synthesized by Trilink Biotechnologies (San Diego, CA, USA). The specific ODN backbone chemistry was consistent within each study. INX-6303, which demonstrates equivalent or enhanced immunomodulatory activity compared with the unmethylated form (INX-6295) when encapsulated in an LN (unpublished data), was used in PK, BD, immune cell activation, and cytotoxicity

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studies. To characterize the PK and BD properties, INX-6303 ODN was spiked with INX-6295 containing two internal tritiated thymidine residues, while for cell uptake studies a 5′-fluorescein isothiocyanate-labeled INX-6295 phosphorothioate ODN was used.

2.2. Animals

Female, 6- to 8-week-old ICR, C3H, and C57BL/6, mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and quarantined for 3 weeks prior to use. All procedures involving animals were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

2.3. Preparation of LN CpG-ODN

ODN were encapsulated in LN containing an ionizable aminolipid using an ethanol dialysis procedure, as described previously by Maurer et al. (2001). Briefly, lipid mixtures consisting of POPC or DSPC/cholesterol/DODMA/PEG-DMG (molar ratio 25:45:20:10) were solubilized in ethanol to give a final ethanol concentration of 36%, passed twice through stacked 200 + 100 nm polycarbonate membranes (Whatman Nuclepore, Clifton, NJ, USA) using a thermobarrel extruder (Northern Lipids, Vancouver, BC, Canada), and then mixed with 50 mM citrate buffer containing either 1.67 or 3.33 mg/mL of ODN. The vesicles were dialyzed against citrate followed by Hepes-buffered saline and unencapsulated ODN removed on DEAE–Sepharose CL-6B columns. ODN and lipid concentrations were determined by UV spectroscopy (260 nm) and an inorganic phosphorus assay after separation of the lipids from the ODN by a Bligh and Dyer extraction (Bligh and Dyer 1959), respectively. The ODN/lipid ratio was typically 0.05 or 0.1 (w/w). Particle size, as determined by quasi-elastic light scattering using a NICOMP submicron particle sizer (model 370; Santa Barbara, CA, USA), was 100 ± 25 nm in diameter.

2.4. Pharmacokinetic and biodistribution studies

ICR mice were injected i.v. at an ODN dose of 20 mg/kg via the lateral tail vein, with free or encapsulated INX-6303. ODN were labeled by spiking with [^{3}H]-labeled INX-6295 (Trilink Biotechnologies) while lipids were labeled with [^{3}H] or [^{14}C]cholesteryl hexadecylether to allow a per mouse dosing of approximately 3 μCi of [^{3}H]ODN and 1 μCi of [^{3}H] or [^{14}C]lipid. Mice were killed by a terminal dose of 3.2% (v/v) ketamine/0.8% (v/v) xylazine 0.5, 1, 2, 4, 6, 8, 16, or 24 h following administration (3 mice per time-point). Blood was collected in Vacutainer (BD Biosciences, Canada) tubes containing EDTA, while the lymph nodes (bilateral, axial, and inguinal), the liver, and spleen were harvested and chemically digested at room temperature using Solvable (Perkin-Elmer, Wellesley, MA, USA) followed by decolorization with hydrogen peroxide (30% w/w). Blood and tissue digests were analyzed by liquid scintillation counting in Pico-Fluor 40 (Perkin-Elmer).

2.5. Immune cell isolation

For immunological assays, mice were killed as described previously. Blood was collected via cardiac puncture into Vacutainer (BD Biosciences) tubes containing EDTA and viable peripheral blood mononuclear cells (PBMCs) were isolated using lympholyte (Cedarlane Laboratories, Canada). Spleens and lymph nodes were harvested and dissociated to single cells by passage through a sterile 100 μm nylon mesh (BD Biosciences) and red blood cells were lysed (0.1 M ammonium acetate, 10 mM potassium bicarbonate, and 70 μM EDTA). The cells were stained with fluorescently labeled antibodies (BD Biosciences) and analyzed on a two-laser, four-color FACSort or FACScalibur flow cytometers (BD Biosciences, San Jose, CA, USA). Dead cells were excluded with propidium iodide and viable cells gated based on forward and side scatter characteristics. Data were acquired and analyzed using CELLQuest Pro software V 4.0.1 (BD Biosciences).

2.6. Uptake studies

Six- to eight-week-old C57BL/6 mice were injected i.v. with 10 mg/kg fluorescein isothiocyanate-labeled INX-6295 ODN-encapsulated within lipid particles. Mice were killed 0.5, 4.0, and 7.0 h after administration and the lymph nodes and the spleen were isolated. Cell suspensions were prepared, stained with antibodies against cell phenotype markers (CD11c for DCs, CD11b, and Mac3 for macrophages, B220/CD45R for B cells, CD4 for CD4 T cells; BD Biosciences) and analyzed by flow cytometry.

2.7. Activation and cytotoxicity assays

C3H mice were injected i.v. with free or encapsulated ODN at a 20 mg/kg dose. As described, mice were euthanized 24, 48, and 72 h following administration. In activation studies, splenocytes and PBMCs were stained with fluorescently labeled antibodies against cell phenotype (CD11c for DCs, DX5 for natural killer cells, CD11b and Mac-3 for macrophages, CD8 and CD4 for T cells, CD45R/B220 for B cells) and cell activation (CD69 and CD86) markers, and analyzed using by flow cytometry. Natural killer (NK) cell killing was assessed in a standard 4-h ^{51}chromium release assay using YAC-1 cells as targets. Splenocytes or PBMC were co-cultured with [^{51}chromium]-labeled target cells at effector-to-target (E/T) cell ratios of 1:1, 5:1, 25:1, and 100:1, and 1:1, 5:1,

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20:1, and 50:1, respectively, for 4 h at 37°C, 5% CO_2, and the amount of ^{51}chromium released to the supernatants was quantitated as a measure of cell killing. Percentage of cytotoxicity was calculated using the equation: [sample counts per minute (cpm) − spontaneous cpm]/(maximum cpm) × 100. Maximum counts were determined using 20% Triton X-100, while spontaneous counts were measured in culture medium. Antibody-dependent cell-mediated cytotoxicity (ADCC) was assessed in a manner similar to assessing lysis of Daudi cells in the presence/absence of Rituxan (100 μg/10^6 cells at a concentration of 10 μg/ml), a monoclonal antibody directed against the CD20 antigen present on the target cells.

3. Results

3.1. LN CpG-ODN clearance from the circulation following intravenous administration is influenced by lipid composition but not D/L ratio

In these studies, LN CpG-ODN formulations were altered in lipid composition (POPC vs. DSPC), or D/L ratio, and the rate of clearance from the circulation following i.v. administration was characterized on the basis of the length of time required for 50% of the initial ODN equivalent dose ([^{3}H]ODN-derived radioactivity representing both intact and metabolized forms) to be removed from the blood. As expected, the POPC-based formulation was cleared more rapidly (Oja et al. 1996; Semple, Klimuk et al. 2000), with half of the injected ODN equivalent dose being cleared within approximately 30 min (Figure 1A). The identical DSPC-based formulation had a 500% longer residence time in the blood, with half of the injected ODN equivalent dose being cleared from the circulation within approximately 2.5 h (Figure 1B). By 4 h, less than 10% of the POPC-based formulation remained in the circulation (Figure 1A) compared with approximately 40% of the DSPC-based formulation (Figure 1B) based on ODN equivalents. By tracking both ODN and lipid equivalents, it was observed that the initial D/L (ODN/lipid) ratio remained essentially constant during the first 4 h for the DSPC-based formulation, indicating that the carrier system remained intact while the D/L ratio for the POPC-based formulation declined to half its initial level (Figure 1A and B), demonstrating a more rapid dissociation and degradation of the ODN payload.

The influence of D/L ratio on the PK profile of the particle was evaluated by comparing LN CpG-ODN with the D/L ratios of 0.05 or 0.10 formulated with DSPC, to ensure that the initial D/L ratios would remain constant during the course of the experiment. No observable impact on the clearance profile of LN CpG-ODN as a function of D/L ratio was observed (Figure 1C).

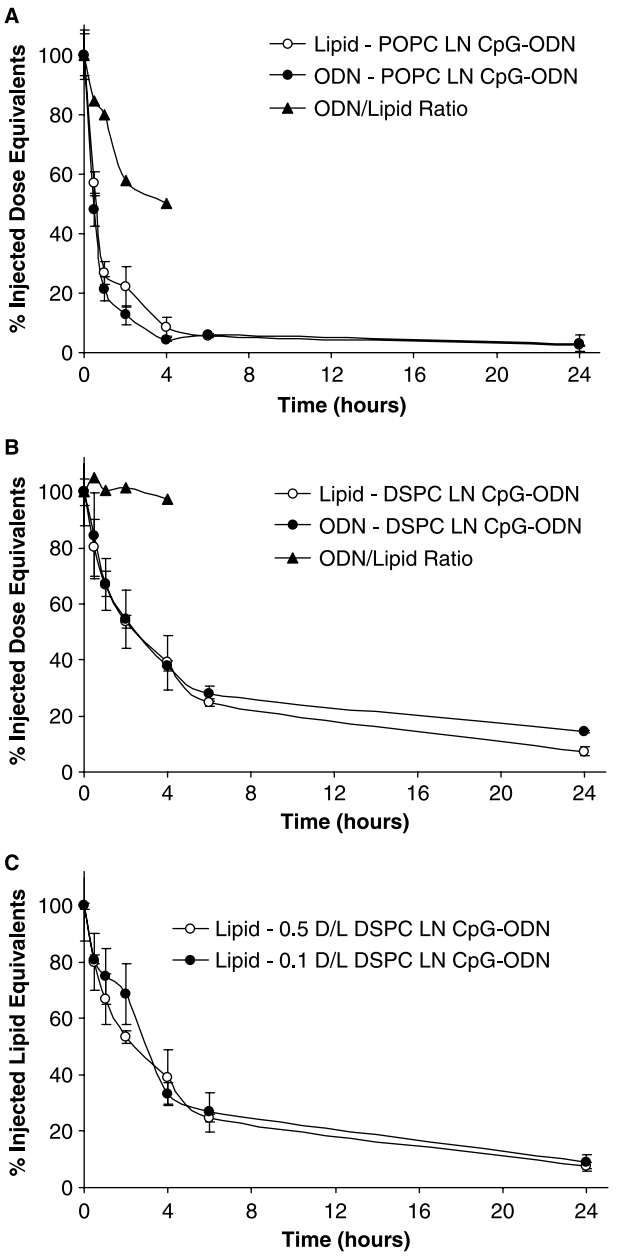

Figure 1. Clearance of LN CpG-ODN from the circulation following systemic administration is influenced by lipid composition but not D/L ratio. LN formulations composed of (A) POPC or (B) DSPC encapsulating CpG-ODN at an ODN/lipid ratio of 0.10 (wt/wt) were administered intravenously to ICR mice at an ODN dose of 20 mg/kg. Formulations were radiolabeled with [^{3}H]ODN, [^{3}H]CHE, or [^{14}C]CHE lipid to monitor clearance of ODN and lipid equivalents, respectively, from the blood and track the ODN/lipid ratio of the particle. (C) Clearance of DSPC-based LN CpG-ODN from the circulation following administration of a 20 mg/kg dose at an ODN/lipid ratio of 0.05 or 0.10 (wt/wt). Each data point represents $n = 3 \pm$ SD.

3.2. Rate of clearance but not D/L ratio influences the biodistribution profiles of LN CpG-ODN following intravenous administration

Biodistribution to the liver, spleen, and lymph nodes (axial and inguinal pooled) was profiled following i.v. administration of LN CpG-ODN formulations

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(Figure 2). Specifically, DSPC- or POPC-based formulations with a D/L ratio of 0.05 (wt/wt) were compared to evaluate the effect of lipid composition (and resultant circulation lifetime) on biodistribution, while DSPC-based formulations with an ODN/lipid ratio of 0.05 or 0.1 (wt/wt) were compared to evaluate the effect of D/L ratio on biodistribution. Consistent with previously reported data (Semple, Klimuk et al. 2000; Wilson, Raney et al. 2007), the majority of the injected ODN equivalent dose was detected in the liver following i.v. administration, while progressively less accumulation was observed in the spleen and lymph nodes. Biodistribution analysis showed maximal liver accumulation levels of ODN dose equivalents within 1–2 h following administration (45% for POPC compared with only 17% for the DSPC-based formulation), after which ODN equivalent levels in the liver rapidly declined, presumably due to ODN degradation.

Maximal accumulation in the spleen (Figure 2B), representing approximately 1–2% of the injected ODN dose for all formulations regardless of lipid composition or D/L ratio, occurred within 2–4 h of administration. Consistent with previous observations, the level of accumulation for all formulations declined over-time. Similar biodistribution profiles were also observed in lymph nodes (Figure 2C), although the levels were >10-fold less on a per organ basis compared with spleen.

In regard to D/L ratio, the pattern of ODN equivalent accumulation in the liver, spleen, and lymph node were similar for both the 0.05 and 0.10 D/L formulations; however, the level of ODN equivalent accumulation was found to be consistently lower for the 0.1 D/L formulation in all tissues examined, with the exception of the lymph nodes (Figure 2C).

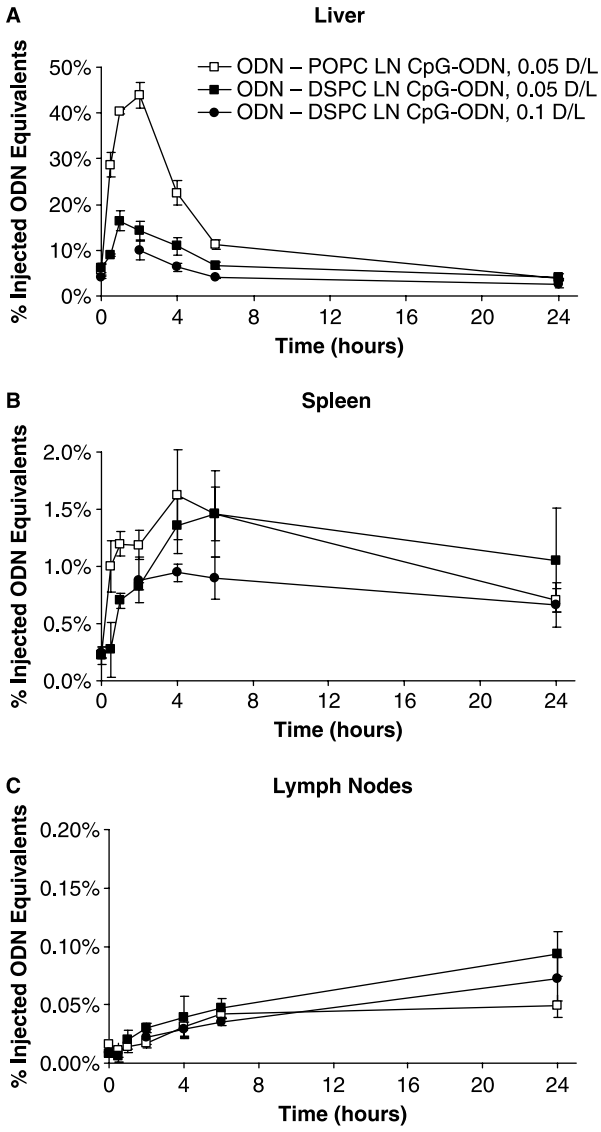

Figure 2. Biodistribution of LN CpG-ODN to the liver, spleen, and lymph nodes following systemic administration is influenced by lipid composition but not D/L ratio. LN CpG-ODN formulations composed of POPC or DSPC encapsulating CpG-ODN at an ODN/lipid ratio of 0.05 or 0.10 (wt/wt) were administered intravenously to ICR mice at an ODN dose of 20 mg/kg. Biodistribution to the (A) liver, (B) spleen, and (C) lymph nodes were monitored by tracking [^{3}H]ODN equivalents. Each data point represents $n = 3 \pm$ SD.

3.3. Long- and short-circulating LN CpG-ODN are accumulated by immune cells in the spleen and lymph nodes following intravenous administration

Consistent with previous findings (Wilson, Raney et al. 2007), i.v. administered LN CpG-ODN were found to be effectively taken up by immune effector cells, predominantly phagocytic APCs (i.e. macrophages and DCs), in both the spleen and the lymph node compartments following i.v. administration (Figure 3). As expected, the relative rates of uptake were impacted by lipid composition with the more rapidly clearing POPC-based LN CpG-ODN having a higher initial rate of uptake by splenic macrophages and DCs (Figure 3). Although the longer circulating DSPC-based formulation showed a lower initial rate of uptake by splenic APCs, by 4-h uptake of these LN by macrophages and DCs invariably exceeded that seen with POPC-based nanoparticles, reaching maximum levels 4–7 h following administration. While a relatively large proportion of B cells were found to take up LN CpG-ODN (up to 30 and 40% for DSPC- and POPC-based formulations, respectively), the uptake was dramatically reduced (5- to 8-fold) on a per cell basis, as determined by mean fluorescence intensity (MFI), compared with other APC populations. Similar LN CpG-ODN uptake patterns were observed both in terms of the total percentage of cells positive for uptake (Figure 3B) and the amount taken up on a per cell basis (Figure 3A). Similar uptake profiles were also observed in the lymph nodes (Figure 3C).

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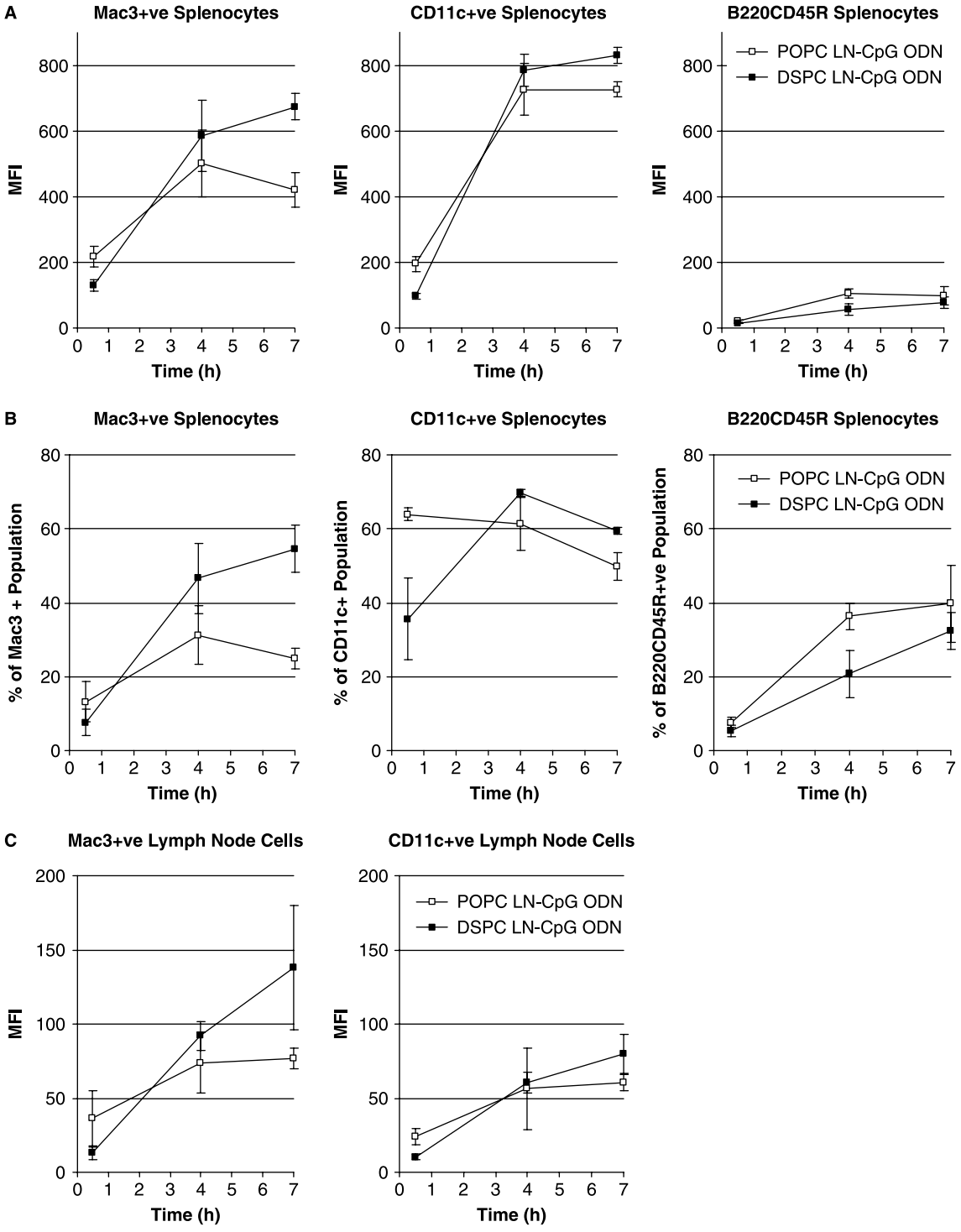

Figure 3. Long- and short-circulating LN CpG-ODN formulations are accumulated by antigen-presenting cells in the spleen and lymph nodes following intravenous administration. LN CpG-ODN formulations composed of POPC or DSPC encapsulating fluorescently labeled CpG-ODN at a D/L ratio of 0.10 (wt/wt) were administered intravenously to C57BL/6 mice at an ODN dose of 10 mg/kg. The uptake of POPC and DSPC-based LN CpG-ODN by Mac3 + (macrophages), CD11c + (DCs), and CD45R/B220 + (B cells) cells in the (A and B) spleen and (C) lymph node compartments are represented based on mean fluorescence intensity (A and C) and the percent positive for uptake (B).

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3.4. Intravenously administered LN CpG-ODN formulations induce similar levels of immune cell activation regardless of differences in the circulation lifetime or D/L ratio

Immune cell activation was assessed following i.v. administration of LN CpG-ODN by evaluating up-regulation of the early activation marker CD69 on splenic and peripheral blood immune cell populations. As shown in Figure 4, the activation of macrophages, DCs, and NK cells in the spleen (Figure 4A) and the PBMC (Figure 4B) compartments was essentially identical to POPC- and DSPC-based formulations; however in PBMC-derived macrophages, DSPC-based LN CpG-ODN did not appear to induce $CD69^+$ activation above control levels. In all other respects, both POPC- and DSPC-based LN CpG-ODN induced an increase in the percentage of $CD69^+$ NK cells, macrophages, and DCs relative to controls, with the greatest activation being observed 24 h following administration.

Likewise, the D/L ratio of the formulation did not appear to influence the activation of splenic NK cells that exhibited similar activation following the administration of the 0.05 (at both 10 and 20 mg/kg) and 0.10 (at 20 mg/kg) D/L ratio formulations (Figure 4C). While both formulations were also effective in activating PBMC-derived NK cells, the 0.05 D/L formulation delivered at 20 mg/kg appeared to provide maximal CD69 up-regulation compared with both a 10 mg/kg dose and the 0.10 D/L ratio formulation delivered at 20 mg/kg.

3.5. Intravenously administered LN CpG-ODN formulations induce similar levels of direct and antibody-dependent cytolytic activity regardless of differences in the circulation lifetime or D/L ratio

Immune cell activation was also assessed following i.v. administration of LN CpG-ODN by measuring NK cytolytic activity *ex vivo* against the NK-sensitive cell line Yac-1, and enhancement of ADCC using the B-cell lymphoma cell line Daudi in combination with the anti-CD20 monoclonal antibody Rituxan (Figures 5 and 6). Despite substantial differences in the circulation lifetimes of POPC- or DSPC-based LN CpG-ODN formulations, the observed cytolytic activity against the NK-sensitive target YAC-1 was similar for both formulations. Maximal cytolytic activity representing 15–20% lysis, when compared with 5% lysis for free CpG-ODN controls was observed in splenocytes 24 h following the i.v. administration of both formulations (Figure 5). With respect to PBMCs, maximal cytolytic activity of 60–70% when compared with only 30–40% for free CpG-ODN controls was observed 24–48 h following administration, and had also declined by 72 h. While relatively similar levels of cytolytic activity against YAC-1 targets were observed *ex vivo* following the administration of DSPC- and POPC-based formulations, splenocytes isolated from animals treated with short-circulating POPC-based LN CpG-ODN appeared to have a slight, but consistently higher, cytolytic activity (Figure 5A). The inverse was observed in PBMCs where the DSPC-based formulation demonstrated a slightly elevated cytolytic activity (Figure 5B). Consistent with that observed for direct cytolytic activity (Figure 5), both POPC- and DSPC-based formulations resulted in enhanced levels of ADCC against the B-cell lymphoma line Daudi in the presence of Rituxan (Figure 6). Also consistent with direct cytolytic activity data, the rapidly cleared POPC-based formulation maximally enhanced ADCC in the splenocytes, while longer circulating DSPC-based LN CpG-ODN maximally enhanced ADCC in PBMCs.

In regard to the influence of D/L ratio on NK cytolytic activity against YAC-1 cells, elevated activity was observed following the administration of LN CpG-ODN at both the D/L ratios investigated with maximal activity being observed at 24 and 48 h in the spleen and peripheral blood compartments (17 and 34%, respectively) when compared with control animals treated with free CpG-ODN (3 and 4%, respectively; Figure 7A and B). While both formulations enhanced cytolytic activity against YAC-1 targets, data indicate that D/L ratio does impact specific aspects the response. In splenocytes (Figure 7A), the 0.05 D/L formulation was found to induce consistently lower levels of cytolytic activity than equivalent doses of the 0.10 D/L formulation, at all time points and E/T ratios investigated. Surprisingly, however, administration of LN CpG-ODN with a D/L ratio of 0.05 at a lower ODN dose (10 mg/kg, therefore an equivalent lipid dose) was found to be more potent, inducing cytolytic activity comparable to that observed for the 0.10 D/L ratio and superior to that observed for the 0.05 D/L formulation at a 20 mg/kg ODN dose. By contrast, for PBMCs (Figure 7B), the 0.05 D/L ratio LN CpG-ODN was most potent when administered at 20 mg/kg, inducing greater cytolytic activity than either 0.05 or 0.10 D/L ratio LN CpG-ODN administered at 10 and 20 mg/kg, respectively.

In correlation with direct cytolytic activity data (Figure 7), administration of LN CpG- ODN at the D/L ratios of 0.05 and 0.10 both induced enhanced ADCC activity (Figure 8), when compare with that observed for free CpG-ODN controls. The effect of D/L ratio on ADCC was consistent with that described for direct cytolytic activity. Splenocyte-mediated ADCC was found to be strongly induced by a 10 mg/kg dose of LN CpG-ODN at the 0.05 D/L ratio, resulting in cytoxicity levels similar and superior to those following treatment with 20 mg/kg of 0.10 and 0.05 D/L ratio LN CpG-ODN, respectively. For PBMCs, however, the 0.05 D/L ratio formulations at 20 mg/kg were found to be most potent, inducing

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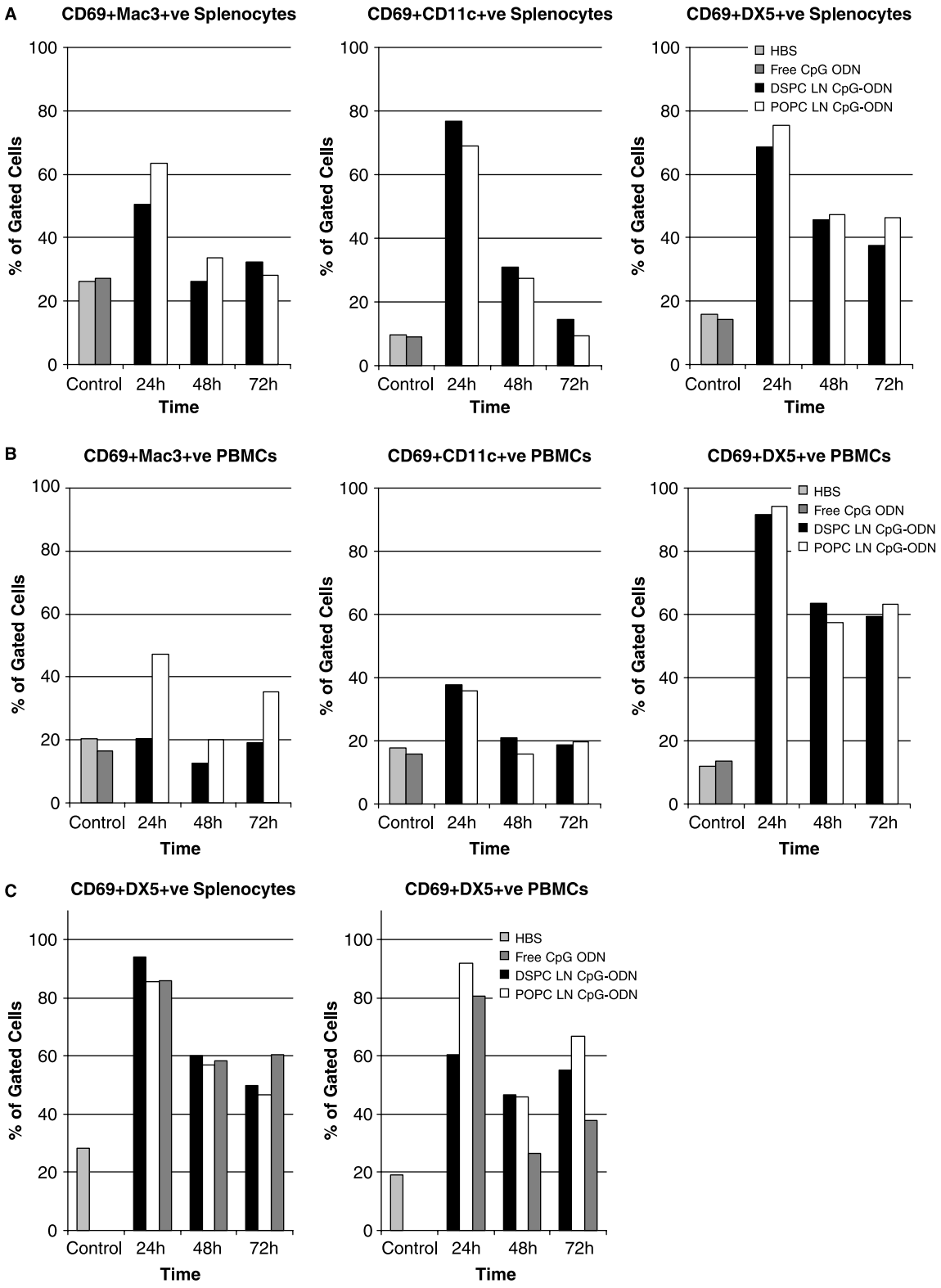

Figure 4. Intravenously administered LN CpG-ODN formulations induce similar levels of immune cell activation despite differences in circulation lifetime or D/L ratio. LN CpG-ODN formulations composed of POPC or DSPC encapsulating CpG-ODN at an ODN/lipid ratio of 0.05 (wt/wt) were administered intravenously to C3H mice at an ODN dose of 20 mg/kg, and 24, 48, and 72 h later the cells from the (A) spleens and (B) peripheral blood (B) compartments were collected. The activation status of NK cells (DX5 +), macrophages (Mac3 +), and dendritic cells (CD11c +) was assessed by flow cytometry based on the up-regulation of the activation marker CD69.(C) Activation status of immune cells following the systemic administration of DSPC-based LN CpG-ODN at an ODN/lipid ratio of 0.05 or 0.10 (wt/wt). Results are representative of at least five independent studies.

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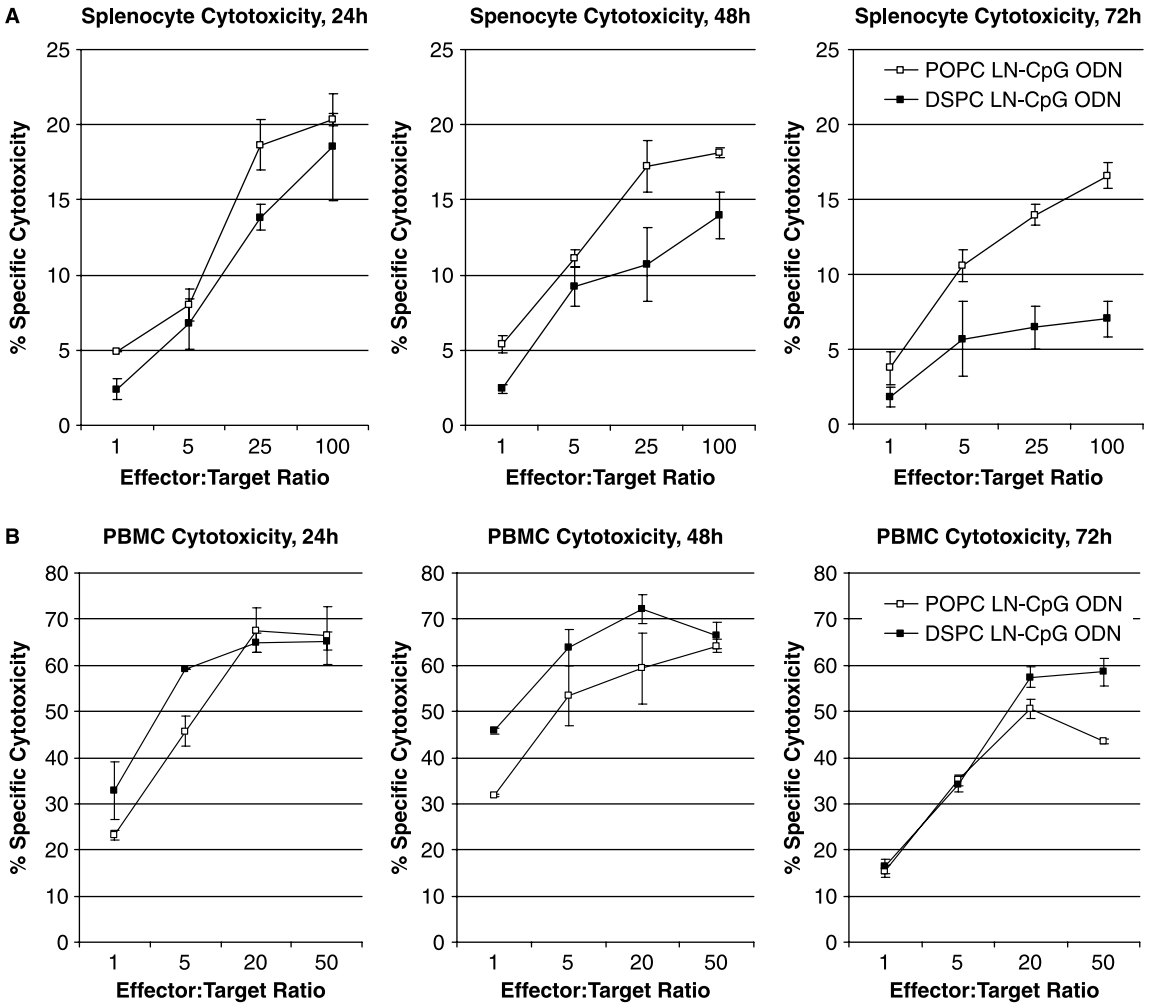

Figure 5. Splenic and peripheral blood mononuclear cells exhibit enhanced cytolytic activity of following systemic administration of long- and short-circulating LN CpG-ODN. LN CpG-ODN formulations composed of POPC or DSPC encapsulating CpG-ODN at an ODN/lipid ratio of 0.05 (wt/wt) were administered intravenously to C3H mice at a 20 mg/kg ODN dose. Cells isolated after 24, 48, and 72 h from the (A) spleens and (B) peripheral blood of treated animals were tested for cytolytic activity a standard 4-h chromium release assay against the NK-sensitive target cell line YAC-1 at effector-to-target ratios of 1, 5, 25, and 100 for splenocytes and 1, 5, 20, and 50 for PMBCs. Background cytotoxicity levels in splenocytes and PBMCs were ≤ 5 and 30%, respectively, in control animals treated with HBS and ≤ 7 and 40%, respectively, in control animals treated with free CpG-ODN. Each data point represents the findings from four mice pooled $\pm$ SD. Results are representative of at least five independent studies.

higher ADCC levels than either 0.05 or 0.10 D/L ratio LN CpG-ODN administered at 10 and 20 mg/kg, respectively.

4. Discussion

Over the past several decades, substantial attention has been focused on modifying the design of nanoparticulate drug delivery systems to better enhance their efficacy. Typical goals include increased capacity for encapsulating the drug, and methods to prolong circulation lifetime by inhibiting clearance. The ability to encapsulate drugs to high concentrations within LN has proved to have two potential benefits: the delivery of higher concentrations of drugs to target tissues as well as a means by which to control their rate of release (Johnston, Semple et al. 2006). With regard to the benefit of extended circulation lifetime, considerable data support a correlation between circulation half-lives and improved delivery of the drug to target tissues (Jain 1987; Maeda 2001; Allen and Cullis 2004). Conventionally, clearance of nanoparticulate systems from the circulation by the immune system has been undesirable, and stearic stabilization has provided an effective means by which to hide nanoparticles from the immune system, and facilitate longer circulation half-lives (Allen et al. 1991; Harris and Chess 2003). The data presented here elucidate key distinctions to be considered in the design of nanoparticulate drug delivery systems that are intended to deliver 'conventional' drugs such as chemotherapeutic agents and antibiotics when compared with those intended for the delivery of immunostimulatory agents to cells of the immune

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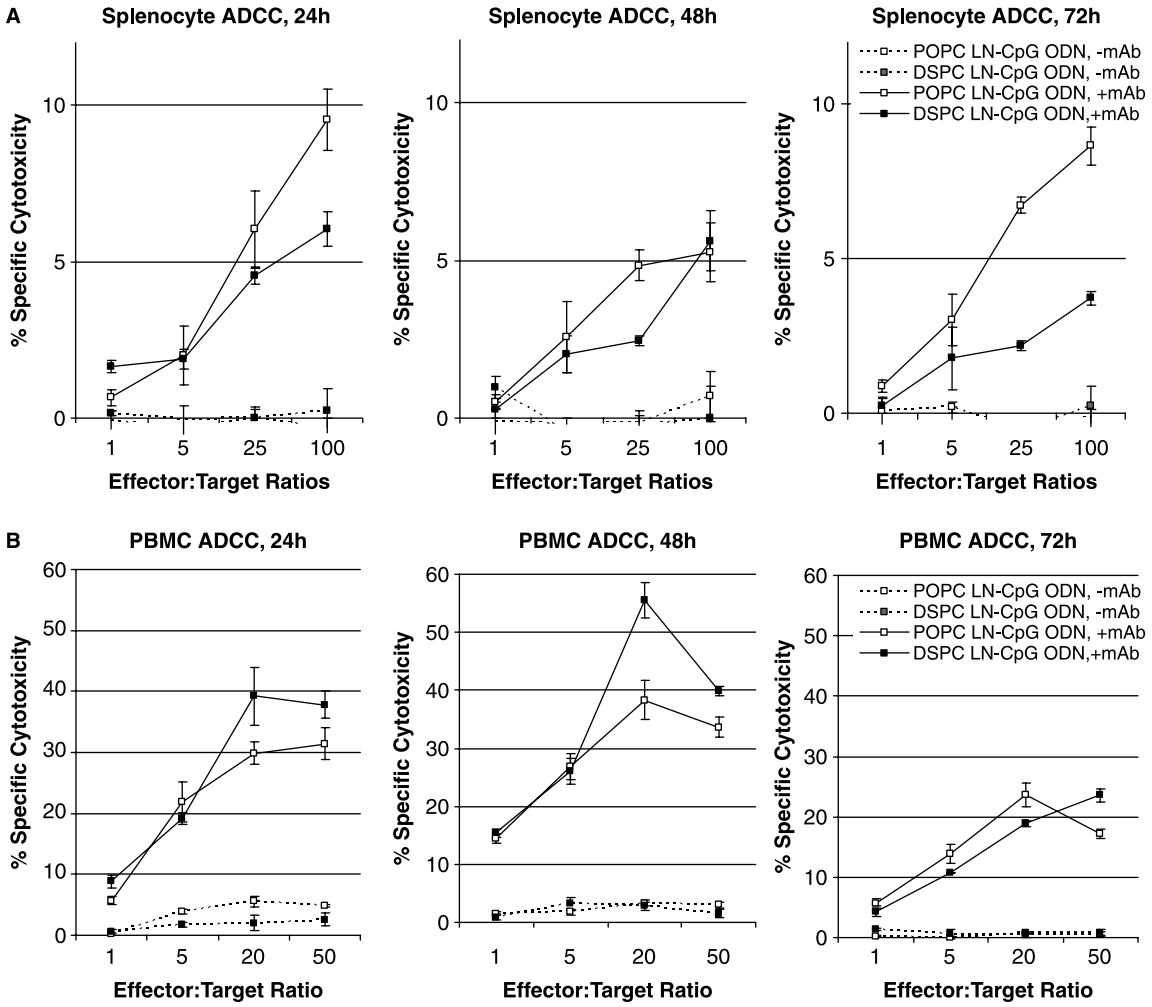

Figure 6. Splenic and peripheral blood mononuclear cells exhibit enhanced cytolytic activity following systemic administration of LN CpG-ODN at ODN/lipid ratios of 0.05 and 0.1 (wt/wt). LN CpG-ODN formulations composed of DSPC encapsulating CpG-ODN at an ODN/lipid ratio of 0.05 or 0.10 (wt/wt) were administered intravenously to C3H mice at an ODN dose of 10 or 20 mg/kg. Cells isolated after 24, 48, and 72 h from the (A) spleens and (B) peripheral blood of treated animals were tested for cytolytic activity a standard 4-h chromium release assay against the NK-sensitive target cell line YAC-1 at effector-to-target ratios of 1, 5, 25, and 100 for splenocytes and 1, 5, 20, and 50 for PMBCs. Background cytotoxicity levels in splenocytes and PBMCs were $< 4\%$ and 5%, respectively, in control animals treated with HBS. Each data point represents the findings from four mice pooled $\pm$ SD. Results are representative of at least five independent studies.

system. Our observations demonstrate that the strong correlation between long circulation lifetime and improved efficacy, which is the foundation for the improved efficacy of liposomal encapsulated conventional drugs, is not supported in the latter case. Indeed, although encapsulation within an LN dramatically enhances the potency of the immunostimulatory agent CpG-ODN, the activity of LN CpG-ODN is on a whole insensitive to alterations in the circulation lifetime or D/L ratio of the delivery vehicle.

Long-circulating LNs are specifically designed to avoid recognition and removal from the circulation by phagocytic cells of the RES, which are represented predominantly by macrophages and DCs of the liver and the spleen (Juliano 1986; Allen, Hansen et al. 1991; Alving 1991; Harris and Chess 2003). These cell types, however, represent the very immune effector population responsible for the recognition of CpG-ODN and are, therefore, the target of this immunomodulatory agent. Thus, in effect, for LN CpG-ODN, rapid clearance represents efficient targeting. As expected, longer circulating DSPC-based nanoparticles initially showed a lower level of uptake by splenic APCs; however, within 4 h of systemic administration, their uptake matched and even slightly exceeded that observed for the shorter circulating POPC-based formulation. Our observations demonstrate that with regard to uptake by APCs in disease-free animals, the circulation half-life of LN CpG-ODN does not influence the fate of the particle, but only the rate at which uptake occurs. In view of this, the relative insensitivity of the immunostimulatory activity of LN CpG-ODN to circulation lifetime in these experiments is not surprising.

Another characteristic of encapsulated drugs, which is the subject of extensive study, is the determination

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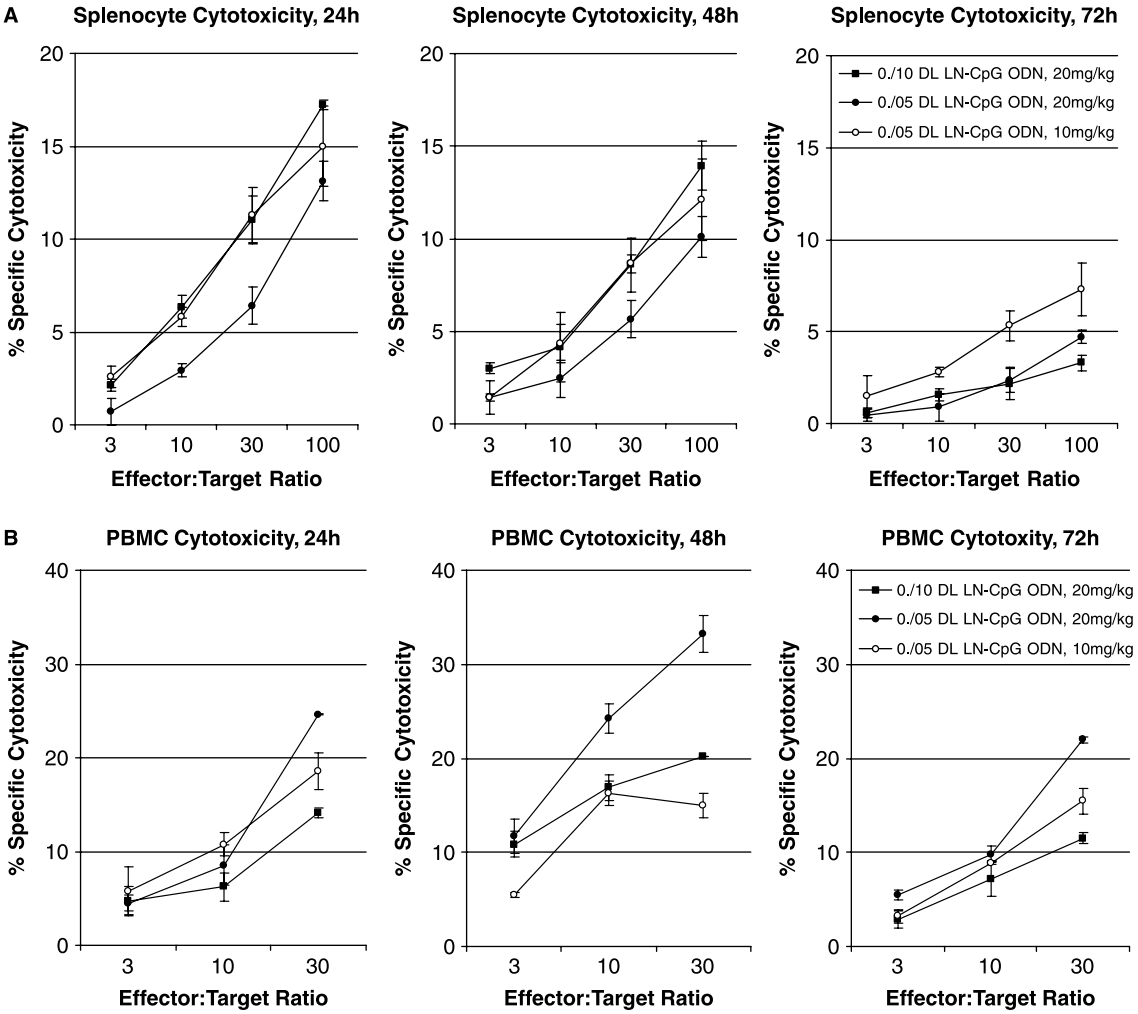

Figure 7. Splenic and peripheral blood mononuclear cells exhibit enhanced ADCC activity of following systemic administration of long- and short-circulating LN CpG-ODN. LN CpG-ODN formulations composed of POPC or DSPC encapsulating CpG-ODN at an ODN/lipid ratio of 0.05 (wt/wt) were administered intravenously to C3H mice at a 20 mg/kg ODN dose. Cells isolated after 24, 48, and 72 h from the (A) spleens and (B) peripheral blood of treated animals were tested for ADCC against the human B-cell lymphoma target cell line Daudi in the presence of the CD20-specific monoclonal antibody Rituxan using a standard 4-h chromium release assay at effector-to-target ratios of 3, 10, 30, and 100 for splenocytes and 3, 10, and 30 for PMBCs. Background ADCC against Daudi cells (in the presence of Rituxan) in splenocytes and PBMCs were ≤ 1 and 9%, respectively, in control animals treated with HBS and $\leq 3\%$ and 10%, respectively, in control animals treated with free CpG-ODN. Each data point represents the findings from four mice pooled $\pm$SD. Results are representative of at least five independent studies.

of an optimal D/L ratio for a particular payload. Traditionally, it is viewed that higher D/L ratios, by virtue of possessing a greater payload per nanoparticle, are advantageous for the delivery of drugs based on their ability to deliver an increased concentration of drug to the site of disease. More recently, it has been demonstrated that D/L ratio can also have a profound effect on the rate of drug release from LN (Johnston, Semple et al. 2006), a characteristic that can also strongly influence the therapeutic activity of conventional encapsulated drugs. As expected, rapid drug release counteracts the benefit of encapsulation as LN are unable to accumulate at disease sites prior to release, thus providing little or no benefit compared with free drug, while very slow rates of release limits the drug bioavailability and thus its ability to exert a therapeutic benefit (Cabanes et al. 1998; Meerum Terwogt et al. 2002). In our studies, it was observed that within the range tested, the D/L ratio did not have a considerable influence on the immunostimulatory activity of LN CpG-ODN, as measured by profiling expression of the activation marker CD69 on APCs and the enhancement of direct and antibody-dependent cytolytic activity.

Our finding that the activity of LN CpG-ODN is relatively insensitive to the D/L ratio of the LN can be rationalized by considering a number of factors that are unique to the target of LN CpG-ODN. In particular, immunotherapeutic drugs such as CpG-ODN differ from conventional chemotherapeutics in that they act through a saturable receptor-mediated process (i.e. via TLR9), for which once all receptors

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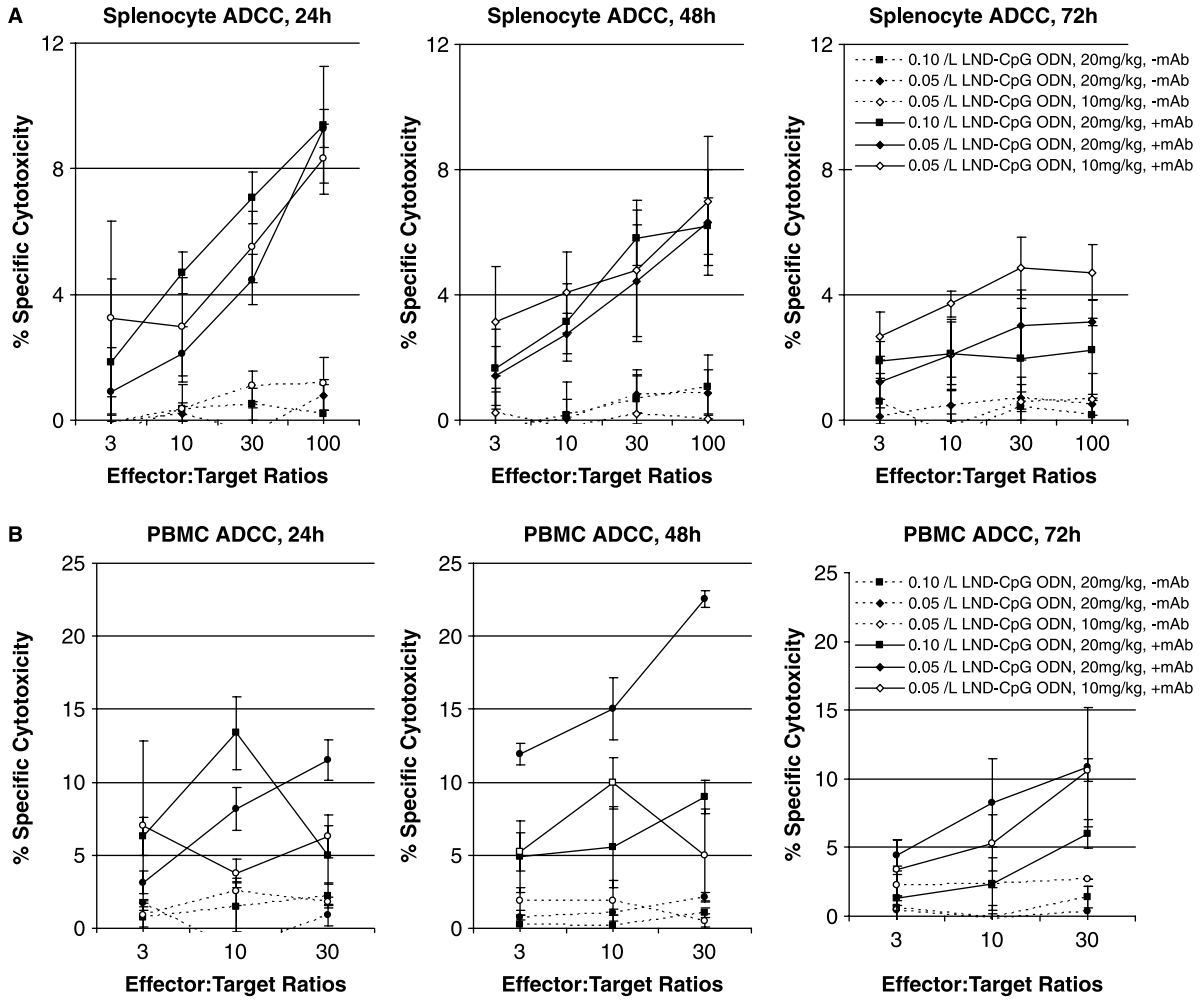

Figure 8. Splenic and peripheral blood mononuclear cells exhibit enhanced ADCC activity following systemic administration of LN CpG-ODN at ODN/lipid ratios of 0.05 and 0.1 (wt/wt). LN CpG-ODN formulations composed of DSPC encapsulating CpG-ODN at an ODN/lipid ratio of 0.05 or 0.10 (wt/wt) were administered intravenously to C3H mice at an ODN dose of 10 or 20 mg/kg. Cells isolated after 24, 48, and 72 h from the (A) spleens (A) and (B) peripheral blood of treated animals were tested for ADCC against the human B-cell lymphoma target cell line Daudi in the presence of the CD20-specific monoclonal antibody Rituxan using a standard 4-h chromium release assay at effector-to-target ratios of 3, 10, 30, and 100 for splenocytes and 3, 10, and 30 for PMBCs. Background ADCC levels against Daudi cells (in the presence of Rituxan) in splenocytes and PBMCs were <3 and 1%, respectively, in control animals treated with HBS. Each data point represents the findings from four mice pooled $\pm$ SD. Results are representative of at least five independent studies.

are engaged, the delivery of additional ligands would not be expected to elicit additional responsiveness. This is particularly relevant in view of the fact that a single LN CpG-ODN nanoparticle delivers up to 2000 CpG motifs to a single cell (Semple et al. 2001). Furthermore, the mammalian immune system is subject to numerous feedback mechanisms designed to prevent uncontrolled and/or inappropriate immune responses that could severely harm the host (Kobayashi and Flavell 2004; Lang and Mansell 2007). It is therefore understandable that for the majority of immunostimulatory agents, including CpG-ODN, a ceiling dose exists above which responses either plateau or decline. Finally, the parameters effecting CpG-ODN release from the nanoparticulate delivery systems are different, and the large hydrophilic nature of CpG-ODN would be expected to inhibit leakage from the LD, irrespective of D/L ratio. Thus, it is understandable that the advantage of high D/L ratio nanoparticles in delivering LN CpG-ODN is not as profound as for conventional chemotherapeutics.

While the circulation lifetime and D/L characteristics of LN CpG-ODN do not have a dramatic impact on the immunostimulatory activity of the particle, they do appear to have the potential to shape specific aspects of the immune response, particularly the compartment in which the maximal response occurs. Specifically, our findings demonstrate that long-circulating (DSPC-based) formulations induce greater direct and antibody-mediated cytolytic activity in the peripheral blood compartment, while shorter circulating (POPC-based) formulations preferentially enhance cytolytic activity in the spleen. Furthermore, although D/L ratio did not have a strong influence on

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the immunostimulatory activity of LN CpG-ODN, the D/L ratio was observed to impact the relative potency of the particle in regard to the cytolytic activity of splenic and peripheral blood immune cells when dosed at 10 and 20 mg/kg.

The tendency of shorter circulating LN CpG-ODN to favor the development of more potent direct and antibody-dependent cytolytic responses in the spleen is thought to stem from the more efficient clearance of these nanoparticles into the spleen where they can access resident immune cells. Along the same line, enhanced responses in the peripheral blood to longer circulating particles is proposed to stem from increased exposure to PMBCs due to a longer residence time in this compartment. The more potent cytolytic responses observed in the peripheral blood compartment in response to lower D/L ratio LN CpG-ODN formulations administered at equivalent ODN doses (i.e. higher lipid doses) may be a result of altered PK/BD behavior based on differential lipid doses (Gregoriadis 1988) as saturation of uptake into the liver can promote extended circulation lifetimes and enhance access to other APC-containing compartments.

Overall, despite dramatic differences in the circulation lifetime and tissue accumulation observed for POPC- and DSPC-based LN CpG-ODN, the immune response was very similar for both formulations as a function of cytolytic activity and immune cell activation in splenocytes and PBMCs. Ultimately, our findings indicate that the circulation lifetime and D/L ratio of immunostimulatory nanoparticles do not directly influence their immunostimulatory activity. This phenomena is hypothesized to stem from the very effective delivery of LN CpG-ODN to its target cell population (macrophages and DCs), regardless of administration route or circulation lifetime. It must, however, be borne in mind that the parameters investigated did have the capacity to 'fine-tune' components of the immune response, particularly the specific compartments that demonstrate maximal response. This naturally has implications for the rational design of future immunostimulatory nanoparticulate systems, and further study will be required to determine whether clearance rate plays a role in the ability of LN CpG-ODN to act as an adjuvant for specific immunotherapies such as monoclonal antibody therapies or systemically administered vaccines.

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Declaration of interest: Both Y.K. Tam and P.R. Cullis are currently affiliated with, and have financial interests in, Tekmira Pharmaceuticals Corporation, Burnaby, BC, CANADA. Tekmira is involved in development of liposomal nanoparticulate nucleic acid therapeutics including encapsulated CpG oligonucleotides as an immunotherapeutic agent.

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