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Effects of intravenous and subcutaneous administration on the pharmacokinetics, biodistribution, cellular uptake and immunostimulatory activity of CpG ODN encapsulated in liposomal nanoparticles

Kaley D. Wilson [a,1,2], Sameersingh G. Raney [b,1], Laura Sekirov [b], Ghania Chikh [b], Susan D. deJong [a], Pieter R. Cullis [a], Ying K. Tam [b,*]

[a] *Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada*
[b] *Tekmira Pharmaceuticals Corporation, 8900 Glenlyon Pkwy, Burnaby, British Columbia, Canada V5J 5J8*

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Abstract

We have previously demonstrated that the immune response to an unmethylated cytidine–guanosine (CpG)-containing oligonucleotide (ODN) is greatly enhanced when encapsulated in a lipid nanoparticle (LN-CpG ODN). In this study, the pharmacokinetics, biodistribution and cellular uptake of LN-CpG ODN following intravenous (i.v.) and subcutaneous (s.c.) administration was characterized and correlated with immunostimulatory activity. It is shown that, despite dramatic differences in tissue distribution profiles and considerable differences in uptake by CD11c-positive, CD11b-positive, Mac-3-positive and CD45R/B220-positive cells following i.v. and s.c. administration, the resultant immune response is very similar with respect to levels of cellular activation (DX5, Mac-3, CD11b, CD45/B220, CD4, CD8 and CD11c) and cytolytic activity of immune cells [natural killer (NK) cells and monocytes/macrophages] in the spleen and blood compartments. Some differences in response kinetics and antibody-dependent cellular cytotoxicity (ADCC) activity were noted in the peripheral blood NK cell population. Analyses of particle biodistribution and cell types involved in uptake leads to the conclusion that the inherent ability of antigen-presenting cells (APCs) to sequester LN-CpG ODN results in efficient uptake of the particle, even when present at very low concentrations, leading to similar responses following i.v. and s.c. administration. These results contrast with the behavior of free CpG ODN, for which distinctly different immune responses are observed following i.v. or s.c. administration.

Keywords: Adjuvant; CpG-oligodeoxynucleotide; Liposomal nanoparticle; Immunostimulatory; Uptake

1. Introduction

Bacterial DNA and synthetic ODN that contain unmethylated CpG motifs activate a wide array of immune effector cells, stimulating potent, T-helper 1-biased immune responses. Upon exposure to APCs, CpG ODNs are rapidly

* Corresponding author. Tel.: +1 604 419 3269; fax: +1 604 419 201.
E-mail address: ytam@tekmirapharm.com (Y.K. Tam).
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internalized into the endosomal compartment where they interact with the Toll-like receptor 9 pattern recognition receptor (TLR9) [1,2]. This leads to activation of cell signaling pathways that result in the up-regulation of co-stimulatory molecules and the secretion of pro-inflammatory and T-helper 1-biased cytokines. Stimulation of these primary immune effects promote the activation of immune cells not directly responsive to CpG ODN, enhancing NK cell cytotoxicity and differentiation of naive CD4 and CD8 T-cells into T-helper 1 cells and cytotoxic lymphocytes, respectively [3,4]. As a result of their potent ability to stimulate and enhance innate and acquired immune responses, synthetic CpG ODN are currently undergoing Phase I–III clinical trials in the treatment and prophylaxis of cancer, allergy and asthma, and infectious disease [5–8]. While the use of chemical modification has effectively reduced the sensitivity of ODN to nuclease degradation, such as with the phosphorothioated CpG ODN currently in clinical trials, and prolonged circulation lifetime, the use of free CpG ODN still faces several significant challenges including unfavorable pharmacokinetics, a lack of specificity for target cells after systemic administration and poor cellular uptake [9–11].

Encapsulation of biologically active agents in liposomal nanoparticles can dramatically enhance their activity by increasing drug delivery to disease sites and by acting as drug reservoirs allowing sustained release of the therapeutic [12]. It is well recognized that liposomal nanoparticles are removed from the circulation by phagocytic cells [i.e. macrophages and dendritic cells (DC)] of the reticular endothelial system, largely in the liver and spleen, or by extravasation in areas where the vascular membrane is more permeable, such as at sites of infection, inflammation and tumors [12–14].

Liposomes have also been widely used in immunotherapeutic applications ranging from adjuvants to carriers of peptide, protein and DNA vaccines [15–18]. We have previously demonstrated that immune responses to a CpG-containing ODN are greatly enhanced when encapsulated in liposomal nanoparticles as determined by plasma cytokine levels (interleukin-12, interferon-gamma, interleukin-6, and macrophage chemoattractant protein-1) [19], immune cell activation and anti-tumor efficacy in animal models [20]. The rationale for encapsulation of CpG ODN is distinctly different than for other therapeutic agents. In particular, we are now taking advantage of the natural ability of APCs to accumulate LN-CpG ODN to provide enhanced delivery, resulting in enhanced immune cell activation and induction of more potent, T-helper 1-biased immune responses.

In the same vein, although i.v. delivery is the most common route of administration for conventional liposomal drugs, it may not represent the optimal route for immunostimulatory agents such as LN-CpG ODN. While i.v. administration would allow accumulation of LN-CpG ODN in circulating and tissue resident macrophages, s.c. administration would result in passage of the particle through the lymphatic capillaries to the regional lymph nodes prior to reaching the general circulation [13,21,22]. Thus the s.c. route of administration would be expected to provide enhanced access to immune cells found in high density in these regions, including DCs, macrophages, as well as T- and B-lymphocytes. Clearly then, the route of administration influences the specific environment and immune cell populations that are exposed to LN-CpG ODN and therefore, has the capacity to exert a potentially significant influence on the nature, degree and duration of the resultant immune response. Here we evaluate the influence of the route of administration on the pharmacokinetics, biodistribution and uptake of lipid-encapsulated CpG ODN and correlate these characteristics with immunopotency.

2. Materials and methods

2.1. Materials

Palmitoyloleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL), cholesterol from Sigma (St. Louis, MO). 1,2-Dioleyloxy-3-*N*,*N*-dimethylaminopropane (DODMA) and polyethylene glycol–dimyristol glycerol (PEG–DMG) were provided by Inex Pharmaceuticals Corporation (Burnaby, BC, Canada). INEX-6295 and 6303, 16-mer phosphorothioate ODN (5′ TAACGTTGAGGGGCAT-3′) containing unmethylated and methylated cytosine residues in the CpG motifs, respectively, were used in pharmacokinetic, biodistribution, immune cell activation and cytotoxicity studies. To assess pharmacokinetics and biodistribution, ODN was spiked with phosphorothioated INX-6295 containing two internal tritiated thymidine residues while for cell uptake studies, an irrelevant 5′-fluoroscene isothiocyanate-bearing 15-mer phosphorothioate ODN (5′-CCGTGGTCATGCTCC-3′) was used. All ODN were synthesized by Trilink Biotechnologies (San Diego, CA).

2.2. Animals

Female, 6- to 8-week-old ICR and C3H mice were obtained from Charles River Laboratories (Wilmington, MA) and were 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 liposomal ODN

ODN were encapsulated in lipid nanoparticles containing an ionisable aminolipid using an ethanol dialysis procedure, as previously described [23]. Briefly, lipid mixtures consisting of POPC/cholesterol/DODMA/PEG-DMG (molar ratio 25/45/20/10) were solubilized in ethanol, mixed with 50 mM citrate buffer containing 3.33 mg/ml of ODN, to give a final ethanol concentration of 36% and passed twice through stacked 200 nm+100 nm polycarbonate membranes (Whatman Nuclepore, Clifton, NJ) using a thermobarrel extruder (Lipex Biomembranes, Vancouver, BC, Canada). The vesicles were dialyzed against citrate followed by HEPES-buffered saline and unencapsulated ODN removed on DEAE-Sepharose CL-6B columns. Oligonucleotide 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 [24], respectively. Studies similar to those previously described [23,25] were performed to determine the optimal final ODN-to-lipid ratio of 0.1 (w/w) for this liposome formulation in terms of initial ratio and encapsulation efficiency (data not shown). Particle size, as determined by quasi-elastic light scattering using a NICOMP submicron particle sizer (model 370, Santa Barbara, CA), was 100±25 nm in diameter. All liposomal formulations used in these studies, including those containing radiolabeled and fluorescently labeled ODN, were assumed to have similar circulation/distribution properties based on identical lipid composition and biophysical characteristics (i.e. size, ODN/lipid ratio, etc).

2.4. Pharmacokinetic and biodistribution studies

ICR mice were injected i.v. or s.c. at ODN doses of 20 mg/kg or 360–440 μg/animal (140–160 μl administered via the lateral tail vein) and 4.5–5.5 mg/kg or 100 μg/animal (100 μl administered into the hind flank), respectively, with free or LN-CpG ODN. Doses for these initial experiments were derived from studies evaluating the effect of CpG dose on immunological response (unpublished data) and selected from the linear portion of the response curve inducing maximal pharmacodynamic responses. ODN were labeled by spiking with ^{3}H-labeled phosphorothioate INX-6295 (Trilink Biotechnologies) while lipids were labeled with ^{14}C-cholesterol-hexadecylether (CHE) to allow a per-mouse dosing of approximately 3 μCi of ^{3}H-ODN and 1 μCi of ^{14}C-lipid. Mice were euthanized 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 lymph nodes (bilateral, axial and inguinal), liver and spleen were harvested and chemically digested at room temperature using Solvable (Perkin-Elmer, Wellesley, MA) followed by de-colorization with hydrogen peroxide (30% w/w). Blood and tissue digests were analyzed by liquid scintillation counting in Picofluor-40 (Perkin Elmer).

2.5. Immune cell isolation

For immunological assays, mice were euthanized 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, 70 μM EDTA). Cells were stained with fluorescently labeled antibodies (BD Biosciences) and analyzed on 2 laser, 4-colour FACSort or FACScaliber flow cytometers (BD Biosciences, San Jose, CA). Dead cells were excluded with propidium iodide and viable cells were gated based on forward and side scatter characteristics. Data was acquired and analyzed using CELLQuest Pro software V 4.0.1 (BD Biosciences).

2.6. Uptake studies

6- to 8-week-old ICR mice were injected i.v. or s.c. with 10 mg/kg fluoroscene isothiocyanate-labeled ODN encapsulated within lipid particles (140–160 μl delivered into the lateral tail vein and hindflank, respectively). Mice were euthanized 1.5, 4, 7.5 and 24.5 h after administration and blood, lymph nodes and spleen were isolated. Cell suspensions were prepared, stained with antibodies against cell phenotype markers (CD11c for DCs, CD11b and Mac-3 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. or s.c. with free or encapsulated ODN at a 20 mg/kg dose (140–160 μl delivered into the lateral tail vein and hindflank, respectively). 24, 48 and 72 h following administration, mice were euthanized as described. In activation studies, splenocytes and PBMCs were stained with fluorescently labeled antibodies against cell phenotype (CD11c for DCs, DX5 for NK 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. NK killing was assessed in a standard 4-h ^{51}Cr release assay using YAC-1 cells as targets. Splenocytes or PBMC were co-cultured with ^{51}Cr-labeled target cells at E/T cell ratios of 1:1, 5:1, 25:1, 100:1 and 1:1, 5:1, 20:1 and 50:1, respectively, for 4 h at 37 °C, 5% CO_2 and the amount of ^{51}Cr released to the supernatants was quantitated as a measure of cell killing. Percentage of cytotoxicity was calculated using the equation: (sample counts per minute − spontaneous counts per minute)/(maximum counts per minute) × 100. Maximum counts were determined using 20% Triton X-100 while spontaneous counts were measured in culture medium. Activation of ADCC was assessed in a similar manner assessing lysis of Daudi cells in

the presence/absence of Rituxan (100 μg/10^6 cells at a concentration of 10 μg/ml), a mAb directed against the CD20 Ag present on the target cells.

2.8. Statistical analyses

A one-way analysis of variance (ANOVA) was used to evaluate the differences between treatment groups. In the case of statistically significant results, the differences between treatment groups were assessed using Bonferroni adjusted *t*-tests. Probability values less than 0.05 were considered significant.

3. Results

INX-6295 and INX-6303 are 16-mer ODNs with identical nucleotide sequences, the latter containing a methylated cytosine in the CpG motif. When encapsulated within a lipid nanoparticle, the immune activity of the methylated sequence is equivalent to or greater than the unmethylated sequence as determined through plasma cytokine profiling, *ex vivo* immune cell activation and anti-tumor efficacy in animal models [20]. As a result, INX-6303 was used for all immunological studies.

3.1. Blood levels of LN-CpG ODN differ dramatically following i.v. or s.c. administration

Pharmacokinetic and biodistribution characteristics were assessed using [^{3}H]-ODN and [^{14}C]-CHE to indicate the fate of the ODN and the lipid delivery system, respectively. Cholesterol-hexadecylether has been used extensively as a marker and has been shown to be non-exchangeable and non-metabolizable [26]. Similarly, internally labeled phosphorothioate ODN have been extensively documented as having good in vivo stability accompanied by slow metabolism [10].

Blood clearance profiles were compared over a 24-h time course following i.v. or s.c. administration of encapsulated and free ODN (Fig. 1). As expected [27,28], ODN- and lipid-equivalents (^{3}H-ODN- and ^{14}C-lipid-derived radioactivity, representing both intact and metabolite forms) show similar clearance profiles following both i.v. and s.c. administration, suggesting that ODN remains encapsulated within the carrier system (data not shown). Following i.v. administration of LN-CpG ODN, approximately half of the injected ODN-equivalent dose is cleared from the circulation within 30 min, with 0.5% of the injected ODN-equivalent dose remaining after 24 h. Conversely, only low levels of ODN-equivalents enter the circulation after s.c. administration, reaching maximum levels representing only 0.2±0.03% of injected ODN-equivalent dose 24 h after administration. Similar trends are observed following administration of free ODN although, based on ODN equivalents, clearance from the circulation following i.v. administration is 2–3 times quicker than with LN-CpG ODN (20.5% of the injected ODN-equivalent dose present 30 min after administration) while s.c. administration of free ODN results in at least 3-fold higher levels of ODN-equivalent dose reaching the circulation, compared to LN-CpG ODN-equivalent dose, at all time points investigated.

3.2. I.V. administration of LN-CpG ODN results in enhanced delivery to liver and spleen, whereas s.c. administration enhances accumulation in regional lymph nodes

Following i.v. administration of LN-CpG ODN, the majority of the ODN-equivalents accumulate in the liver,

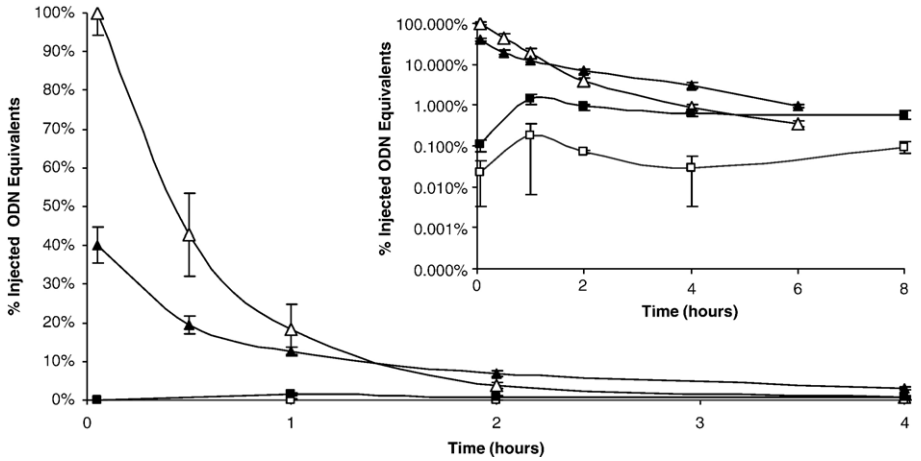

Fig. 1. Blood levels of free and LN-CpG ODN differ dramatically following i.v. or s.c. administration. Blood clearance profiles following intravenous or subcutaneous administration of encapsulated (△ – i.v., □ – s.c.) and free (▲ – i.v., ■ – s.c.) CpG ODN, tracking ^{3}H-labeled ODN, expressed as the percent of the injected dose detected±S.D. (3 mice/group). LN-CpG ODN and free ODN were administered intravenously at an ODN dose of 20 mg/kg or 360–440 μg/animal, or subcutaneously at a dose of 4.5–5.5 mg/kg or 100 μg/animal. Data are shown on both linear (main) and log (insert) scales.

with peak levels (71.1±4.7% of injected does) occurring 4 h post-injection (Fig. 2). Accumulation in the spleen follows a similar pattern with maximal ODN-equivalent accumulation of 1.57±0.36% of the injected dose occurring 4 h post-injection. Encapsulation enhances hepatic and splenic accumulation following i.v. administration compared to free ODN due to clearance by the reticular endothelial system. ODN-equivalent levels in both the liver and spleen remain relatively constant after 4 h with no significant decline over 24 h, likely due to uptake of the stable phosphorothioated ODN by tissue-resident phagocytic cells in the liver and spleen. Low, but detectable levels of ODN-equivalents are observed in the lymph nodes over the entire time course with both encapsulated and free forms.

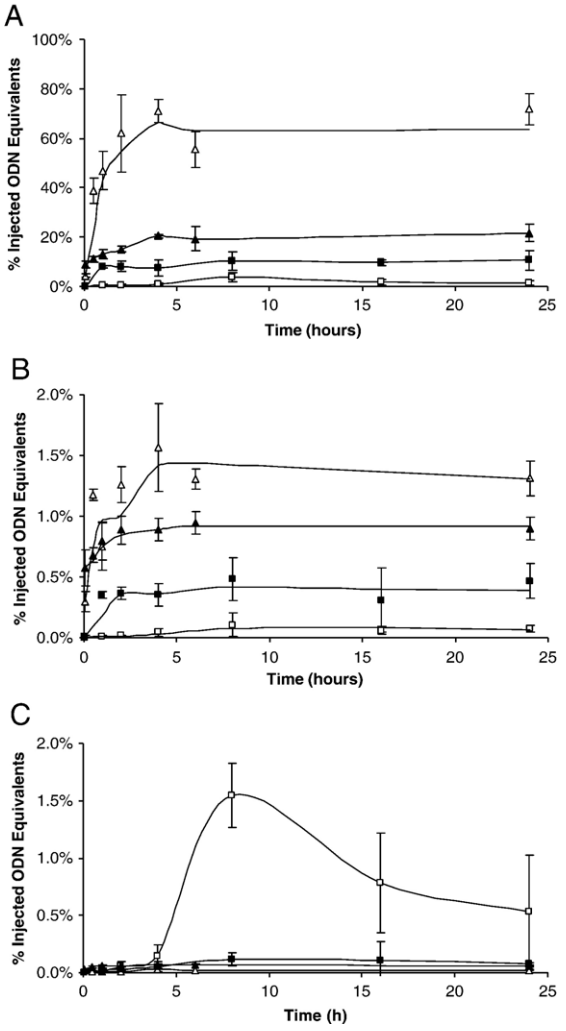

Fig. 2. Biodistribution of i.v.- and s.c.-administered free and LN-CpG ODN to liver, spleen and lymph nodes. Tissue accumulation of CpG ODN following intravenous or subcutaneous administration of encapsulated (△ – i.v., □ – s.c.) and free (▲ – i.v., ■ – s.c.) CpG ODN, tracking ^{3}H-labelled ODN, in the liver (A), spleen (B) and lymph nodes (C), expressed as the percent of the injected dose detected±S.D. (3 mice/group). LN-CpG ODN and free ODN were administered intravenously at an ODN dose of 20 mg/kg or 360–440 μg/animal, or subcutaneously at a dose of 4.5–5.5 mg/kg or 100 μg/animal.

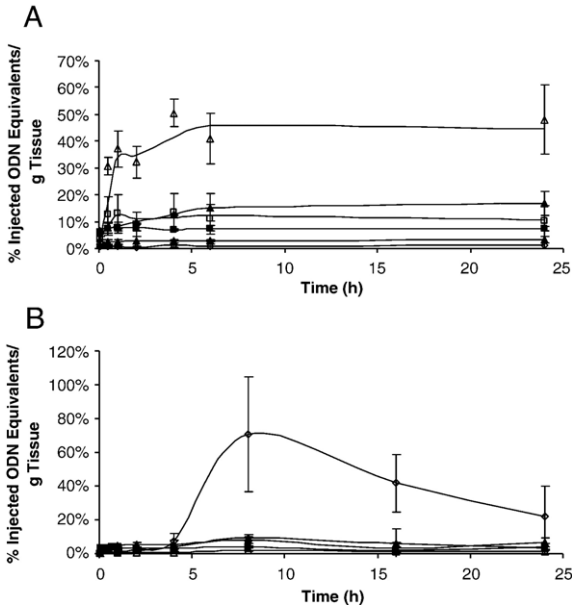

Fig. 3. Preferential accumulation of LN-CpG ODN in the lymph nodes following s.c. administration. Tissue accumulation of encapsulated and free CpG ODN in the liver (LN-CpG ODN △; free ODN ▲), spleen (LN-CpG ODN □; free ODN ■) and lymph nodes (LN-CpG ODN ◇; free ODN ◆) tracking ^{3}H-labelled ODN following intravenous (A) and subcutaneous (B) administration, expressed as the percent of the injected dose per gram of tissue±S.D., each data point represents three mice. Encapsulated and free CpG ODN was administered intravenously at an ODN dose of 20 mg/kg or 360–440 μg/animal, or subcutaneously at a dose of 4.5–5.5 mg/kg or 100 μg/animal.

When LN-CpG ODN is administered s.c., relatively little ODN-equivalents accumulation is observed in the liver and spleen with reductions of approximately 18- and 14-fold, respectively, compared to i.v. administration. Maximum ODN-equivalents accumulation (3.9±1.84% and 0.11±0.10%, respectively) is not reached until 8 h post-administration. However, although ODN-equivalents levels are considerably lower, relative biodistribution patterns in the blood, liver and spleen are similar to those observed following i.v. administration, indicating that when the particles reach the peripheral circulation they are subject to similar clearance mechanisms as those administered i.v.

In contrast, s.c. delivery of LN-CpG ODN results in levels of accumulation in lymph nodes (1.54±0.28%) that are 50-fold greater than following i.v. administration, with maximal ODN-equivalents accumulation occurring by 8 h post-injection. Levels declined thereafter to approximately 0.6% by 24 h, likely due to ODN uptake by highly mobile phagocytic cells in the injection site and migration to and from the draining lymph nodes. Also, in contrast to i.v.

delivery, liposomal delivery results in reduced CpG ODN-equivalents accumulation in the liver and spleen, with free ODN demonstrating enhanced hepatic and splenic ODN-equivalent localization following s.c. administration. Interestingly, enhanced accumulation in the lymph nodes is not observed for s.c. administered free ODN.

Comparison of organ accumulation based on tissue weight demonstrates that when administered s.c., LN-CpG ODN-equivalents accumulate preferentially in the lymph nodes, with very low levels of ODN-equivalents being observed in the spleen and liver (Fig. 3). Conversely, s.c. administration of free ODN or i.v. administration of free or encapsulated ODN resulted in low levels of ODN-equivalents in the lymph nodes.

3.3. LN-CpG ODN are accumulated by immune cells in the blood, spleen and lymph nodes following i.v. or s.c. administration

In the spleen, maximum levels of uptake by CD11c-positive, Mac-3-positive, CD11b-positive and B220/CD45R-positive cells are observed 7.5 h following both i.v. and s.c. administration (Fig. 4). Not surprisingly, based on pharmacokinetic and biodistribution data, enhanced uptake is seen in the spleen and peripheral blood compartments following i.v. administration, both in terms of the total number of cells and on a per cell basis. In the spleen, i.v. administration results on average in 10% to 30% more APCs taking up LN-CpG ODN

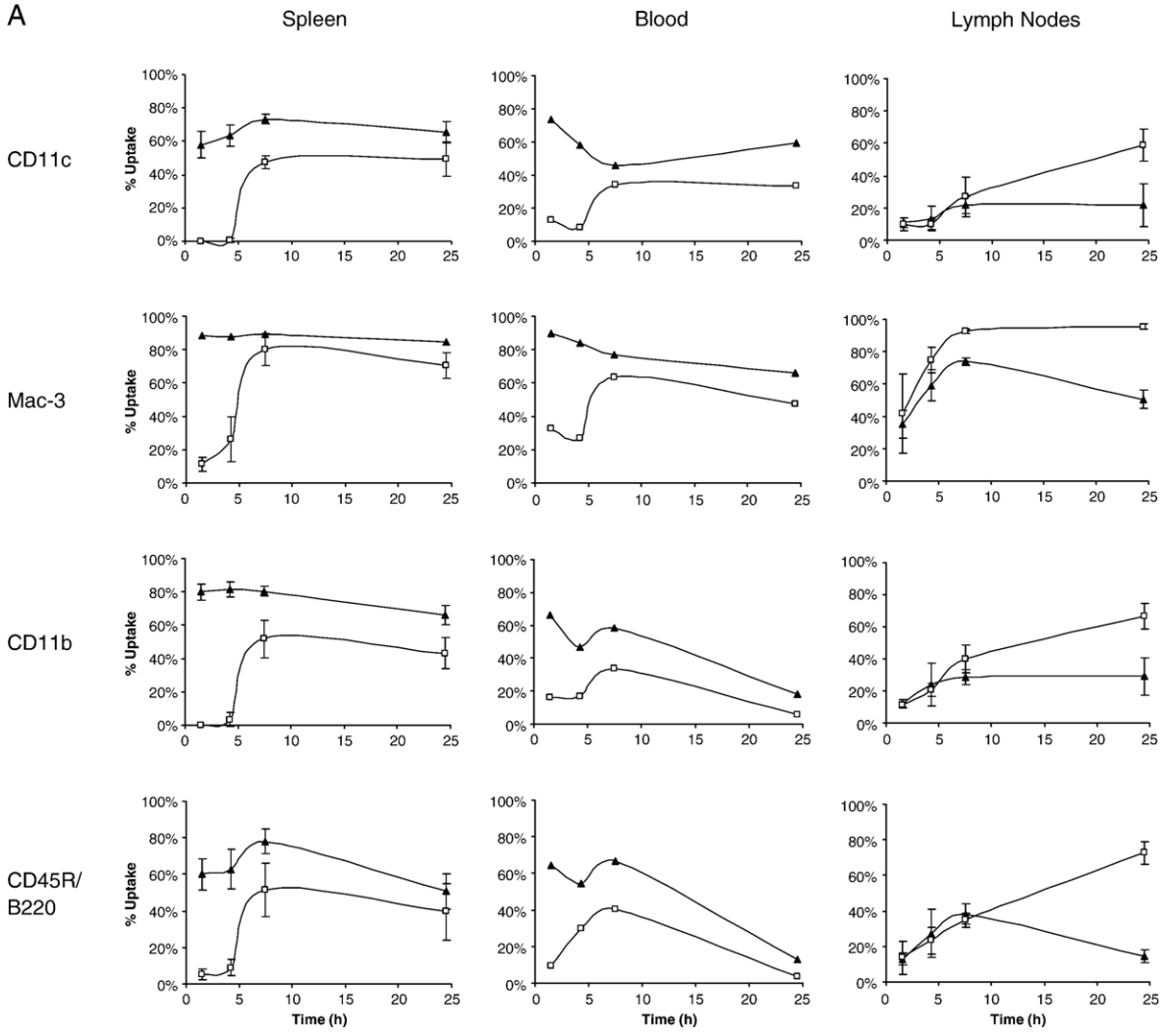

Fig. 4. LN-CpG ODN are accumulated by CD11c-positive, Mac3-positive, CD11b-positive and B220/CD45R-positive cells in the blood, spleen and lymph nodes compartments following i.v. and s.c. administration. Uptake of LN-CpG ODN containing FITC-labeled ODN by CD11c-, Mac3-, CD11b- and B220/CD45R-positive cells in the spleen, blood and lymph node compartments following intravenous (▲) or subcutaneous (□) administration at an ODN dose of 10 mg/kg. Results are expressed as (A) the percent of cells positive for uptake and (B) MFI±S.D., as determined by flow cytometry. Each data point represents four mice, blood results represent 4 mice pooled.

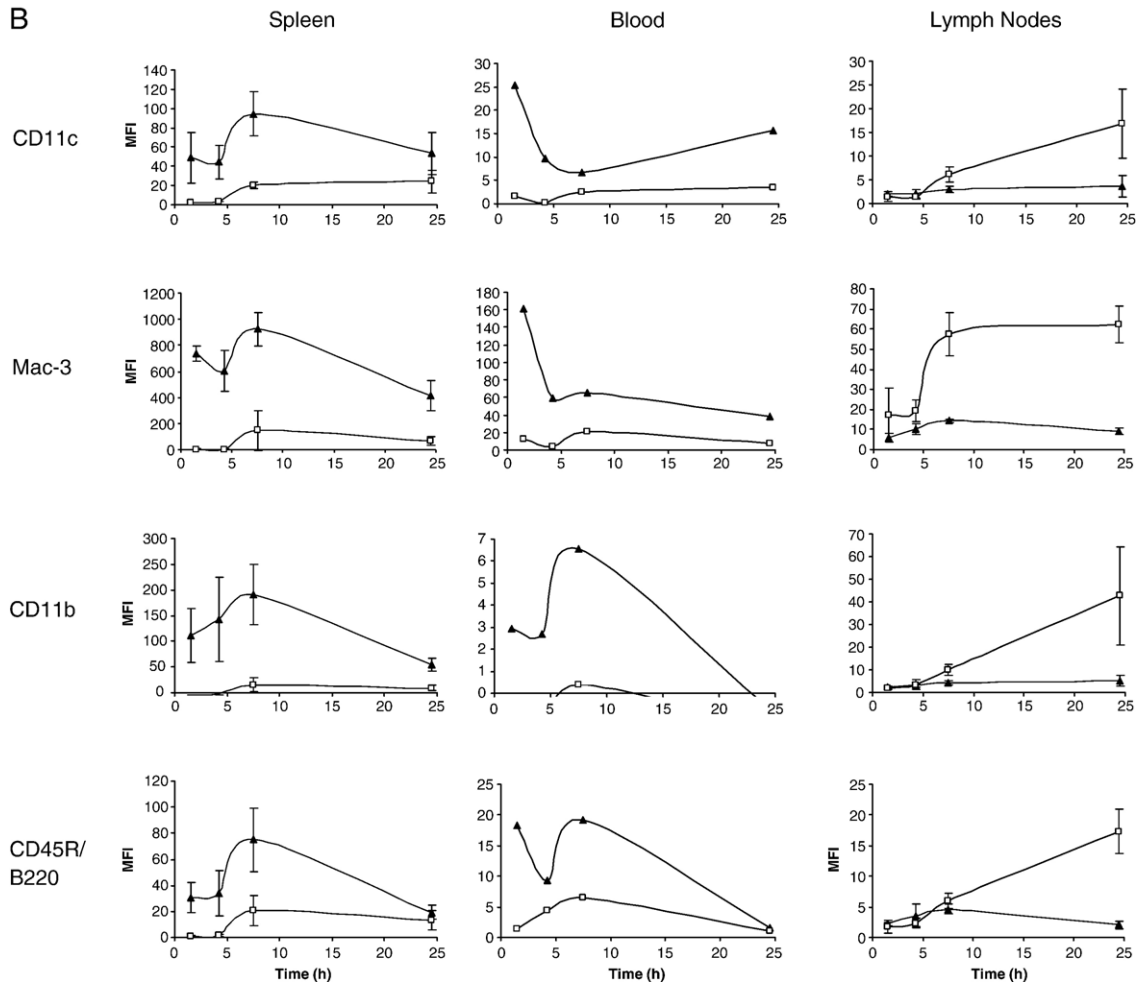

Fig. 4 (*continued*).

compared to s.c. administration. Furthermore, mean fluorescence intensity is 4- to 13-fold higher following i.v. administration, indicating enhanced uptake on a per cell basis. Similarly, 10 – 50% more peripheral blood CD11c-positive, Mac-3-positive, CD11b-positive or B220/CD45R-positive cells take up LN-CpG ODN following i.v. administration (Fig. 4), concomitant with 3- to 18-fold higher uptake on a per cell basis.

Greater accumulation in the lymph nodes is seen following s.c. administration which is also consistent with pharmacokinetic and biodistribution data. 2- to 5-fold greater numbers of APCs positive for uptake and 5- to 10-fold higher uptake per cell is observed compared to i.v. administration. However, unlike the peripheral blood and splenic compartments that exhibit similar biodistribution and uptake patterns, APCs in the lymph nodes demonstrate significantly divergent LN-CpG ODN uptake patterns based on route of administration. Uptake in lymph node APCs, both on a total cell number and a per cell basis, increase throughout the first 24.5 h after s.c. administration for all cells with the exception of Mac-3 cells which reach maximal levels by 7.5 h and plateau thereafter. In contrast, after i.v. administration, maximum lymph node accumulation is observed for all cell types by 7.5 h, after which uptake levels either plateau or decline.

3.4. Similar levels of immune cell activation are observed following s.c. or i.v. administration of LN-CpG ODN

Administration of LN-CpG ODN by either route results in significant up-regulation of the activation markers CD69 or CD86 on NK cells, monocyte/macrophages, B- and T-lymphocytes and DCs (Fig. 5). For all cells types, i.v. and s.c. treatment with LN-CpG ODN results in at least a 3-fold enhancement of activation marker expression compared to levels 48 h after treatment with free ODN, which, in turn, is slightly elevated compared to control animals. We have previously found that maximal cell activation occurs 24–48 h after administration of free CpG ODN (unpublished data). Similar levels of activation marker expression are observed following i.v. and s.c. administration for almost all cell types

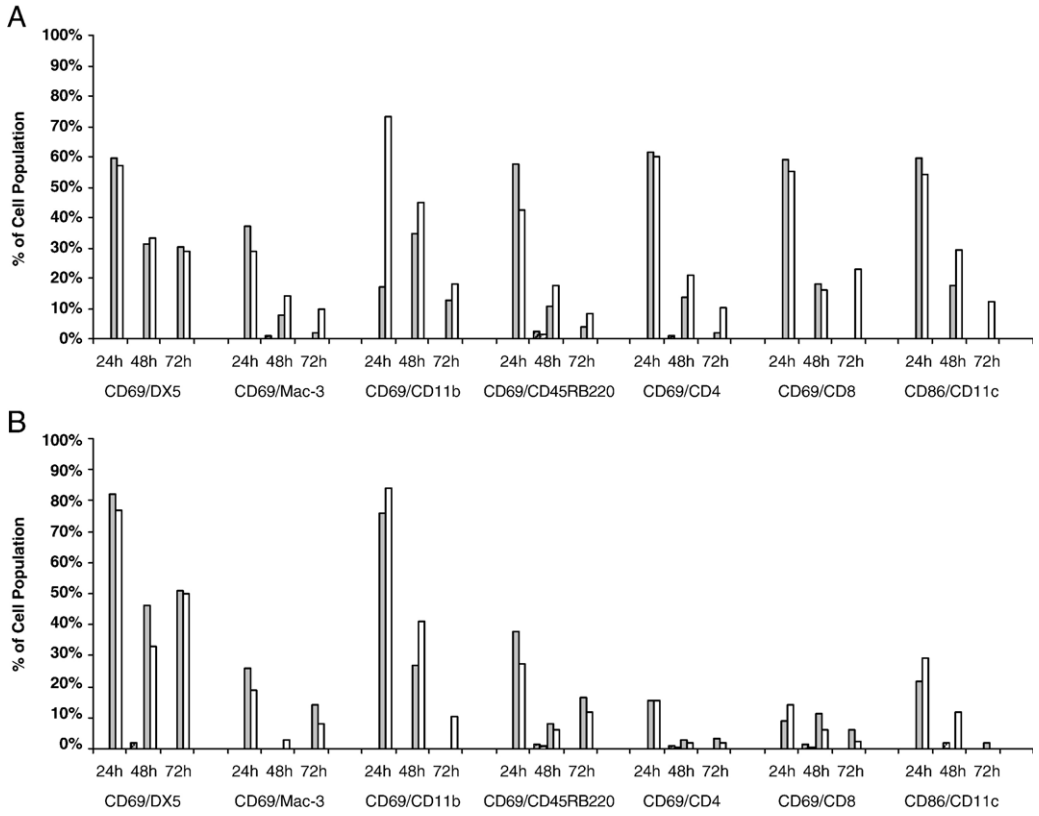

Fig. 5. Similar levels of splenic and peripheral blood immune cell activation following i.v. and s.c. administration of LN-CpG ODN. Expression of cell activation markers CD69 or CD86 on splenocytes (A) and PBMCs (B) following intravenous (LN-CpG ODN ▒; free ODN ▨) (5 mice/group) or subcutaneous (LN-CpG ODN □; free ODN ▨) (4 mice/group) administration of LN-CpG ODN at an ODN dose of 20 mg/kg. Data is expressed as the percent of CD69- or CD86-positive cells in each specific cell-type population, background fluorescence levels (1–20% depending on cell type) were subtracted.

investigated, with the exception of splenic CD11b-positive cells, where a notable increase in CD69 expression is observed following s.c. administration of LN-CpG ODN at the 24-h time point. Similar trends are observed in regards to the level of activation marker expression on a per cell basis (data not shown). Of note is that s.c. administration appears to result in a more prolonged activation of splenic APCs (Mac-3-, CD11b-, CD45R/B220- and CD11c-positive cells) and T-lymphocytes compared to i.v. as judged by CD69 and CD86 activation marker expression at the 48- and 72-h time points. This same phenomenon, however, is not observed in PBMCs.

3.5. Similar levels of NK cytolytic activity and ADCC are observed following s.c. or i.v. administration of LN-CpG ODN

Levels of NK cytolytic activity, against the cell line Yac-1, are at least 2-fold greater in splenocytes and PBMCs from mice treated with LN-CpG ODN than in animals 48 h after treatment with free ODN administered by either route at all E/T ratios investigated (Fig. 6). We have previously found that maximal NK activity occurs 24–48 h after administration of free CpG ODN (unpublished data). Overall, similar levels of NK activity are detected in splenocyte and PBMC populations following i.v. and s.c. administration of LN-CpG ODN resulting in elevated cytotoxicity against Yac-1 cells throughout the entire 72-h period following administration. Although levels of NK activity are similar, the kinetics do vary, with i.v. delivery resulting in peak cytolytic activity in both PBMC and splenocyte populations 24 h after i.v. administration and declining thereafter. Following s.c. administration, the activity remains relatively constant over the 72-h time course in PBMCs but peaks at 48 h and declines thereafter, in splenocyte populations.

Furthermore, for splenocyte populations, enhanced levels of ADCC are observed ex vivo using the anti-CD20 monoclonal antibody Rituxan and the B-cell lymphoma line Daudi, following both i.v. and s.c. administration of LN-CpG ODN, with similar levels seen following delivery via both routes (Fig. 7). In the case of PBMCs, although the NK activity is similar, analysis reveals that i.v. administration of LN-CpG ODN induces a statistically significant 10%–15% increase in ADCC levels compared to s.c. administration at all E/T ratios investigated at the 24- and 48-h time points,

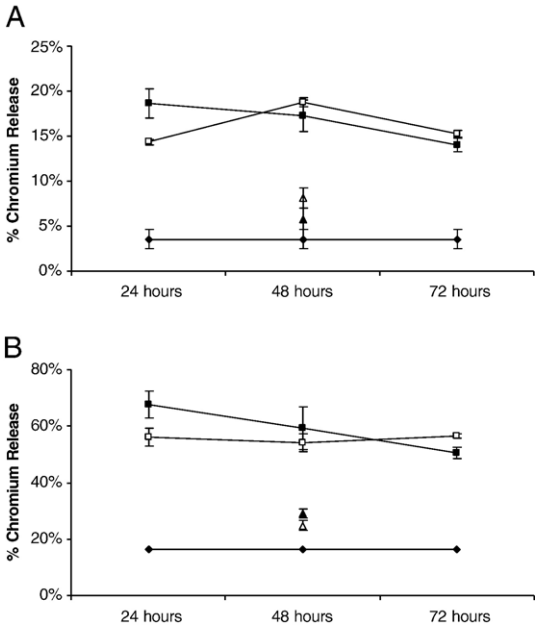

Fig. 6. Similar levels of NK cytolytic activity of splenic and peripheral blood immune cells are observed following i.v. and s.c. administration of LN-CpG ODN. Activation of NK-specific cytolytic activity in splenocyte (A) and PBMC (B) populations was assessed in a standard 4-h Cr51 release assay using YAC-1 cells as targets following intravenous (LN-CpG ODN ■; free ODN ▲) (5 mice/group) and subcutaneous (LN-CpG ODN □; free ODN △) (4 mice/group) administration of LN-ODN at a dose of 20 mg/kg or intravenous administration of an HBS control (♦). Splenocyte data is reported at an E/T ratio of 25:1, PBMC 20:1.

with a consistent but non-significant difference by 72 h (Fig. 7) (Bonferroni adjusted *t*-tests; 24 h: $t(4)=3.772$, $p<0.05$; and 48 h: $t(3)=4.155$, $p<0.05$). At all time points and E/T ratios, Daudi cell killing in the absence of Rituxan is equal to or less than 5.0% for PBMCs and 1.0% for splenocytes and Daudi cell killing in the presence of Rituxan following treatment with free ODN by either route of administration was only slightly greater than control at the 48-h time point.

Of relevance to both the immune cell activation and NK/ADCC activity studies is the fact that dose titrations of LN-CpG ODN, including doses exceeding those administered in these studies, demonstrate a strong response between dose and immune cell activation and activity confirming that the similar immunostimulatory effects following i.v. and s.c. administration are not due delivery of excessive doses of CpG ODN (data not shown).

4. Discussion

The results of this investigation show that despite large differences in LN-CpG ODN pharmacokinetics and biodistribution following i.v. or s.c. administration, the immunostimulatory effects are largely similar. Four aspects of this work of particular interest concern: first, the relation between the pharmacokinetics and biodistribution observed for LN-CpG ODN as they relate to the mode of administration; second, the mechanism whereby similar immune responses are observed despite large differences in biodistribution; third, the areas in which the mode of administration does cause differences in immune response; and finally, the ways in which the immune response to LN-CpG ODN administered i.v. or s.c. differ from those observed by others for free ODN administered by either route.

The pharmacokinetics and biodistribution characteristics of liposomally encapsulated drugs following i.v. [16,27–29], and to a lesser extent, s.c. administration [13,21,30], have been well characterized and are consistent with the results presented here for LN-CpG ODN. In particular, half of the injected LN-CpG ODN dose is cleared from the circulation approximately 30 min following i.v. injection, much longer than observed for free ODN. While the circulation lifetime of LN-CpG ODN is relatively short compared to liposomal formulations of drugs such as vincristine, where values

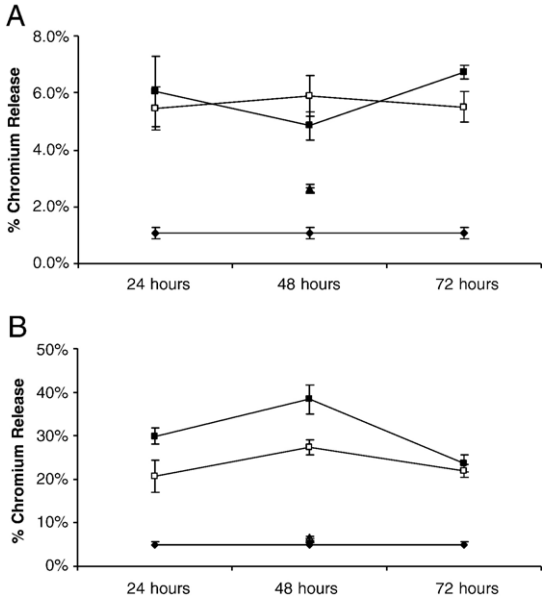

Fig. 7. Similar levels of ADCC of splenic and peripheral blood immune cells against the human B-cell lymphoma cell Daudi are observed following i.v. and s.c. administration of LN-CpG ODN. Activation of ADCC in splenocyte (A) and PBMC (B) populations was assessed in a standard 4-h Cr51 release assay using Daudi cells as targets in the presence of Rituxan following intravenous (LN-CpG ODN ■; free ODN ▲) (5 mice/group) and subcutaneous (LN-CpG ODN □; free ODN △) (4 mice/group) administration of LN-CpG ODN at a dose of 20 mg/kg or intravenous administration of an HBS control (♦). Splenocyte data is reported at an E/T ratio of 25:1, PBMC 20:1.

of 10 h or more are commonly observed [31], this can be attributed to the different lipid composition of the LN-CpG ODN system. The shorter circulation lifetime for the LN-CpG ODN reflect the fact that they are optimized for maximum immunostimulatory activity and are intended to be taken up by the APCs of the reticular endothelial system.

For s.c. delivery, pharmacokinetic studies demonstrate that LN-CpG ODN appears in the circulation within 1 h of administration and blood concentrations remain near constant below 0.1% of the injected dose over 24 h. This behavior is consistent with that previously observed for liposomes [21], where a continual supply of particles drain from the injection site through the regional lymph nodes into the bloodstream. The effective delivery of LN-CpG ODN to APCs in the lymph nodes following s.c. administration is dramatically illustrated by the increasing levels of uptake over the 24-h period following injection. This is in contrast to uptake by splenic and peripheral blood APCs following i.v. and s.c. administration, as well as lymph node APCs following i.v. administration, where maximal levels were reached at 7.5 h after administration and declined thereafter. It is interesting to note that levels associated with the blood, liver and spleen were proportionately reduced relative to the i.v. route, indicating that when particles reach the blood they are removed in the liver and spleen by a mechanism similar to that following i.v. administration.

The pharmacokinetic and biodistribution patterns of LN-CpG ODN indicate that particles reach APCs in the spleen, peripheral blood and lymph following both i.v.. and s.c. injection, albeit at very different levels. The fact that similar levels of immunostimulatory activity are observed is consistent with two features of APCs and their interactions with immunostimulatory particles. First, it has been long established that APCs such as macrophages and DCs avidly accumulate exogenous particles such as liposomes [13,22,32] which is consistent with results presented here. For example, although only a very small portion (approximately 0.1–0.2%) of the injected LN-CpG ODN dose reaches the blood and spleen compartments following s.c. administration, the percentage of APCs positive for uptake is similar (50–95%) to that observed following i.v. administration. Similarly, although levels of LN-CpG ODN reaching the lymph nodes are some 50-fold lower for i.v. injection versus s.c., the percentage of APCs positive for uptake are still more than 20% of s.c. levels. The second feature concerns the fact that stimulation of immune activity requires only threshold levels of CpG ODN, and higher levels of uptake do not to lead to more activity. The fact that different effector cells from various target organs exhibit very similar levels of immune activation in spite of significant differences in LN-CpG ODN uptake most likely stems from the fact that the uptake of even a single LN-CpG ODN results in the effective delivery of greater than 2000 CpG ODNs [25].

The final area of discussion concerns differences between pharmacokinetics and tissue distribution of LN-CpG and free phosphorothioate ODN following s.c. and i.v. administration, and whether this results in significant differences in immune response. Stimulation of the immune system has been investigated following the i.v. and s.c., epicutaneous, intragastric, intramuscular and intradermal administration of free or lipid complexed CpG ODN, alone or combined with peptide, protein or DNA vaccination [33–36]. Few studies, however, directly compare immune stimulation following different routes of CpG ODN administration. Comparative studies that have been performed investigated effects of administering free ODN by i.v. and s.c. routes in both mice [37] and humans [38] and indicate a lack of immune stimulation following i.v. administration, as determined by cytotoxic T-lymphocyte activation and cytokine/chemokine profiling, respectively. This is consistent with results reported here, where relatively low levels of immune stimulation are observed for i.v. and s.c. administered free ODN. In contrast, comparison of expression levels of the activation markers CD69 and CD86 on splenocyte and PBMCs populations, 24, 48 and 72 h following administration of LN-CpG ODN by either route indicates that there is no observable difference in the levels of cellular activation (DX5, Mac-3, CD11b, CD45/B220, CD4, CD8 and CD11c). Treatment by either route resulted in at least a 3-fold enhancement of activation marker expression over peak levels observed after administration of free ODN, indicating that lipid encapsulation of the ODN enhances its immunostimulatory potential over that of free for all cell types investigated, with the exception of those expressing Mac-3.

On a functional level, it has been previously demonstrated that CpG-containing ODN are effective at inducing NK cell lytic activity [39]. In our investigation, similar levels of NK-specific cytolytic activity were observed in splenocyte and PBMC populations of mice treated by both routes at all time points investigated, although some differences in response kinetics was observed. Therefore, based on this measure, the data again indicates that both i.v. and s.c. administration of LN-CpG ODN results in potent,

largely similar NK responses, despite disparate pharmacokinetic, biodistribution and uptake behavior. Further, levels of NK-specific cytolytic activity observed were at least 2-fold greater than with free ODN.

Given that CpG ODNs activate two effector cell populations that are effective mediators of ADCC (NK cells and monocytes/macrophages), activation of ADCC resulting from administration of LN-CpG ODN was studied in detail. Stimulation of ADCC is important as tumor-specific monoclonal antibodies can have significant clinical activity [40] and most employ ADCC as part of their mechanism of action [41]. While similar levels of ADCC were observed for splenocytes isolated from mice treated with LN-CpG ODN by both routes, PBMCs exhibited a statistically significant increase in ADCC following i.v. administration, as compared to s.c. at all E/T ratios investigated at the 24-h, 48-h and, to a lesser extent, 72-h time points. This suggests that i.v. administration of LN-CpG ODN could offer improved therapeutic benefit, compared with s.c., when used to improve the potency of monoclonal antibodies via ADCC.

In conclusion, results presented here indicate that LN-CpG ODN elicit similar immunostimulatory responses following i.v. and s.c. administration in spite of dramatic differences in biodistribution and cellular uptake. It is concluded that the inherent ability of APCs to accumulate liposomal nanoparticles results in very efficient uptake of LN-CpG ODN, even when present at very low concentrations, resulting in enhanced immune responses as compared to free ODN. Any differences that are observed are more of degree than of kind, indicating, for example, that i.v. administration may be preferred to s.c. administration if LN-CpG ODN are used to increase the potency of monoclonal antibodies via ADCC. Compared to free CpG ODN, LN-CpG ODN offers the dual benefits of significantly enhanced immune responses as well as a relative insensitivity of the immune response to route of administration.

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