



Lipid-based delivery of CpG oligonucleotides enhances immunotherapeutic efficacy [☆]

Kaley D. Wilson ^{a,*}, Susan D. de Jong ^b, Ying K. Tam ^b

^a Centre for Drug Research and Development, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

^b Tekmira Pharmaceuticals Corporation, Burnaby, British Columbia, Canada V5J 5J8

ARTICLE INFO

Article history:

Received 22 July 2008

Accepted 15 December 2008

Available online 13 February 2009

Keywords:

CpG

Lipid nanoparticle

Encapsulation

Immunotherapy

Adjuvant

Vaccine

Innate

Adaptive

ABSTRACT

There has been significant interest in the potential of cytosine–guanine (CpG) containing oligodeoxynucleotides (ODN) as an immunotherapy for malignant, infectious and allergic diseases. While human trials have yielded promising results, clinical use of free CpG ODN still faces several challenges which limit their effectiveness. These include suboptimal *in vivo* stability, toxicity, unfavorable pharmacokinetic/biodistribution characteristics, lack of specificity for target cells and the requirement for intracellular uptake. To overcome these challenges, optimized lipid-based delivery systems have been developed to protect the CpG ODN payload, modify their circulation/distribution so as to enhance immune cell targeting and facilitate intracellular uptake. Ultimately, lipid-mediated delivery has the capacity to increase the immunopotency of CpG ODN and enhance their prophylactic or therapeutic efficacy in a range of diseases. Lipid-encapsulation provides a feasible strategy to optimize the immunostimulatory activity and immunotherapeutic efficacy of CpG ODN, thereby allowing their full clinical potential to be realized.

© 2009 Elsevier B.V. All rights reserved.

Contents

1. Introduction	233
2. Free CpG ODN as a therapeutic	234
3. Lipid based delivery systems for the <i>in vivo</i> administration of CpG ODN	234
3.1. Lipid encapsulation of ODN	234
3.2. Liposomes and lipoplexes for ODN delivery	235
3.3. Formulation of lipid-nanoparticulate CpG ODN	235
4. The benefits of lipid-based delivery	236
4.1. Lipid-based delivery protects the nucleic acid payload	236
4.2. Lipid nanoparticle delivery systems improve the pharmacokinetic characteristics of CpG ODN	236
4.3. Lipid nanoparticle delivery systems enhance intracellular uptake	236
4.4. Lipid-based delivery systems enhance delivery to target APCs	236
4.5. Lipid encapsulation enhances the immunostimulatory potency of CpG ODN	237
4.6. Lipid nanoparticle delivery confers activity to non-immunostimulatory sequences	237
5. Immunotherapeutic efficacy of LN-CpG ODN	238
5.1. Lipid-encapsulated CpG ODN as a standalone agent	238
5.2. LN-CpG ODN to enhance monoclonal antibody therapy	238
5.3. LN-CpG ODN as an adjuvant for prophylactic and therapeutic vaccination	238
6. Concluding remarks	239
References	239

[☆] This review is part of the *Advanced Drug Delivery Reviews* theme issue on “CpG Oligonucleotides as Immunotherapeutic Adjuvants: Innovative Applications and Delivery Strategies”.

* Corresponding author. Centre for Drug Research and Development, Suite 364-2259 Lower Mall University of British Columbia, Vancouver, BC, Canada V6T 1Z4. Tel.: +1 604 221 7750x132; fax: +1 604 221 7753.

E-mail address: kwilson@cdrrd.ca (K.D. Wilson).

1. Introduction

Although a wide variety of immunological adjuvants are currently under investigation, only a small handful have, to date, been approved for use in humans. Those which have been approved function largely to stimulate humoral immune responses which are effective at

protecting against extracellular pathogens but fail to elicit the cellular responses critical for protection against intracellular pathogens and cancer [1,2]. In contrast to currently licensed adjuvants, ODN containing unmethylated CpG dinucleotide motifs have demonstrated the potential to elicit strong cellular and humoral immune responses against a wide variety of antigens (Ags) and provide protection against an extensive range of viral, bacterial and some parasitic pathogens in numerous animal models [3–6]. The therapeutic potential of CpG ODN is further supported by their demonstrated activity as stand-alone agents, adjunct therapy and vaccine adjuvants for the prevention or treatment of cancer [7]. Finally, their strong Th1-bias has been shown to be able to redirect allergic Th2 responses and prevent inflammatory disease manifestations in animal models, even with established disease [6].

Encouraged by the results from a number of promising preclinical and clinical studies, a tremendous level of interest surrounds the development of immunostimulatory ODN-based therapeutics. Clinically, the safety, immunopotency and efficacy of CpG ODN have been evaluated in healthy human volunteers [8] and a broad range of disease indications in a number of Phases I–III clinical trials, for the treatment and prophylaxis of cancer, allergy and asthma, and infectious disease [9–13]. Despite successes demonstrated in early clinical trials, the clinical use of free CpG ODN still faces several significant challenges including poor stability, unfavorable pharmacokinetic (PK; the absorption, distribution, metabolism and excretion of a drug in the body) and biodistribution (BD; localization of a drug within the body), a lack of specificity for target cells and poor cellular uptake. This review will focus on investigating the immunotherapeutic efficacy of CpG ODN and the enhancement in activity that can be achieved through the use of lipid nanoparticles as delivery vehicles.

2. Free CpG ODN as a therapeutic

It has been well established that CpG motifs are recognized by the innate immune system through interaction with the pattern recognition receptor Toll Like Receptor 9 (TLR9) which is located inside the endocytic compartment of professional antigen presenting cells (APCs) [14,15]. Due to their ability to stimulate potent innate immunity, CpG ODN are effective standalone bio-response modifiers against a number of viral and bacterial infections as well as against malignant disease, and their activity can be further enhanced by co-administration with adjunct therapies such as monoclonal antibodies (mAbs) [11,16], and chemo- and radiation therapy [17]. The utility of the unmodified natural phosphodiester (PO) form of CpG ODN *in vivo* has, however, been limited by their extreme susceptibility to nuclease degradation which renders them inactive in the free form [18,19]. To overcome this problem, a variety of stabilizing chemical modifications have been employed to minimize DNA degradation, including replacement of a non-bridging oxygen atom in the PO bond with a sulphur atom (phosphorothioate or PS) and incorporation of substituted phosphate linkages or nucleotides with chemically substituted furanose rings [18].

Using chemically modified forms of CpG ODN, cancer clinical studies have been conducted in solid tumors including melanoma, non-small cell lung cancer, breast, renal cell and hematological malignancies such as chronic T-cell leukemia and non-Hodgkin's lymphoma. Furthermore, CpG ODN have been evaluated as an adjuvant, with specific tumor associated Ags (TAAs) in cancer vaccines and have been shown to be effective in inducing adaptive, Ag-specific cellular anti-tumor immune responses and anti-cancer efficacy [12,20]. CpG ODN have also shown potential as adjuvants for infectious disease vaccines in humans including hepatitis B, anthrax and influenza [9,10,13,21]. These latter studies indicate that CpG ODN can induce significant humoral immunity, including secretory IgA mucosal responses [22], against even poorly immunogenic viral and bacterial Ags [23]. This high potency raises the prospects of CpG-adjuvanted

vaccines for chronic and latent infections and studies in Hepatitis B [24] and C [25], Herpes [26] and HIV [27] have been undertaken. Finally, in addition to *immunostimulatory* applications, CpG ODN can also be used to modulate dysregulated immune responses as seen in chronic inflammation, allergic disease and autoimmunity [28]. Results from clinical programs have been, in large part, promising, yielding data supporting the potent immunomodulatory activity of chemically modified CpG ODNs in a range of clinical applications.

While chemical modifications, such as the PS backbone for CpG ODN used in current clinical trials, have effectively reduced the sensitivity of ODN to nuclease degradation and thus increased their potency and circulation lifetime, the use of free CpG ODN still faces several other significant challenges. Despite backbone stabilization, PS-modified ODN are still susceptible to nuclease degradation, albeit at a reduced rate [29]. More critically, PS modification is associated with several inherent disadvantages including non-sequence specific toxicity, stemming from the promiscuous binding of the PS backbone to a variety of polyanions and serum proteins, unfavorable PK and BD, a lack of specificity for target cells and poor cellular uptake [30–32]. Administration of high doses of PS ODN has been demonstrated to result in significant acute toxicity in primates due to transient complement activation and other hemodynamic changes which, in extreme cases, can result in cardiovascular collapse and death [29] although these severe toxicities are only observed at doses exceeding those necessary to induce immune responsiveness. Free, stabilized CpG ODN also demonstrate rapid clearance from the circulation following *i.v.* administration owing to rapid and broad tissue distribution, and to a lesser extent, metabolism [33] which occurs almost immediately following *i.v.* administration [29,34]. Furthermore, the necessity for intracellular uptake into endosomal compartments represents a significant hurdle since charged macromolecules such as free DNA have difficulty crossing hydrophobic membranes.

Finally, recent evidence collected from numerous groups indicates that PS-modified ODN do not faithfully mimic the interaction of natural PO ODN with TLR9. Evidence in support of this comes from surface plasmon resonance studies which demonstrate a reduced affinity of TLR9 for PS modified ODN, compared to the natural PO backbone ODN, [35] and the finding that the strict species and sequence-specific requirements for the activation of TLR9 by CpG ODN [34,36] are largely artifacts of PS-modified ODN [37–39]. In fact, a recent report has even suggested that even the requirement for a CpG motif is artifactual with the ability of PO ODN to activate TLR9 being based on its deoxyribose backbone [37]. Although these data remain to be confirmed by other groups, they raise a number of very interesting questions regarding the mechanisms by which the immunostimulatory activity of DNA may be regulated.

3. Lipid based delivery systems for the *in vivo* administration of CpG ODN

3.1. Lipid encapsulation of ODN

In an attempt to overcome the issues associated with the use of free CpG ODN *in vivo*, significant effort has been devoted to the design and development of methods to allow for improved delivery of CpG ODN and enhanced immunotherapeutic efficacy. Foremost among these has been the development of optimized lipid-based delivery systems. Lipid-based delivery systems represent one of the most advanced drug delivery technologies to date with 5 liposomal drugs currently approved in the United States and numerous others in late-stage clinical development [40,41]. These systems have been used primarily to enhance the potency of a broad range of systemically administered small molecule drugs by extending their circulation lifetime, promoting passive accumulation at sites of disease [42,43] and/or by acting as drug reservoirs allowing for the sustained release of the therapeutic payload [40]. Based on these characteristics, it has

been proposed that delivery in lipid-based nanoparticles could be used to improve ODN stability, enhance their delivery to and cellular uptake by target cells and reduce toxicity of ODN therapeutics.

3.2. Liposomes and lipoplexes for ODN delivery

The high solubility of synthetic ODN in aqueous solutions allows them to be encapsulated within lipid particles using a broad range of methods including thin film and reverse-phase hydration as well as detergent dialysis, minimal volume entrapment and enhanced lipophilicity [44]. These passive encapsulation strategies have been used to produce anionic and neutral liposomes as well as modified liposomes tailored to promote cellular targeting and uptake. However, a major limitation of these approaches is their poor ODN encapsulation efficiencies which are generally only in the range of 3–10%. To overcome this drawback, methods of “actively” encapsulating ODN, which employ cationic lipids and can achieve high encapsulation efficiencies based on the association of the positively charged lipids with the negatively charged DNA backbone, have been developed [43,45]. Although widely referred to as cationic liposomes, the majority of work in this area involves, what is more appropriately termed, cationic complexes or lipoplexes as a result of their extremely heterogeneous structure, morphology and size [46]. In addition to their ability to spontaneously condense negatively charged ODN, cationic lipids possess a number of advantages as ODN delivery vehicles, including their natural ability to interact with negatively charged cell surface membranes and promote cellular uptake, and their ability to partially or fully protect associated ODN from degradation by serum nucleases [44,47].

While cationic lipid-containing ODN complexes demonstrate enhanced levels of immunostimulatory activity *in vitro* [48], a number of obstacles limit their use *in vivo*. Cationic complexes have a large,

heterogeneous size distribution (100–2000 nm) and carry a strong net positive charge, which can lead to non-specific interactions with cells, proteins and other macromolecules in the circulation following systemic administration, resulting poor PK/BD characteristics and in the formation of large aggregates (>300 nm in diameter) which become trapped in the capillary beds of the lung [49,50]. Furthermore, a range of adverse side-effects have been reported following their systemic administration including hepatic [51,52] and pulmonary toxicity [53,54] as well as erythrocyte agglutination [55], complement activation [56], the depletion of several blood components and cell types and aggregation-mediated micro infarction [57] all of which can result in lethality [58–61].

3.3. Formulation of lipid-nanoparticulate CpG ODN

In order to achieve high levels of ODN encapsulation and avoid the problems associated with the *in vivo* administration of large cationic particles, our group has developed a method of encapsulation which relies on the presence of an ionizable cationic lipid which is neutral at physiological pH (Fig. 1A). This method of encapsulation relies on the electrostatic interaction of polyanionic ODN with cationic lipid which occurs at the low pH employed during formulation, driving the formation of uni- and/or multi-lamellar lipid nanoparticles that trap ODN within the bilayers (Fig. 1B). Following encapsulation, the system is raised to physiologic pH, removing the cationic surface charge and resulting in the generation of a particle with a neutral surface charge. To achieve small, stable, homogenous systems, the lipid nanoparticles (LN) are formulated with an exchangeable poly (ethylene glycol)-conjugated lipid which provides a surface steric barrier that inhibits vesicle aggregation and fusion during LN-CpG ODN formation but leaves the delivery system rapidly after administration to allow for effective intracellular delivery [62].

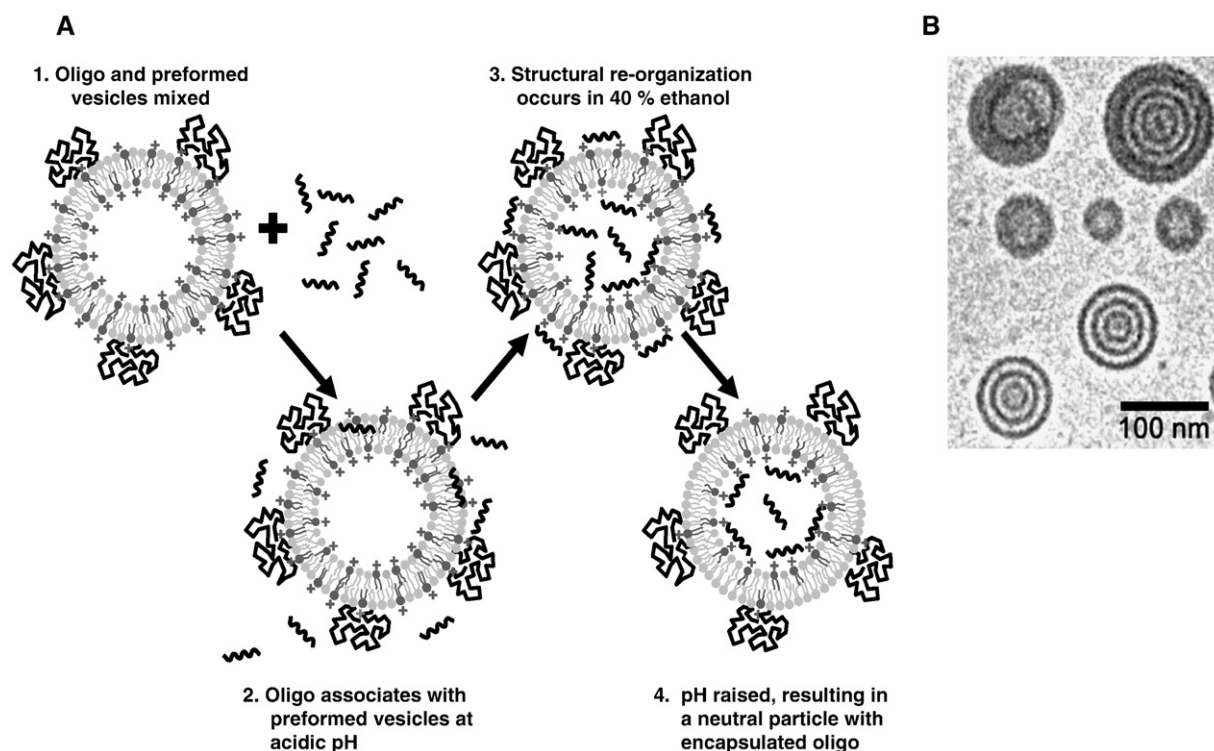


Fig. 1. Production of LN-CpG ODN using pre-formed vesicle technology. Panel A: 1. Preformed LN containing an ionizable lipid are mixed with CpG ODN at subphysiological pH causing the lipid to assume a cationic configuration; 2. Anionic CpG-ODN associates with cationic LN due to electrostatic interaction; 3. In the presence of 40% ethanol, the LN are destabilized allowing structural reorganization of the vesicles, resulting in encapsulation of CpG-ODN; 4. Raising the pH of the finished product causes the ionizable lipid to assume a neutral configuration and unencapsulated CpG-ODN can then be removed. Resultant product is a homogeneous LN population (100 nm ± 20 nm) with high ODN incorporation efficiency of up to 95% and ODN:lipid ratio of up to 0.20 w/w with long circulation properties based on its small size and neutral charge. Adapted from Future Lipidology (2006) 1(1), 35–46 with permission of Future Medicine Ltd [135]. Panel B: Electron micrograph of LN-CpG particles. Particles are uni- or multi-lamellar, largely dependent on ODN:lipid ratio.

LN-CpG ODN consist of a homogeneous population of small (<200 nm diameter), neutral, well tolerated particles that completely encapsulate and fully protect their nucleic acid payload from nuclease degradation [44,63]. LN-CpG ODN possess a high encapsulation efficiency (up to 95%), net neutral charge and high ODN to lipid ratio capable of entrapping up to ~2200 ODN per 100 nm lipid nanoparticle [43]. As detailed below, these systems have demonstrated great potential as immune response modifiers with applications ranging from vaccine adjuvants to enhancing cell mediated antibody-dependent cellular cytotoxicity (ADCC).

4. The benefits of lipid-based delivery

4.1. Lipid-based delivery protects the nucleic acid payload

While the extreme sensitivity to nuclease degradation renders PO ODN inactive *in vivo* [18,19], lipid encapsulation offers an alternative to chemical modification to endow CpG ODN with immunostimulatory activity. Formulation in cationic complexes has been shown to significantly reduce ODN degradation and extend their activity, although this protection is often incomplete [64]. In contrast, while cationic lipid complexes can partially protect the ODN payload, *in vitro* analysis confirms that ODN are completely protected from degradation by serum nucleases when contained within LN [15,44] with metabolites being virtually undetectable following *in vivo* administration. Importantly, this efficient protection enables the use of unmodified PO CpG ODN as an immunotherapeutic *in vivo*. In addition to completely eliminating any PS-associated toxicities, previous research with lipoplexes and LN-CpG ODN have shown that the PO form possesses greater immune potency [65] and induces enhanced anti-tumor responses compared to the equivalent PS form [66]. Enhanced potency of the PO backbone is likely due to more efficient interactions with TLR-9 and possibly through TLR-9 independent pathways [39,67].

4.2. Lipid nanoparticle delivery systems improve the pharmacokinetic characteristics of CpG ODN

One of the predominant rationales for formulating drugs into lipid nanoparticle delivery systems is to optimize the PK and BD characteristics of the free drug. In the free form, i.v. administered PS ODN demonstrates a very rapid clearance from the general circulation and a very broad tissue distribution [33] which is thought to contribute to the observed failure of systemically administered free CpG ODN to elicit appreciable immune responsiveness in human volunteers [8] when compared to the s.c. administration. While delivery of free CpG ODN via the s.c. route generally induces more potent immune responses, likely due to a depot effect in which persistence of CpG ODN at the site of delivery allows uptake by local immune cells, this route suffers from similar degradation and metabolic characteristics as i.v. with lower maximal concentrations [68].

Similarly, traditional cationic lipid complexes containing CpG ODN are also not ideal for systemic delivery in humans as they are also rapidly eliminated from the blood and manifest non-specific immunological activity due to complement activation via the alternative pathway that can result in damage to the liver and other tissues [29]. However, it is well recognized that optimally designed lipid-based carrier systems characterized by minimal surface charge and small particle size, such as the LN delivery systems developed by our group, possess relatively long circulation lifetimes, increasing the half-life from minutes for free CpG ODN to hours [62] or days [69].

4.3. Lipid nanoparticle delivery systems enhance intracellular uptake

Due to its polyanionic charge and large size, free DNA requires an active system to allow intracellular uptake, and although a number of candidates have been proposed to mediate this process, the identity of

the ODN uptake receptor remains elusive [32,70,71]. While uptake of free ODN has been demonstrated to be inefficient and highly saturable, thus limiting the capacity to deliver CpG ODN to TLR9 [67], lipid-based delivery can facilitate the uptake of relatively large amounts of CpG ODN by immune effector cells [72–75] (Fig. 2); the uptake of a single LN-CpG ODN particle is capable of delivering hundreds or thousands of CpG ODN molecules [43].

Although the exact mechanisms have not been completely elucidated, several factors which play a role in the uptake of lipid-based delivery systems have been identified. For example, cationic lipid particles have the ability to interact with negatively charged biological surfaces via electrostatic interactions, to passively promote uptake [76]. Furthermore, various research has demonstrated that clathrin-mediated endocytosis (CME), caveolae-mediated uptake, phagocytosis, macropinocytosis and clathrin- and caveolae-independent endocytosis can all play a role in lipid carrier uptake, with the specific contributions being dictated by cell type [77–79] and physical/chemical properties of the lipid delivery vehicle [80,81]. Similarly, our own research specifically focused on the uptake of LN-CpG ODN has also indicated that multiple pathways, specifically CME and macropinocytosis, are involved.

4.4. Lipid-based delivery systems enhance delivery to target APCs

The increased circulation lifetime of lipid delivery vehicles represents an optimal characteristic for the delivery of conventional small molecule drugs as it allows for extravasation and passive accumulation at sites of disease (i.e. infection, inflammation and tumors) where the vasculature is more permeable [40,82,83]. Extended circulation lifetimes are primarily a function of the ability of lipid nanoparticles to avoid removal from the circulation by phagocytic cells [i.e. macrophages and dendritic cells (DC)] of the reticular endothelial system (RES), largely in the liver and spleen. The rationale for the encapsulation of CpG ODN is therefore, at least on one level, distinctly different than for other lipid formulated therapeutic agents as the primary targets of CpG ODN are the very cells that conventional lipid nanoparticles are designed to avoid. Indeed, it is proposed that the enhanced activity of CpG ODN, when delivered in a lipid-based system is due, in large part, to the preferential tendency of LN delivery systems to naturally target to and be taken up by macrophages and DCs [84]. Work performed by numerous groups, including our own, support this hypothesis and have demonstrated

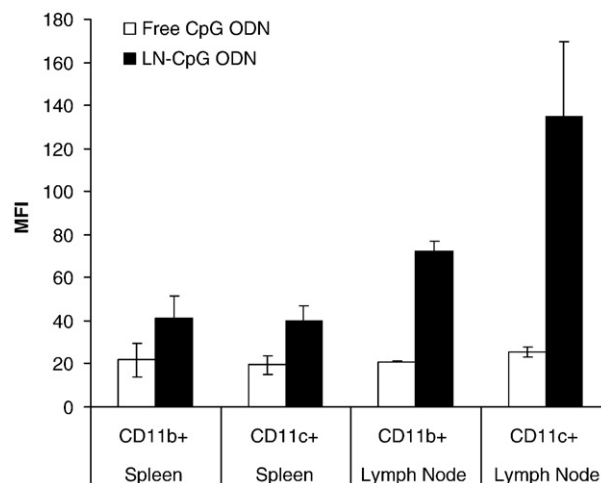


Fig. 2. Encapsulation in lipid nanoparticles enhances uptake of CpG ODN. Five mg/kg of fluorescently labeled free (white bars) and encapsulated (black bars) CpG ODN was administered s.c. to ICR mice (4 animals/group). After 24 h, animals were euthanized and spleens and lymph nodes were harvested and processed to single cell suspensions. Macrophage (CD11b) and dendritic cells (CD11c) were analyzed for uptake (MFI $\pm$ SD) in conjunction with phenotype markers by flow cytometry.

that LN-CpG ODN containing fluorescently labeled ODN are rapidly taken up by APCs in the blood, spleen and lymph node (Fig. 2) compartments following administration by the intravenous or subcutaneous route [74,85].

An interesting characteristic of lipid-based delivery stemming from improved uptake by APCs is that the immune response is relatively insensitive to the route of administration. In this regard, we have demonstrated that despite dramatic differences in tissue distribution profiles following i.v. and s.c. administration, the resultant immune response is very similar. This is demonstrated to be due to efficient uptake of LN-CpG ODN by immune cells, even when present at very low concentrations [85]. These findings are particularly relevant to the therapeutic application of CpG ODN as studies investigating the effects of i.v. and s.c. routes in mice [86] and humans [10] have indicated a lack of immune stimulation following i.v. administration of free ODN. Similarly, we have shown that immune responses induced by LN-CpG ODN, are also relatively insensitive to circulation half-life inducing similar responses, as judged by plasma cytokine levels, immune cell activation and direct and antibody-mediated cytotoxicity, regardless of their clearance characteristics (Raney et al., manuscript in press).

It is important, however, to bear in mind that while we have found a minimal effect of route of administration and PK/BD on LN-CpG ODN activity, these conclusions are based on parameters indicative of systemic immune responses, specifically plasma cytokines and activation/function of peripheral blood and splenic immune cells. While these give an indication of the overall activity of encapsulated CpG ODN, they do not provide insight into localized immune effects prevalent in the tumor or at sites of inflammation and autoimmunity. Therefore, the more traditional benefits of lipid delivery should not be discounted. Accumulation of long circulating lipid particles at sites of disease has the potential to deliver CpG ODN directly to tumors and sites of inflammation and may be able to impact the immune response in these microenvironments, particularly in view of the numerous studies that have demonstrated the activity and efficacy of CpG ODN administered directly to sites of disease [87,88].

Importantly, unlike the majority of lipid based nucleic acid delivery systems used to date where release of the payload from the endosomal compartment is a desired and necessary component of drug activity (i.e. for gene therapy, antisense and siRNA applications), delivery of CpG ODN to late endosomal/lysosomal compartments is required for the induction of immunostimulatory signaling. It is well established that particulates such as lipid nanoparticles allow more efficient access to endocytosis-mediated uptake compared to free ODN, consistent with facilitating CpG-TLR9 interaction in the late endosomal compartment [72]. Importantly, unlike cationic lipoplexes, which are normally

used for intracellular delivery of DNA *in vitro* [89], LN-CpG ODN do not appear to disrupt endosomal structure to release ODN into the cytoplasm. Therefore, they are expected to promote prolonged exposure of CpG ODN to TLR9 in the endosomal compartment.

4.5. Lipid encapsulation enhances the immunostimulatory potency of CpG ODN

Lipid-based nanoparticles have been used in a number of immunostimulatory applications, ranging from carriers of nucleic acid based adjuvants to the delivery of peptide, protein and genetic vaccines, and their ability to enhance resultant immune responses are well documented [90,91]. With respect to CpG ODN, lipid encapsulation dramatically enhances the activation of numerous immune effector cell populations (macrophage, DCs, NK, B-cells and T-cells) and results in a significant elevation in the plasma levels of a number of cytokines including IL-12, IFN- γ , IL-6, MCP-1 and TNF- α , both in terms of magnitude (Fig. 3) and the duration of their expression when compared to free ODN [65,73,74,92]. As detailed above, the ability of lipid-based delivery to enhance the immunopotency of CpG ODN is believed to stem from a number of factors including protection of the nucleic acid payload, improved PK/BD and increased cellular uptake by target APCs as empty cationic lipid particles themselves are well demonstrated to be immunologically inert [92–94].

4.6. Lipid nanoparticle delivery confers activity to non-immunostimulatory sequences

While lipid encapsulation of CpG ODN provides the predicted benefits of protection of the nucleic acid payload, improved PK/BD and enhanced cellular uptake, the unexpected observation that lipid encapsulation can confer immunostimulatory activity on sequences, including methylated (m)CpG ODN, which are non-immunostimulatory (non-IS) in their free-form, has also been made. To this effect, it has been reported, by ourselves [95] and others [39,67], that lipid encapsulation endows mCpG ODN with immunostimulatory activity (Fig. 3) through a TLR9-mediated mechanism. Yasuda et al. hypothesize that while inefficient natural uptake allows only ligands with high affinity (i.e. unmethylated CpG ODN) and not low affinity (i.e. mCpG ODN), to initiate immune responses, delivery in lipid complexes enables accumulation of sufficiently high levels of mCpG ODN to overcome its low affinity for TLR9. In contrast, our results suggest that mCpG ODN is fully capable of interacting with and triggering TLR9 signaling to induce responses that often exceed those seen with the equivalent free or encapsulated, unmethylated CpG ODN. More recently we have reported the novel

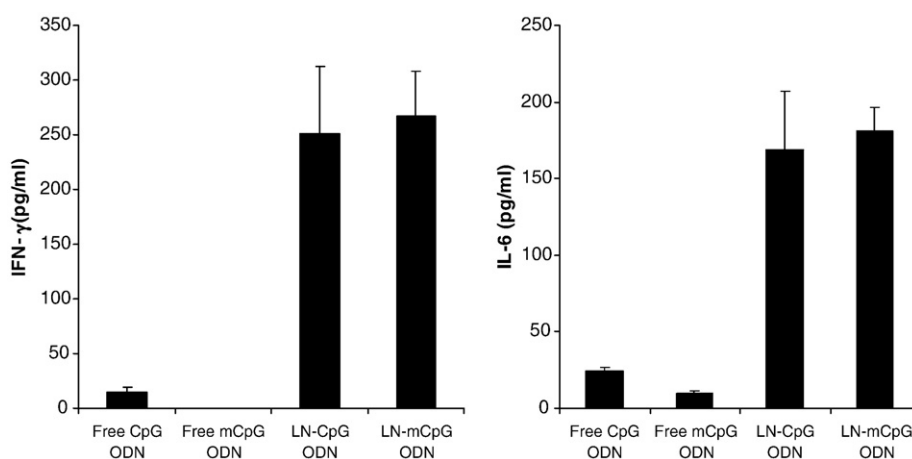


Fig. 3. Encapsulation of CpG ODN in lipid nanoparticles enhances plasma cytokine levels and endows methylated CpG with immunostimulatory activity. Five mg/kg of free or encapsulated, unmethylated or methylated CpG ODN was administered s.c. to ICR mice. Blood was collected from animals by cardiac puncture and processed to collect plasma. Cytokine levels (pg/ml $\pm$ SD) were determined by ELISA. Peak cytokine concentrations at 7 h for free and 24 h for LN-CpG ODN are shown here.

finding that uptake of LN, regardless of payload, promotes TLR9 mobilization to the endosome thus allowing both methylated and unmethylated CpG ODN to co-localize with and engage TLR9 [96]. Based on our data, a model has been proposed whereby LN encapsulation confers immunostimulatory activity to sequences which are non-IS in their free form, and possibly enhance the immunostimulatory activity of CpG ODN, by effectively inducing TLR9 mobilization to the endosomal compartment allowing for co-localization (de Jong et al., manuscript submitted).

5. Immunotherapeutic efficacy of LN-CpG ODN

5.1. Lipid-encapsulated CpG ODN as a standalone agent

Importantly, the potent immunostimulatory activity of encapsulated CpG ODN translates to enhanced efficacy in a range of immunotherapeutic applications. As a standalone immune response modifier, encapsulated CpG ODN stimulates enhanced innate immune responses that can provide generalized protection against infectious, parasitic and malignant disease in a variety of animal models ranging from mice to non-human primates [97]. Lipid-encapsulated CpG ODN has demonstrated the potential to induce superior and more durable protection in murine models of acute respiratory viral infections [98] where up to 80% of animals challenged with ten times the lethal dose of influenza A were protected against challenge, compared to only 40% for the same dose of free ODN. Similarly, lipid-encapsulated CpG ODN has been demonstrated to be highly effective in a murine model of Leishmaniasis, reducing disease burden by 81% compared to 62% for free ODN [99]. The ability of lipid-encapsulation to enhance the activity of CpG ODN as an immune response modifier is of particular interest in view of recent concerns regarding rapidly evolving and newly emergent infectious diseases for which effective therapies do not exist.

Lipid-encapsulated CpG ODN also exert improved immunotherapeutic activity over free ODN as a standalone agent in a number of tumor models. As demonstrated by Whitmore and Dow using a mouse lung metastatic model, systemic administration of DNA or ODN containing lipid complexes can trigger significant anti-tumor activity against established tumors, resulting in inhibition of growth and increased survival [66,100,101]. When employed as a standalone agent, anti-tumor activity exerted by encapsulated CpG ODN is a combination of both innate and subsequently acquired Ag-specific immune responses [66,102]. Enhanced immunotherapeutic efficacy of encapsulated CpG ODN has been confirmed in a number of animal models of cancer including pulmonary metastases [66,103] colon adenocarcinoma [104], intraperitoneal sarcoma [102] human head and neck xenografts [105] and orthotopic glioblastoma [106], indicating that improved efficacy of encapsulated CpG ODN is not restricted to specific cancer or tissue types.

5.2. LN-CpG ODN to enhance monoclonal antibody therapy

In addition to activity as a standalone agent, CpG ODN can also function as an adjunct therapy to enhance the activity of therapeutic agents such as monoclonal antibodies (mAbs; i.e. Herceptin® and Rituxan®) which have become a standard treatment option for a number of cancers due to their significant clinical activity [107]. It has been previously demonstrated that CpG ODN can enhance direct and antibody-mediated (i.e. ADCC) lytic activity as well as induce cell activation and cytokine secretion in both human and murine NK cells, largely in response to IL-12 as well as IFN- α and β and TNF- α secreted from other immune effector cells [108,109]. The ability of CpG ODN to stimulate ADCC is important in view of the fact that most mAbs employ ADCC as part of their mechanism of action [110].

Functionally, free CpG ODN have been shown to enhance the anti-tumor activity of the therapeutic mAbs Herceptin® and Rituxan® in animal models of breast cancer [111], and B-cell lymphoma [112–114], respectively (Fig. 4A). As the expression of TLR9 in humans is restricted

to plasmacytoid (p)DCs and B-cells and is absent from myeloid lineage cells, there have been some questions raised around the likelihood of the ability of CpG ODN to induce parallel activity in humans as NK activation has largely been attributed to IL-12 produced predominantly in macrophages and monocytes. However, significant data exists to show that type I IFNs produced by pDCs are effective activators of NK cells [28,115], and the safety [116] and activity [11,117] of CpG ODN in combination with mAbs has recently been confirmed in humans.

Based on the increased immunopotency of lipid-based CpG ODN, it should not be surprising that LN-CpG ODN are capable of inducing enhanced NK activity and mAb-mediated anti-tumor efficacy when compared to free ODN. Work in our laboratory has demonstrated in murine models that systemic administration of LN-CpG ODN effectively activates NK cells resulting in enhanced cytolytic activity against tumor cells *in vitro*, particularly in the presence of tumor targeted mAbs. Furthermore, LN-CpG ODN also acts to increase the NK cell effector population through increased proliferation, as assessed by BrdU incorporation and homing of activated NK cells to sites of malignant disease. Overall, these effects translate to enhanced anti-tumor efficacy for anti-cancer therapeutic mAbs (Fig. 4A) in a wide range of tumor models as evidenced by increased survival and inhibition of tumor growth [118,119].

5.3. LN-CpG ODN as an adjuvant for prophylactic and therapeutic vaccination

Not surprisingly, a significant body of work also supports the ability of lipid-encapsulated CpG ODN to act as an effective vaccine adjuvant, mediating a 10–100 fold enhancement of Ag-specific

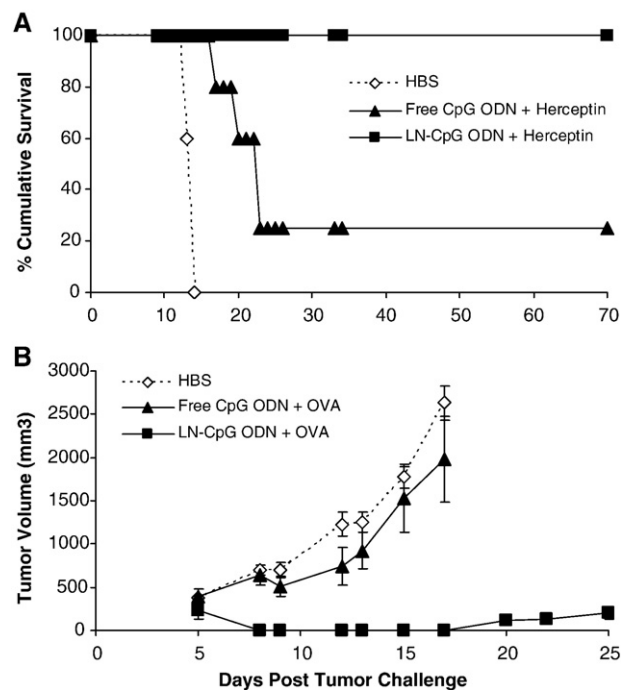


Fig. 4. The anti-tumor activity of CpG ODN as an adjunct therapy to monoclonal antibodies and a cancer vaccine adjuvant is enhanced by encapsulation in lipid nanoparticles. Panel A: Anti-tumor activity of the monoclonal antibody Herceptin® in combination with free or encapsulated CpG ODN in a 38C13-Her2/neu syngeneic tumor model. C3H mice were challenged i.v. with 3×10^3 38C13 B-cell lymphoma cells transduced to express human Her2/neu antigen. Mice were treated with Herceptin® in combination with free or encapsulated CpG ODN and monitored for survival. Panel B: Antigen-specific anti-tumor activity following immunization with OVA adjuvanted with free or encapsulated CpG ODN in an E.G7-OVA xenogeneic tumor model. C57BL/6 mice were immunized prophylactically s.c. with OVA adjuvanted with 100 μ g free or encapsulated CpG ODN. Mice were then challenged s.c. with 2.5×10^6 E.G7-OVA thymoma cells and tumor growth was monitored.

humoral [120] and cellular [74,121,122] immune responses against co-administered Ags compared to free ODN. Similar results obtained with other delivery systems such as synthetic virus like particles [123] serve to emphasize the importance of delivery for the optimal activity of CpG ODN based adjuvants.

As a vaccine adjuvant, lipid-encapsulated CpGs have been shown to be effective for prophylactic vaccination against infectious disease. Vaccination with encapsulated CpG ODN has been found to induce up to 30× higher humoral (Ag-specific serum and Th1-biased IgG2a and IgA Abs) and cellular (splenocyte proliferation, cytotoxicity, IFN- γ production, delayed type hypersensitivity) immune responses against viral Ags compared to free ODN which is concomitant with improved protection against a viral challenge [120]. Importantly, formulated CpG ODN have been shown to be an effective adjuvant for mucosal vaccines, inducing high titres of neutralizing IgA Abs following mucosal immunization [25,120,124–126]. This is particularly important in view of the long held goal of developing vaccines capable of inducing immunity at mucosal surfaces where the majority of pathogens gain access to the body.

Similarly, lipid-formulated CpG ODN has been found to support the generation of protective immunity in a number of animal models of cancer, promoting effective tumor immunoprophylaxis compared to free CpG ODN or other potent adjuvants such as complete Freund's adjuvant. In our own work, the generation of higher frequencies of functional Ag-specific CTLs, as assessed using MHC tetramer (5× increase), IFN- γ secretion (3–4× increase) and cytotoxicity assays (7–20× increase) compared to free ODN, directly supports the characterization of LN-CpG ODN as a highly effective adjuvant that is able to promote generation of TAA-specific immune responses [74]. Importantly, this strategy appears to be capable of not only providing protection against xenogeneic model Ags (Fig. 4B), but also against syngeneic TAAs such as TRP2 in a B16 lung metastasis model, an aggressive tumor expressing only very low levels of MHC class I [74,122,127].

Lipid-based delivery has been routinely used to enhance CD8⁺ T cell responses by improved protein Ag uptake and loading into MHC I by APCs [122]. Within the context of lipid based delivery of CpG ODN, these systems offer the additional advantage of providing a convenient means by which to associate the Ag with the CpG ODN, either through Ag conjugation or co-encapsulation with the CpG ODN, thereby further enhancing the resultant immune response through co-delivery to the same APC [15,128,129]. The simultaneous delivery of Ag and CpG ODN to the same APCs has been extensively shown to enhance the resultant adaptive responses to a number of cancer [128,130,131] and infectious [124,126] and parasitic [132] disease vaccines [130,131,133,134].

6. Concluding remarks

Extensive preclinical and clinical evaluation of CpG ODNs has confirmed their potential as immune response modifiers in a wide variety of applications ranging from standalone agents to vaccine adjuvants for prophylaxis and treatment of infectious and malignant disease and as immunomodulatory agents for the amelioration of allergic and autoimmune disorders. Despite their promise, the clinical utility of free CpG ODN is still faced with a number of significant hurdles. The development of lipid-based systems for the *in vivo* delivery of CpG ODN has successfully addressed a number of these shortcomings by providing an efficient means to reduce toxicity, improve PK/BD characteristics, direct CpG ODN to target cells and increase uptake of CpG ODN. Most importantly, numerous animal studies performed by multiple groups unequivocally demonstrate that these factors translate to dramatically enhanced immunotherapeutic efficacy against a variety of disease indications.

Although the data discussed here provides strong support for the potential of lipid encapsulation to significantly improve the clinical

activity of CpG ODN, a complete understanding of the clinical utility lipid-encapsulated CpG ODN and how its full potential can be harnessed have yet to be fully established. Furthermore, effective preclinical and even early clinical strategies often do not translate to successful human therapies and it is advanced clinical trials that will be needed to determine the clinical utility of free and encapsulated CpG ODN. However, given the validation of CpG ODN activity and efficacy in human trials and of the principles supporting the clinical benefits of liposomal drugs, evidence supports the possibility that encapsulated CpG ODN may represent an optimal strategy by which to modulate the immune system.

References

- [1] E.B. Lindblad, Aluminium compounds for use in vaccines, *Immunol. Cell Biol.* 82 (2004) 497–505.
- [2] N. Petrovsky, J.C. Aguilar, Vaccine adjuvants: current state and future trends, *Immunol. Cell Biol.* 82 (2004) 488–496.
- [3] K.L. Elkins, T.R. Rhinehart-Jones, S. Stibitz, J.S. Conover, D.M. Klinman, Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria, *J. Immunol.* 162 (1999) 2291–2298.
- [4] R.A. Gramzinski, D.L. Doolan, M. Sedegah, H.L. Davis, A.M. Krieg, S.L. Hoffman, Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice, *Infect. Immun.* 69 (2001) 1643–1649.
- [5] D.G. Rees, A.J. Gates, M. Green, L. Eastaugh, R.A. Lukaszewski, K.F. Griffin, A.M. Krieg, R.W. Titball, CpG-DNA protects against a lethal orthopoxvirus infection in a murine model, *Antivir. Res.* 65 (2005) 87–95.
- [6] A.M. Krieg, Therapeutic potential of Toll-like receptor 9 activation, *Nat. Rev., Drug Discov.* 5 (2006) 471–484.
- [7] A.M. Krieg, Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides, *Curr. Oncol. Rep.* 6 (2004) 88–95.
- [8] A.M. Krieg, S.M. Efler, M. Wittpoth, M.J. Al Adhami, H.L. Davis, Induction of systemic Th1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist, *J. Immunother.* 27 (2004) 460–471.
- [9] C.L. Cooper, H.L. Davis, J.B. Angel, M.L. Morris, S.M. Efler, I. Seguin, A.M. Krieg, D.W. Cameron, CPG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults, *Aids* 19 (2005) 1473–1479.
- [10] C.L. Cooper, H.L. Davis, M.L. Morris, S.M. Efler, A.M. Krieg, Y. Li, C. Laframboise, M.J. Al Adhami, Y. Khaliq, I. Seguin, D.W. Cameron, Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluorix influenza vaccine, *Vaccine* 22 (2004) 3136–3143.
- [11] J.W. Friedberg, H. Kim, M. McCauley, E.M. Hessel, P. Sims, D.C. Fisher, L.M. Nadler, R.L. Coffman, A.S. Freedman, Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin's lymphoma: increased interferon- α /beta-inducible gene expression, without significant toxicity, *Blood* 105 (2) (2005) 489–495.
- [12] D.E. Speiser, D. Lienard, N. Rufer, V. Rubio-Godoy, D. Rimoldi, F. Lejeune, A.M. Krieg, J.C. Cerottini, P. Romero, Rapid and strong human CD8⁺ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909, *J. Clin. Invest.* 115 (2005) 739–746.
- [13] C.L. Cooper, H.L. Davis, M.L. Morris, S.M. Efler, M.A. Adhami, A.M. Krieg, D.W. Cameron, J. Heathcote, CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Enderix-B HBV vaccine in healthy adults: a double-blind phase I/II study, *J. Clin. Immunol.* 24 (2004) 693–701.
- [14] H. Hemmi, O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, S. Akira, A Toll-like receptor recognizes bacterial DNA, *Nature* 408 (2000) 740–745.
- [15] F. Takeshita, C.A. Leifer, I. Gursel, K.J. Ishii, S. Takeshita, M. Gursel, D.M. Klinman, Cutting edge: role of toll-like receptor 9 in CpG DNA-induced activation of human cells, *J. Immunol.* 167 (2001) 3555–3558.
- [16] D.M. Klinman, S. Kamstrup, D. Verthelyi, I. Gursel, K.J. Ishii, F. Takeshita, M. Gursel, Activation of the innate immune system by CpG oligodeoxynucleotides: immunoprotective activity and safety, *Springer Semin. Immunopathol.* 22 (2000) 173–183.
- [17] B.J. Weigel, D.A. Rodeberg, A.M. Krieg, B.R. Blazar, CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma, *Clin. Cancer Res.* 9 (2003) 3105–3114.
- [18] J. Kurreck, Antisense technologies. Improvement through novel chemical modifications, *Eur. J. Biochem.* 270 (2003) 1628–1644.
- [19] F. Shi, D. Hoekstra, Effective intracellular delivery of oligonucleotides in order to make sense of antisense, *J. Control. Release* 97 (2004) 189–209.
- [20] G.J. Weiner, Immunostimulatory DNA sequences and cancer therapy, *Springer Semin. Immunopathol.* 22 (2000) 107–116.
- [21] S.A. Halperin, G. Van Nest, B. Smith, S. Abtahi, H. Whitley, J.J. Eiden, A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant, *Vaccine* 21 (2003) 2461–2467.

- [22] A.A. Horner, N. Cinman, A. Ronaghy, E. Raz, Mucosal adjuvant activity of immunostimulatory DNA sequences, *Springer Semin. Immunopathol.* 22 (2000) 133–146.
- [23] G. Hartmann, R.D. Weeratna, Z.K. Ballas, P. Payette, S. Blackwell, I. Suparto, W.L. Rasmussen, M. Waldschmidt, D. Sajuthi, R.H. Purcell, H.L. Davis, A.M. Krieg, Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses *in vitro* and *in vivo*, *J. Immunol.* 164 (2000) 1617–1624.
- [24] D. Verthelyi, V.W. Wang, J.D. Lifson, D.M. Klinman, CpG oligodeoxynucleotides improve the response to hepatitis B immunization in healthy and SIV-infected rhesus macaques, *Aids* 18 (2004) 1003–1008.
- [25] X. Jiao, R.Y. Wang, Q. Qiu, H.J. Alter, J.W. Shih, Enhanced hepatitis C virus NS3 specific Th1 immune responses induced by co-delivery of protein antigen and CpG with cationic liposomes, *J. Gen. Virol.* 85 (2004) 1545–1553.
- [26] A.M. Harandi, K. Eriksson, J. Holmgren, A protective role of locally administered immunostimulatory CpG oligodeoxynucleotide in a mouse model of genital herpes infection, *J. Virol* 77 (2003) 953–962.
- [27] L. Deml, R. Schirmbeck, J. Reimann, H. Wolf, R. Wagner, Immunostimulatory CpG motifs trigger a T helper-1 immune response to human immunodeficiency virus type-1 (HIV-1) gp 160 envelope proteins, *Clin. Chem. Lab. Med.* 37 (1999) 199–204.
- [28] J.D. Marshall, S. Abtahi, J.J. Eiden, S. Tuck, R. Milley, F. Haycock, M.J. Reid, A. Kagey-Sobotka, P.S. Creticos, L.M. Lichtenstein, G. Van Nest, Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes T(H)1 cytokine expression while downregulating T(H)2 cytokine expression in PBMCs from human patients with ragweed allergy, *J. Allergy Clin. Immunol.* 108 (2001) 191–197.
- [29] A.A. Levin, A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides, *Biochim. Biophys. Acta* 1489 (1999) 69–84.
- [30] S. Agrawal, J. Temsamani, W. Galbraith, J. Tang, Pharmacokinetics of antisense oligonucleotides, *Clin. Pharmacokinet.* 28 (1995) 7–16.
- [31] H. Sands, L.J. Gorey-Feret, A.J. Cocuzza, F.W. Hobbs, D. Chidester, G.L. Trainor, Biodistribution and metabolism of internally 3H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate, *Mol. Pharmacol.* 45 (1994) 932–943.
- [32] Q. Zhao, S. Matson, C.J. Herrera, E. Fisher, H. Yu, A.M. Krieg, Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides, *Antisense Res. Dev.* 3 (1993) 53–66.
- [33] R.Z. Yu, R.S. Geary, J.M. Leeds, T. Watanabe, M. Moore, J. Fitchett, J. Matson, T. Burckin, M.V. Templin, A.A. Levin, Comparison of pharmacokinetics and tissue disposition of an antisense phosphorothioate oligonucleotide targeting human Ha-ras mRNA in mouse and monkey, *J. Pharm. Sci.* 90 (2001) 182–193.
- [34] A.M. Krieg, CpG motifs in bacterial DNA and their immune effects, *Annu. Rev. Immunol.* 20 (2002) 709–760.
- [35] J. Kindrachuk, J.E. Potter, R. Brownlie, A.D. Ficzyz, P.J. Griebel, N. Mookherjee, G.K. Mutwiri, L.A. Babiuk, S. Napper, Nucleic acids exert a sequence-independent cooperative effect on sequence-dependent activation of Toll-like receptor 9, *J. Biol. Chem.* 282 (2007) 13944–13953.
- [36] S. Bauer, C.J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, G.B. Lipford, Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition, *PNAS* 98 (2001) 9237–9242.
- [37] T. Haas, J. Metzger, F. Schmitz, A. Heit, T. Muller, E. Latz, H. Wagner, The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation, *Immunity* 28 (2008) 315–323.
- [38] E. Latz, A. Schoenemeyer, A. Visintin, K.A. Fitzgerald, B.G. Monks, C.F. Knetter, E. Lien, N.J. Nilsen, T. Espevik, D.T. Golenbock, TLR9 signals after translocating from the ER to CpG DNA in the lysosome, *Nat. Immunol.* 5 (2004) 190–198.
- [39] K. Yasuda, M. Rutz, B. Schlatter, J. Metzger, P.B. Luppa, F. Schmitz, T. Haas, A. Heit, S. Bauer, H. Wagner, CpG motif-independent activation of TLR9 upon endosomal translocation of “natural” phosphodiester DNA, *Eur. J. Immunol.* 36 (2006) 431–436.
- [40] T.M. Allen, P.R. Cullis, Drug delivery systems: entering the mainstream, *Science* 303 (2004) 1818–1822.
- [41] V. Wagner, A. Dullaart, A.K. Bock, A. Zweck, The emerging nanomedicine landscape, *Nat. Biotechnol.* 24 (2006) 1211–1217.
- [42] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, *Cancer Res.* 46 (1986) 6387–6392.
- [43] N. Maurer, K.F. Wong, H. Stark, L. Louie, D. McIntosh, T. Wong, P. Scherrer, S.C. Semple, P.R. Cullis, Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-stabilized cationic liposomes, *Biophys. J.* 80 (2001) 2310–2326.
- [44] S.C. Semple, S.K. Klimuk, T.O. Harasym, M.J. Hope, Lipid-based formulations of antisense oligonucleotides for systemic delivery applications, *Methods Enzymol.* 313 (2000) 322–341.
- [45] M.B. Bally, P. Harvie, F.M. Wong, S. Kong, E.K. Wasan, D.L. Reimer, Biological barriers to cellular delivery of lipid-based DNA carriers, *Adv. Drug Deliv. Rev.* 38 (1999) 291–315.
- [46] C.R. Dass, Liposome-mediated delivery of oligodeoxynucleotides *in vivo*, *Drug Deliv. Rev.* 9 (2002) 169–180.
- [47] A.R. Thierry, A. Dritschilo, Intracellular availability of unmodified, phosphorothioate and liposomally encapsulated oligodeoxynucleotides for antisense activity, *Nucleic Acids Res.* 20 (1992) 5691–5698.
- [48] K. Yasuda, Y. Ogawa, M. Kishimoto, T. Takagi, M. Hashida, Y. Takakura, Plasmid DNA activates murine macrophages to induce inflammatory cytokines in a CpG motif-independent manner by complex formation with cationic liposomes, *Biochem. Biophys. Res. Commun.* 293 (2002) 344–348.
- [49] O. Meyer, D. Kirpotin, K. Hong, B. Sternberg, J.W. Park, M.C. Woodle, D. Papahadjopoulos, Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides, *J. Biol. Chem.* 273 (1998) 15621–15627.
- [50] R.I. Mahato, K. Anwer, F. Tagliaferri, C. Meaney, P. Leonard, M.S. Wadhwa, M. Logan, M. French, A. Rolland, Biodistribution and gene expression of lipid/plasmid complexes after systemic administration, *Hum. Gene Ther.* 9 (1998) 2083–2099.
- [51] J.D. Tosi, A.L. Gates, L.A. Ingram, C.L. Johnson, J.B. Nietupski, S.H. Cheng, S.J. Eastman, R.K. Scheule, Comprehensive analysis of the acute toxicities induced by systemic administration of cationic lipid:plasmid DNA complexes in mice, *Hum. Gene Ther.* 11 (2000) 2493–2513.
- [52] S. Loisel, C. Le Gall, L. Doucet, C. Ferec, V. Floch, Contribution of plasmid DNA to hepatotoxicity after systemic administration of lipoplexes, *Hum. Gene Ther.* 12 (2001) 685–696.
- [53] Y. Tan, F. Liu, Z. Li, S. Li, L. Huang, Sequential injection of cationic liposome and plasmid DNA effectively transfects the lung with minimal inflammatory toxicity, *Mol Ther* 3 (2001) 673–682.
- [54] S. Dokka, D. Toledo, X. Shi, V. Castranova, Y. Rojanasakul, Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes, *Pharm. Res.* 17 (2000) 521–525.
- [55] H. Eliyahu, N. Serval, A.J. Domb, Y. Barenholz, Lipoplex-induced hemagglutination: potential involvement in intravenous gene delivery, *Gene Ther.* 9 (2002) 850–858.
- [56] L.G. Barron, K.B. Meyer, F.C. Szoka Jr., Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid–DNA complexes, *Hum. Gene Ther.* 9 (1998) 315–323.
- [57] M.J. Wright, E. Rosenthal, L. Stewart, L.M. Wightman, A.D. Miller, D.S. Latchman, M.S. Marber, Beta-galactosidase staining following intracoronary infusion of cationic liposomes in the *in vivo* rabbit heart is produced by microinfarction rather than effective gene transfer: a cautionary tale, *Gene Ther.* 5 (1998) 301–308.
- [58] H.C. Chiou, M.V. Tangco, S.M. Levine, D. Robertson, K. Kormis, C.H. Wu, G.Y. Wu, Enhanced resistance to nuclease degradation of nucleic acids complexed to asialoglycoprotein-polylysine carriers, *Nucleic Acids Res.* 22 (1994) 5439–5446.
- [59] P.R. Dash, M.L. Read, L.B. Barrett, M.A. Wolfert, L.W. Seymour, Factors affecting blood clearance and *in vivo* distribution of polyelectrolyte complexes for gene delivery, *Gene Ther.* 6 (1999) 643–650.
- [60] S.J. Hwang, M.E. Davis, Cationic polymers for gene delivery: designs for overcoming barriers to systemic administration, *Curr. Opin. Mol. Ther.* 3 (2001) 183–191.
- [61] H. Faneca, S. Simoes, M.C. de Lima, Evaluation of lipid-based reagents to mediate intracellular gene delivery, *Biochim. Biophys. Acta* 1567 (2002) 23–33.
- [62] S.C. Semple, S.K. Klimuk, T.O. Harasym, N. Dos Santos, S.M. Ansell, K.F. Wong, N. Maurer, H. Stark, P.R. Cullis, M.J. Hope, P. Scherrer, Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures, *Biochim. Biophys. Acta* 1510 (2001) 152–166.
- [63] S.K. Klimuk, S.C. Semple, P. Scherrer, M.J. Hope, Contact hypersensitivity: a simple model for the characterization of disease-site targeting by liposomes, *Biochim. Biophys. Acta* 1417 (1999) 191–201.
- [64] S. Capaccioli, G. Di Pasquale, E. Mini, T. Mazzei, A. Quattrone, Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum, *Biochem. Biophys. Res. Commun.* 197 (1993) 818–825.
- [65] B. Mui, S.G. Raney, S.C. Semple, M.J. Hope, Immune stimulation by a CpG-containing oligodeoxynucleotide is enhanced when encapsulated and delivered in lipid particles, *J. Pharmacol. Exp. Ther.* 298 (2001) 1185–1192.
- [66] M.M. Whitmore, S. Li, L. Falo Jr., L. Huang, Systemic administration of LPD prepared with CpG oligonucleotides inhibits the growth of established pulmonary metastases by stimulating innate and acquired antitumor immune responses, *Cancer Immunol. Immunother.* 50 (2001) 503–514.
- [67] K. Yasuda, P. Yu, C.J. Kirschning, B. Schlatter, F. Schmitz, A. Heit, S. Bauer, H. Hochrein, H. Wagner, Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways, *J. Immunol.* 174 (2005) 6129–6136.
- [68] J.M. Leeds, S.P. Henry, R. Geary, T. Burckin, A.A. Levin, Comparison of the pharmacokinetics of subcutaneous and intravenous administration of a phosphorothioate oligodeoxynucleotide in cynomolgus monkeys, *Antisense Nucleic Acid Drug Dev.* 10 (2000) 435–441.
- [69] R.Z. Yu, R.S. Geary, J.M. Leeds, T. Watanabe, J.R. Fitchett, J.E. Matson, R. Mehta, G.R. Hardee, M.V. Templin, K. Huang, M.S. Newman, Y. Quinn, P. Uster, G. Zhu, P.K. Working, M. Horner, J. Nelson, A.A. Levin, Pharmacokinetics and tissue disposition in monkeys of an antisense oligonucleotide inhibitor of Ha-ras encapsulated in stealth liposomes, *Pharm. Res.* 16 (1999) 1309–1315.
- [70] H. Wagner, The immunobiology of the TLR9 subfamily, *Trends Immunol.* 25 (2004) 381–386.
- [71] A.H. Dalpke, K. Heeg, CpG-DNA as immune response modifier, *Int. J. Med. Microbiol.* 294 (2004) 345–354.
- [72] K. Yasuda, Y. Ogawa, I. Yamane, M. Nishikawa, Y. Takakura, Macrophage activation by a DNA/cationic liposome complex requires endosomal acidification and TLR9-dependent and -independent pathways, *J. Leukoc. Biol.* 77 (2005) 71–79.
- [73] I. Gursel, M. Gursel, K.J. Ishii, D.M. Klinman, Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides, *J. Immunol.* 167 (2001) 3324–3328.
- [74] S. de Jong, G. Chikh, L. Sekirov, S. Raney, S. Semple, S. Klimuk, N. Yuan, M. Hope, P. Cullis, Y. Tam, Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN, *Cancer Immunol. Immunother.* 56 (2007) 1251–1264.

- [75] K.D. Lee, S. Nir, D. Papahadjopoulos, Quantitative analysis of liposome–cell interactions *in vitro*: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes, *Biochemistry* 32 (1993) 889–899.
- [76] S. Chesnoy, L. Huang, Structure and function of lipid–DNA complexes for gene delivery, *Annu. Rev. Biophys. Biomol. Struct.* 29 (2000) 27–47.
- [77] L.K. Medina-Kauwe, J. Xie, S. Hamm-Alvarez, Intracellular trafficking of nonviral vectors, *Gene Ther.* 12 (2005) 1734–1751.
- [78] T.K. Prasad, N. Rangaraj, N.M. Rao, Quantitative aspects of endocytic activity in lipid-mediated transfections, *FEBS Lett.* 579 (2005) 2635–2642.
- [79] U.S. Huth, R. Schubert, R. Peschka-Suss, Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging, *J. Control. Release* 110 (2006) 490–504.
- [80] J.M. Brewer, K.G. Pollock, L. Tetley, D.G. Russell, Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles, *J. Immunol.* 173 (2004) 6143–6150.
- [81] J. Rejman, M. Conese, D. Hoekstra, Gene transfer by means of lipo- and polyplexes: role of clathrin and caveolae-mediated endocytosis, *J. Liposome Res.* 16 (2006) 237–247.
- [82] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review, *J. Control. Release* 65 (2000) 271–284.
- [83] C. Oussoren, M. Velinova, G. Scherphof, J.J. van der Want, N. van Rooijen, G. Storm, Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. IV. Fate of liposomes in regional lymph nodes, *Biochim. Biophys. Acta* 1370 (1998) 259–272.
- [84] F. Bonte, R.L. Juliano, Interactions of liposomes with serum proteins, *Chem. Phys. Lipids* 40 (1986) 359–372.
- [85] K.D. Wilson, S.G. Raney, L. Sekirov, G. Chikh, S.D. deJong, P.R. Cullis, Y.K. Tam, Effects of intravenous and subcutaneous administration on the pharmacokinetics, biodistribution, cellular uptake and immunostimulatory activity of CpG ODN encapsulated in liposomal nanoparticles, *Int. Immunopharmacol.* 7 (2007) 1064–1075.
- [86] G. Wingender, N. Garbi, B. Schumak, F. Jungerkes, E. Endl, D. von Bubnoff, J. Steitz, J. Striegler, G. Moldenhauer, T. Tuting, A. Heit, K.M. Huster, O. Takikawa, S. Akira, D.H. Busch, H. Wagner, G.J. Hammerling, P.A. Knolle, A. Limmer, Systemic application of CpG-rich DNA suppresses adaptive T cell immunity via induction of IDO, *Eur. J. Immunol.* 36 (2006) 12–20.
- [87] K. Heckelsmiller, S. Beck, K. Rall, B. Sipos, A. Schlamp, E. Tuma, S. Rothenfusser, S. Endres, G. Hartmann, Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy, *Eur. J. Immunol.* 32 (2002) 3235–3245.
- [88] Y. Kawarada, R. Ganss, N. Garbi, T. Sacher, B. Arnold, G.J. Hammerling, NK- and CD8(+) T cell-mediated eradication of established tumors by peritumoral injection of CpG-containing oligodeoxynucleotides, *J. Immunol.* 167 (2001) 5247–5253.
- [89] M.J. Hope, B. Mui, S. Ansell, Q.F. Ahkong, Cationic lipids, phosphatidylethanolamine and the intracellular delivery of polymeric, nucleic acid-based drugs (review), *Mol. Membr. Biol.* 15 (1998) 1–14.
- [90] C.R. Alving, V. Koulchin, G.M. Glenn, M. Rao, Liposomes as carriers of peptide antigens: induction of antibodies and cytotoxic T lymphocytes to conjugated and unconjugated peptides, *Immunol. Rev.* 145 (1995) 5–31.
- [91] G. Gregoriadis, Liposomes as immunoadjuvants and vaccine carriers: antigen entrapment, *Immunomethods* 4 (1994) 210–216.
- [92] Y. Suzuki, D. Wakita, K. Chamoto, Y. Narita, T. Tsuji, T. Takeshima, H. Gyobu, Y. Kawarada, S. Kondo, S. Akira, H. Katoh, H. Ikeda, T. Nishimura, Liposome-encapsulated CpG oligodeoxynucleotides as a potent adjuvant for inducing type 1 innate immunity, *Cancer Res.* 64 (2004) 8754–8760.
- [93] E. Khazanov, D. Simberg, Y. Barenholz, Lipoplexes prepared from cationic liposomes and mammalian DNA induce CpG-independent, direct cytotoxic effects in cell cultures and in mice, *J. Gene Med.* 8 (2006) 998–1007.
- [94] D.J. Marciani, Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity, *Drug Discov. Today* 8 (2003) 934–943.
- [95] S.G. Raney, L. Sekirov, S.D. DeJong, I.I. Brodsky, G. Chikh, Y.K. Tam, Methylated CpG ODNs are potent adjuvants when encapsulated in stabilized lipid particles, 12th International Conference of Immunology and 4th Annual Conference of FOCIS, Montreal, Canada, 2004.
- [96] Y.K. Tam, S.D. deJong, G. Basha, K.D. Wilson, M. Kazem, P. Cullis, Lipid encapsulation promotes co-localization of methylated CpG ODN and TLR9 in late endosomes: a new model for the immunostimulatory activity of CpG DNA, 99th Annual AACR Meeting, San Diego, CA, USA, 2008.
- [97] G.K. Mutwiri, A.K. Nichani, S. Babiuk, L.A. Babiuk, Strategies for enhancing the immunostimulatory effects of CpG oligodeoxynucleotides, *J. Control. Release* 97 (2004) 1–17.
- [98] J.P. Wong, L.P. Nagata, M.E. Christopher, A.M. Salazar, R.M. Dale, Prophylaxis of acute respiratory virus infections using nucleic acid-based drugs, *Vaccine* 23 (2005) 2266–2268.
- [99] N. Datta, S. Mukherjee, L. Das, P.K. Das, Targeting of immunostimulatory DNA cures experimental visceral leishmaniasis through nitric oxide up-regulation and T cell activation, *Eur. J. Immunol.* 33 (2003) 1508–1518.
- [100] S.W. Dow, L.G. Fradkin, D.H. Liggitt, A.P. Willson, T.D. Heath, T.A. Potter, Lipid–DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously, *J. Immunol.* 163 (1999) 1552–1561.
- [101] M. Whitmore, S. Li, L. Huang, LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth, *Gene Ther.* 6 (1999) 1867–1875.
- [102] M. Lanuti, S. Rudginsky, S.D. Force, E.S. Lambright, W.M. Siders, M.Y. Chang, K.M. Amin, L.R. Kaiser, R.K. Scheule, S.M. Albelda, Cationic lipid:bacterial DNA complexes elicit adaptive cellular immunity in murine intraperitoneal tumor models, *Cancer Res.* 60 (2000) 2955–2963.
- [103] F. Sakurai, T. Terada, M. Maruyama, Y. Watanabe, F. Yamashita, Y. Takakura, M. Hashida, Therapeutic effect of intravenous delivery of lipoplexes containing the interferon-beta gene and poly I: poly C in a murine lung metastasis model, *Cancer Gene Ther.* 10 (2003) 661–668.
- [104] S. Sturlan, A. Schneeberger, M. Fang, B.G. Beinhauer, P. Luhrs, R. Kutil, B. Wessner, L. Huang, A.O. Aasen, M.A. Rogy, Intraperitoneal administration of pMP6/liposome complexes inhibits the growth of co-localized colon-26 adenocarcinoma cells by inducing a tumor-specific immune response, *Anticancer Res.* 23 (2003) 4843–4851.
- [105] K.J. Ishii, K. Kawakami, I. Gursel, J. Conover, B.H. Joshi, D.M. Klinman, R.K. Puri, Antitumor therapy with bacterial DNA and toxin: complete regression of established tumor induced by liposomal CpG oligodeoxynucleotides plus interleukin-13 cytotoxin, *Clin. Cancer Res.* 9 (2003) 6516–6522.
- [106] R.J. Higgins, M. McKisic, P.J. Dickinson, D.F. Jimenez, S.W. Dow, L.D. Tripp, R.A. LeCouteur, Growth inhibition of an orthotopic glioblastoma in immunocompetent mice by cationic lipid–DNA complexes, *Cancer Immunol. Immunother.* 53 (2004) 338–344.
- [107] D.G. Maloney, A.J. Grillo-Lopez, C.A. White, D. Bodkin, R.J. Schilder, J.A. Neidhart, N. Janakiraman, K.A. Foon, T.M. Liles, B.K. Dallaire, K. Wey, I. Royston, T. Davis, R. Levy, IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma, *Blood* 90 (1997) 2188–2195.
- [108] Z.K. Ballas, W.L. Rasmussen, A.M. Krieg, Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA, *J. Immunol.* 157 (1996) 1840–1845.
- [109] G.J. Weiner, H.M. Liu, J.E. Wooldridge, C.E. Dahle, A.M. Krieg, Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization, *Proc. Natl. Acad. Sci. U. S. A.* 94 (1997) 10833–10837.
- [110] N. Villamor, E. Montserrat, D. Colomer, Mechanism of action and resistance to monoclonal antibody therapy, *Semin. Oncol.* 30 (2003) 424–433.
- [111] J.M. Roda, R. Parihar, W.E. Carson III, CpG-containing oligodeoxynucleotides act through TLR9 to enhance the NK cell cytokine response to antibody-coated tumor cells, *J. Immunol.* 175 (2005) 1619–1627.
- [112] H.H. van Ojik, L. Bevaart, C.E. Dahle, A. Bakker, M.J. Jansen, M.J. van Vugt, J.G. van de Winkel, G.J. Weiner, CpG-A and B oligodeoxynucleotides enhance the efficacy of antibody therapy by activating different effector cell populations, *Cancer Res.* 63 (2003) 5595–5600.
- [113] T.L. Warren, C.E. Dahle, G.J. Weiner, CpG oligodeoxynucleotides enhance monoclonal antibody therapy of a murine lymphoma, *Clin. Lymphoma* 1 (2000) 57–61.
- [114] H. Wang, E.R. Rayburn, W. Wang, E.R. Kandimalla, S. Agrawal, R. Zhang, Chemotherapy and chemosensitization of non-small cell lung cancer with a novel immunomodulatory oligonucleotide targeting Toll-like receptor 9, *Mol. Cancer Ther.* 5 (2006) 1585–1592.
- [115] J.B. Swann, Y. Hayakawa, N. Zerafa, K.C. Sheehan, B. Scott, R.D. Schreiber, P. Hertzog, M.J. Smyth, Type I IFN contributes to NK cell homeostasis, activation, and antitumor function, *J. Immunol.* 178 (2007) 7540–7549.
- [116] J.P. Leonard, B.K. Link, C. Emmanouilides, S.A. Gregory, D. Weisdorf, J. Andrey, J. Hainsworth, J.A. Sparano, D.E. Tsai, S. Horning, A.M. Krieg, G.J. Weiner, Phase I trial of toll-like receptor 9 agonist PF-3512676 with and following rituximab in patients with recurrent indolent and aggressive non Hodgkin's lymphoma, *Clin. Cancer Res.* 13 (2007) 6168–6174.
- [117] E. Moga, E. Alvarez, E. Canto, S. Vidal, J.L. Rodriguez-Sanchez, J. Sierra, J. Briones, NK cells stimulated with IL-15 or CpG ODN enhance rituximab-dependent cellular cytotoxicity against B-cell lymphoma, *Exp. Hematol.* 36 (2008) 69–77.
- [118] L. Sekirov, I. Brodsky, S.G. Raney, G. Chikh, Y.K. Tam, Intravenous administration of a liposomal CpG ODN (INX-0167) activates NK cells and potentiates ADCC, 12th International Conference of Immunology and 4th Annual Conference of FOCIS, Montreal, Canada, 2004.
- [119] Y.K. Tam, L. Sekirov, S.G. Raney, I. Brodsky, G. Chikh, Liposomal encapsulation enhances the immunopotency of CpG ODN and potentiates ADCC by activation, expansion and enhanced homing of NK cells 97th Annual AACR Meeting, Washington, D.C., USA, 2006.
- [120] A. Joseph, I. Louria-Hayon, A. Plis-Finarov, E. Zeira, Z. Zakay-Rones, E. Raz, T. Hayashi, K. Takabayashi, Y. Barenholz, E. Kedari, Liposomal immunostimulatory DNA sequence (ISS-ODN): an efficient parenteral and mucosal adjuvant for influenza and hepatitis B vaccines, *Vaccine* 20 (2002) 3342–3354.
- [121] T. Yoshikawa, S. Imazu, J.Q. Gao, K. Hayashi, Y. Tsuda, N. Okada, Y. Tsutsumi, M. Akashi, T. Mayumi, S. Nakagawa, Non-methylated CpG motif packaged into fusogenic liposomes enhance antigen-specific immunity in mice, *Biol. Pharm. Bull.* 29 (2006) 105–109.
- [122] K. Zaks, M. Jordan, A. Guth, K. Sellins, R. Kedl, A. Izzo, C. Bosio, S. Dow, Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes, *J. Immunol.* 176 (2006) 7335–7345.
- [123] T. Storni, C. Ruedl, K. Schwarz, R.A. Schwendener, W.A. Renner, M.F. Bachmann, Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects, *J. Immunol.* 172 (2004) 1777–1785.
- [124] V. Alcon, M. Baca-Estrada, M. Vega-Lopez, P. Willson, L.A. Babiuk, P. Kumar, R. Hecker, M. Foldvari, Mucosal delivery of bacterial antigens and CpG oligonucleotides formulated in biphasic lipid vesicles in pigs, *Aaps J.* 7 (2005) E566–E571.
- [125] L.A. Mitchell, A. Joseph, E. Kedari, Y. Barenholz, E. Galun, Mucosal immunization against hepatitis A: antibody responses are enhanced by co-administration of synthetic oligodeoxynucleotides and a novel cationic lipid, *Vaccine* 24 (2006) 5300–5310.
- [126] M. Tafaghodi, M.R. Jaafari, S.A. Sajadi Tabassi, Nasal immunization studies using liposomes loaded with tetanus toxoid and CpG-ODN, *Eur. J. Pharm. Biopharm.* 64 (2006) 138–145.

- [127] P.L. Lollini, C. De Giovanni, G. Nicoletti, A. Bontadini, P.L. Tazzari, L. Landuzzi, K. Scotlandi, P. Nanni, Enhancement of experimental metastatic ability by tumor necrosis factor- α alone or in combination with interferon- γ , *Clin. Exp. Metastasis* 8 (1990) 215–224.
- [128] W.M. Li, W.H. Dragowska, M.B. Bally, M.P. Schutze-Redelmeier, Effective induction of CD8 $^{+}$ T-cell response using CpG oligodeoxynucleotides and HER-2/neu-derived peptide co-encapsulated in liposomes, *Vaccine* 21 (2003) 3319–3329.
- [129] V. Jerome, A. Graser, R. Muller, R.E. Kontermann, A. Konur, Cytotoxic T lymphocytes responding to low dose TRP2 antigen are induced against B16 melanoma by liposome-encapsulated TRP2 peptide and CpG DNA adjuvant, *J. Immunother.* 29 (2006) 294–305.
- [130] G. Chikh, M.P. Schutze-Redelmeier, Liposomal delivery of CTL epitopes to dendritic cells, *Biosci. Rep.* 22 (2002) 339–353.
- [131] G.G. Chikh, S. Kong, M.B. Bally, J.C. Meunier, M.P. Schutze-Redelmeier, Efficient delivery of Antennapedia homeodomain fused to CTL epitope with liposomes into dendritic cells results in the activation of CD8 $^{+}$ T cells, *J. Immunol.* 167 (2001) 6462–6470.
- [132] A. Badiie, M.R. Jaafari, A. Samiei, D. Soroush, A. Khamesipour, Coencapsulation of CpG oligodeoxynucleotides with recombinant *Leishmania* major stress-inducible protein 1 in liposome enhances immune response and protection against leishmaniasis in immunized BALB/c mice, *Clin. Vaccine Immunol.* 15 (2008) 668–674.
- [133] D.T. O'Hagan, M. Singh, Microparticles as vaccine adjuvants and delivery systems, *Expert Rev. Vaccines* 2 (2003) 269–283.
- [134] A. Pashine, N.M. Valiante, J.B. Ulmer, Targeting the innate immune response with improved vaccine adjuvants, *Nat. Med.* 11 (2005) S63–S68.
- [135] Y. Tam, Liposomal encapsulation enhances the activity the immunostimulatory oligonucleotides, *Future Lipidology* 1 (2006) 35–46.