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Liposome–nucleic acid immunotherapeutics

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Cationic liposome–nucleic acid complexes, which were originally developed for use as non-viral gene delivery vectors, may now have an equally important application as immunotherapeutic drugs. Recent studies have highlighted the ability of cationic liposomes to potently activate the innate immune system when used to deliver certain Toll-like receptor (TLR) agonists. The immune-enhancing properties of cationic liposomes have been most clearly demonstrated when combined with nucleic acid agonists for endosomally located TLRs, including TLR3, TLR7/8 and TLR9. Immune potentiation by cationic liposomes likely results from the combined effects of endosomal targeting, protection of nucleic acids from extracellular degradation, and from signaling via newly identified cytoplasmic receptors for nucleic acids. The potent innate immune stimulatory properties of liposome–nucleic acid complexes make them particularly attractive as non-specific immunotherapeutics and as vaccine adjuvants. Liposome–nucleic acid complexes have demonstrated impressive anticancer activity in a number of different animal tumor models. Moreover, liposome–nucleic acid complexes have also been shown to be effective for immunotherapy of acute viral and bacterial infections, as well as chronic fungal infections. When used as vaccine adjuvants, liposome–nucleic acid complexes target antigens for efficient uptake by dendritic cells and are particularly effective in eliciting $CD8^+$ T-cell responses to protein antigens. Thus, liposome–nucleic acid complexes form a potent and versatile immunotherapeutic platform.

Keywords: adjuvants, innate immune, interferons, Toll-like receptors

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1. Introduction

Our understanding of the innate immune system has advanced remarkably over the past decade. Among the more important developments has been the identification of pattern recognition receptors (PRR), including particularly the Toll-like receptor (TLR) family. At the same time, natural ligands for many PRRs were identified and synthetic agonists developed. There is also now a much better understanding of the interconnectedness of innate and adaptive immune systems and how the initial activation of innate immune responses strongly shapes subsequent adaptive immune responses.

As our knowledge of innate immunity has increased, so too have efforts to harness the innate immune system for therapeutic benefit. In this review, the author discusses the components of the innate immune system that have evolved to recognize nucleic acids and related molecules, including the TLRs, NOD-like receptors (NLRs) and intracellular nucleic acid receptors such as RIG-I and DNA-dependent activator of interferon regulatory factors (DAI). The mechanisms by which cationic liposomes enhance the immune stimulatory properties of nucleic acid ligands for TLRs are also discussed. Finally, the use of cationic

liposome–nucleic acid complexes for immunotherapy is covered, including their use in cancer immunotherapy and the development of vaccine adjuvants.

2. Pattern recognition receptors and the Toll-like receptor family

The recognition of pathogens by the innate immune system depends critically on a series of innate immune receptors known as PRRs. These are germ line-encoded receptors that recognize structural features common to broad classes of microorganisms [1-4]. There are at least four families of mammalian PRRs, which include the TLRs, the NLRs, the C-type lectin receptors and the triggering receptors expressed on myeloid cells [2,5,6]. All of the TLRs are membrane bound, as are the C-type lectin receptors (e.g., DC-SIGN) and the triggering receptors expressed on myeloid cells (e.g., macrophage scavenger receptor and the macrophage mannose receptor). In contrast, members of the NLR family, as well as the cytoplasmic nucleic acid receptors, are expressed primarily in the cytoplasm of cells [7-10]. All of these receptors are believed to have evolved primarily to recognize pathogens and trigger their elimination. Examples of pathogen-derived molecules recognized by PRRs include bacterial lipopeptides and lipopolysaccharides, bacterial peptidoglycans, bacterial flagellins, fungal and bacterial mannans and viral and bacterial nucleic acids.

The TLR family of receptors and their agonists have received intense research scrutiny, in part because of their widespread use in immunotherapy. At present, 13 different TLRs have been described in humans and mice [2-4,11,12]. In addition, natural (microbe-derived) or synthetic agonists have been identified for nearly all of the known TLRs [4,10]. In some cases, endogenous (host-derived) ligands have also been identified [11]. Cells of the innate immune system, including macrophages, monocytes, and dendritic cells, typically express the greatest variety and highest density of TLRs, although a variety of other cell types may also express TLRs. For example, epithelial cells often express high levels of certain TLRs, including TLR2 and TLR4. Epithelial cell TLRs may play important roles in the maintenance of epithelial cell integrity, as well as in response to microbial pathogens at mucosal surfaces.

Ligand binding to the ligand recognition domain (leucine-rich region) of the TLR triggers conformational changes in the receptor that result in recruitment of a series of adaptor proteins that interact with the Toll/IL-1 homology region of the TLR [10,11]. Most TLRs signal via a NF-κB-mediated pathway, which ultimately results in the release of pro-inflammatory cytokines such as TNF, IL-1β, IL-12 and IL-6. TLR activation can also signal via an interferon regulatory factor-dependent pathway that is variably dependent on NF-κB signaling, and results in the production of type I interferons. The chemokines and pro-inflammatory cytokines released upon TLR activation recruit immune effector cells to the site of stimulation, stimulating additional local cytokine release and activating adjacent cells and additional immune effector mechanisms, resulting eventually in the generation of adaptive immune responses [13,14].

3. NOD-like receptor family of pattern recognition receptors

The NLR family also plays an important role in regulation of innate immunity [7,9]. The NLR family includes proteins previously identified as members of the CATERPILLAR (CARD, transcription enhancer, R [purine] binding, pyrin, lots of leucine-rich regions) NOD and NALP groups of proteins [15]. Members of this family all share a three-component structure comprised of a leucine-rich region, responsible for ligand binding, a central NOD (nucleotide binding oligomerization domain) region with ATPase activity, and an amino terminal region comprised of protein–protein interaction domains known as CARDs or pyrins.

The intracellular location of NLRs implies that they do not interact directly with pathogens. Instead, these receptors are thought to bind to processed fragments of microorganisms [15]. Indeed, the major NLR agonists identified so far consist primarily of the degradation products of bacterial peptidoglycans [9]. For example, γ-D-glutamyl, meso-diaminopimelic acid and muramyl dipeptide, both of which are bacterial peptidoglycan-derived peptides, activate the NOD1 and NOD2 receptors, respectively. However, there is still considerable uncertainty regarding how NLR ligands, many of which are derived from extracellular bacteria, actually gain access to the cytosolic compartment in order to interact with the receptors and activate cells. The NOD1 and NOD2 molecules are expressed primarily in antigen-presenting cells and epithelial cells – sites where exposure to microbes is likely to occur. Although antigen-presenting cells express both NOD1 and NOD2 molecules, only NOD1 is expressed at high levels in epithelial cells examined so far, including especially intestinal epithelial cells [16].

The activation of NLRs results in triggering in many of the same NF-κB-dependent pathways that are activated by TLR signaling [7,15]. However, it is likely that there are qualitative as well as quantitative differences in the immune responses elicited by the TLR and NLR family of receptors, although as yet this issue has not yet been thoroughly investigated. Undoubtedly, differences in immune responses elicited by these two classes of receptors will have important implications for the development of immunotherapeutics based on the TLR and NLR pathways.

Diseases associated with the dysregulation of NLR-dependent immune responses are presently the subject of considerable research interest. For example, gain-of-function mutations in the NOD2 gene are associated with common inflammatory bowel diseases in humans [16-18]. In addition, mice lacking a critical signaling

molecule (CARD9) in the NOD2 complex develop abnormal immune responses and fail to clear intestinal bacterial infections [18]. The recognition of intestinal bacteria by NOD1 is also proposed to serve an important role in the maintenance of intestinal epithelial cell integrity and protection from infection [19,20].

4. Intracellular receptors for nucleic acids

Recently, a series of cytoplasmic receptors that recognize intracellular nucleic acids have been identified. Activation of these receptors elicits strong production of type I interferons, as typically occurs in response to certain viral and bacterial infections. The molecules RIG-I and MDA5 are intracytoplasmic RNA helicases responsible in part for the induction of interferon responses to intracellular double-stranded viral RNA or to the viral RNA analog polyinosine-polycytidylic acid (polyI:C) [21-23]. These RNA receptors may also play a role in the synergistic activation of innate immunity observed when synthetic double stranded RNA or polyI:C are delivered into the cytoplasm of cells using cationic liposomes. In addition, a cytoplasmic receptor for DNA (DAI) has been described recently [24]. This receptor was identified in part based on its ability to increase the production of IFN-α following the treatment of dendritic cells with CpG oligodeoxynucleotides (ODNs) complexed to cationic liposomes [24-26].

5. Toll-like receptor and NOD-like receptor agonists as immunotherapeutics

The identification of the first TLR (TLR4) led to a remarkable resurgence of interest in innate immunity and prompted the hunt for additional receptors and their agonist. As the field developed, it quickly became apparent that PRR agonists could be used for immunotherapy by targeting the activation of specific TLRs [4,6,27,28]. So far, the greatest success has been achieved using synthetic TLR agonists, which are generally based on modifications of known natural TLR ligands. In some cases, completely synthetic small molecule agonists have also been developed, particularly in the case of TLR7/8 agonists.

Both TLR4 and TLR9-based immunotherapeutics have progressed well into clinical development [6,28,29]. Presently, a TLR4-based vaccine adjuvant (MPL®; Corixa and GlaxoSmithKline) is being developed primarily for use in vaccines against infectious agents. TLR9-based agonists are being evaluated for cancer immunotherapy, allergy and asthma immunotherapy, and for use as vaccine adjuvants [29]. Synthetic ODNs that contain unmethylated CpG dinucleotide repeats are widely used as synthetic TLR9 agonists [6,29]. Both bacterial and plasmid DNA molecules are enriched in CpG sequences, relative to mammalian DNA, which accounts for their ability to also stimulate the innate immune system.

Toll-like receptor 9 is primarily expressed within the endosomal compartment of dendritic and B cells and these cells can be efficiently activated with CpG ODNs. The administration of CpG ODNs results in rapid immune activation, primarily in draining lymph nodes and in the spleen, triggering release of pro-inflammatory cytokines including TNF, IL-1β, IL-6, IL-12, IFN-γ, IFN-β and IFN-α [29,30]. Immunotherapy with TLR9 agonists elicits cytokine responses with a definite Th1-bias, which generally promotes the development of cell-mediated immune responses necessary to control intracellular pathogens and tumor growth [28]. TLR3 agonists such as polyinosine-polycytidylic acid (polyI:C) also promote the development of Th1 immune responses, as do small molecule agonists of TLR7/8, such as the imidazoquinoline compounds [11].

Earlier studies demonstrated that liposomes could be used to enhance the immune stimulatory properties of the NOD2 agonists muramyl dipeptide and muramyl tripeptide (MTP) [31-33]. In fact, complexes of phosphatidylethanolamine liposomes and MTP (MTP-PE) were among the first liposome-based immunotherapeutics developed. These compounds were extensively evaluated for adjuvant therapy of melanoma and sarcoma in dogs and also in humans [32,34-36]. The immunologic mechanism of action of MTP-PE appears to primarily involve macrophage activation and the release of pro-inflammatory cytokines such as TNF [37].

6. Liposomes potentiate immune activation by nucleic acids

Shortly after the immune stimulatory properties of bacterial DNA were described, several groups discovered that cationic liposomes could markedly enhance the immunogenicity of both bacterial DNA and CpG ODN. This discovery occurred largely as a result of research wherein liposome–nucleic acid complexes were used for intravenous non-viral gene delivery for the expression of transgenes in the lung [38]. Cationic liposomes have been extensively used for non-viral gene delivery with plasmid-based gene therapy vectors, and were initially considered safer and less immunogenic than viral-vectored gene therapy [38,39]. However, it was subsequently found that when mice were intravenously injected with high doses of cationic liposome–DNA complexes (CLDCs), they manifested signs consistent with a massive activation of immunity. The immune response elicited by the intravenous injection of high doses of CLDC was sufficient to trigger toxicity. The authors reported that the toxicity and antitumor activity elicited by the intravenous injection of CLDC were both mediated by the innate immune system, particularly by the activation of NK cells and subsequent IFN-γ production [40]. Many of these findings were later confirmed by other groups [41-44].

Cationic liposomes complexed with bacterial DNA are extremely potent immune response activators. For example, on a per-weight basis, CLDCs have been shown to elicit much greater immune stimulation than other conventional activators of innate immunity, including lipopolysaccharide and polyI:C [40,41]. Cationic liposomes also potentiate the activation of innate immunity by CpG ODN, in addition to plasmid DNA [42,45]. Moreover, even non-phosphorothioate modified CpG ODNs are highly stimulatory following systemic delivery with cationic liposomes (S Dow; unpublished data). In fact, even mammalian DNA becomes a potent immune stimulant when combined with cationic liposomes, as recently demonstrated in a murine tumor model [46]. Cationic liposomes can also be used to potentiate immune activation by other nucleic acid TLR agonists, including the TLR3 agonist polyI:C and TLR7/8 agonists such as single-stranded and double stranded RNA [47-50].

Gene delivery using liposome–DNA complexes is relatively inefficient, and high doses are typically required for effective systemic gene transfer. Because systemic non-viral gene therapy requires such high doses of plasmid DNA and liposomes, excessive immune stimulation has been the major barrier to the translation of this technology to humans. Moreover, strong activation of innate immunity and particularly the induction of type I interferon responses also contributes to the very short duration of *in vivo* gene expression usually observed following liposome-mediated gene transfer [51]. Thus, unintended immune activation accounts for many of the problems that continue to plague the non-viral gene therapy field.

In contrast, it is much easier to adapt liposome–nucleic acid complexes for use in immunotherapy. For one, the doses of liposome–nucleic acid complexes required are much lower, as marked systemic activation of innate immunity is not needed for most applications. In addition, clear cut dose response relationships are typically observed between the degree of immune stimulation (e.g., cytokine release) and the dose of liposome–nucleic acid complex administered, thus making it easier to develop safe and effective dosing strategies [52].

Unintended activation of innate immunity has also been encountered when cationic liposomes were used to delivery short interfering RNA (siRNA) molecules [53]. For example, it was noted that the introduction of siRNA molecules into cells using liposomes frequently resulted in the activation of the interferon system [54,55]. This effect was found in most instances to be mediated by the activation of the double stranded RNA-dependent kinase PKR. Thus, activation of innate immunity is likely to occur anytime cationic liposomes are used to deliver any nucleic acid to eukaryotic cells, either *in vitro* or *in vivo*, and this likely side effect should be taken into consideration whenever designing siRNA experiments involving liposomal delivery.

7. The influence of cationic liposomes on intracellular trafficking and immune activation by nucleic acid molecules

Complexes of cationic liposomes and nucleic acids enter cells via endosomal uptake [56-58]. After endocytosis, liposome–nucleic acid complexes are localized initially to the early endosomal compartment. Importantly, the uptake of liposome complexes is an active process and is not mediated to any appreciable degree by liposomal fusion with the cell membrane. Uptake is dependent on the clathrin-mediated pathway and can be inhibited by cholesterol depletion [59]. The localization of complexes in the early endosomal compartment probably accounts for most of the potentiation of immune activation that occurs following liposomal delivery of DNA and other nucleic acids, inasmuch as TLR9 as well as TLR3 and TLR7/8 are all expressed primarily in the endosomal membrane [10,11,60]. Interestingly, the release of DNA from the liposome complex, which is required in order for the DNA to interact with TLR9 and probably the cytoplasmic DNA receptor as well, appears to be mediated primarily by interactions with anionic liposomes released from the endosomal membrane [61]. Thus, this sequence of events probably explains why helper or neutral liposomes are often required along with cationic liposomes for the efficient entry of DNA into the cytoplasm of cells following liposomal delivery.

The physical properties (e.g., charge, higher order molecular structure) of liposome–nucleic acid complexes also affect the nature of the immune response elicited. For example, Guiducci *et al.* [62] investigated the interaction of CpG ODNs with TLR9 in plasmacytoid dendritic cells and demonstrated that the production of IFN-α was strongly upregulated when CpG ODN complexes were localized to the early endosome. In contrast, the localization of the liposome–CpG ODN complexes to the late endosome or lysosome resulted in dendritic cell maturation, but without the production of IFN-α. Thus, the manipulation of the physical properties of cationic liposome–nucleic acid complexes should be considered when optimizing the design of liposome–nucleic acid complexes as immunotherapeutics.

It is also important to note that cationic liposomes do not increase the immune stimulatory properties of all PRR agonists. For example, cationic liposomes are relatively ineffective in augmenting immune activation when combined with ligands or agonists for TLRs expressed on the cell surface (e.g., TLR2 and TLR4) [47]. The delivery of TLR agonists with liposomes probably sequesters the TLR agonist within endosomal and lysosomal compartments, thereby blocking access to the extracellularly expressed TLRs. Thus, as a general rule, cationic liposomes are most effective in augmenting immune activation when complexed with agonists whose receptors are expressed in intracellular compartments, such as the endosomally expressed TLRs

(e.g., TLR3, TLR7/8 and TLR9) and the cytosolically expressed nucleic acid receptors (e.g., RIG-I and DAI).

The interaction of cationic liposomes with nucleic acids elicits both qualitative and quantitative changes in the immune responses elicited by nucleic acids alone. Clearly, liposome–nucleic acid complexes are more potent when total levels of cytokine production are compared. For example, the addition of cationic liposomes to CpG ODNs or plasmid DNA consistently elicits a 10- to 100-fold increase in cytokine release both *in vitro* and *in vivo*, particularly in terms of release of type I and II interferons [40,51]. However, there are also important qualitative differences in immune responses elicited by liposome-complexed agonists. Notably, liposomal delivery of CpG ODNs has been shown to activate several TLR9-independent pathways when compared with responses elicited by CpG ODNs alone [24-26]. In part, these differences may be the result of the binding of DNA to a newly described cytosolic receptor: DAI [24]. This interaction is postulated to occur when liposomes facilitate the escape of DNA from the endosomal compartment and into the cell cytoplasm where DAI is located. One of the major differences noted following the activation of cytosolic nucleic acid receptor pathways by liposome-delivered nucleic acids was a marked increase in the production of type I interferons [24,26,63]. Thus, cationic liposomes may fundamentally alter the immune stimulatory properties of nucleic acids, largely as a consequence of changing their intracellular trafficking pathways.

Other positively charged molecules, in addition to cationic liposomes, can be used to augment the innate immune stimulatory effects of plasmid DNA or CpG ODNs. For example, Cui *et al.* [64] demonstrated increased immune activation as a result of complexing nucleic acids with protamine sulfate. This phenomenon was also recently illustrated in bacterial and viral infection models. Bacteria and viruses that were able to gain entry into the cytoplasm of infected cells induced significantly greater innate immune activation than bacteria or viruses that failed to enter the cell cytoplasm, presumably because the intracellular microbes released DNA into the cytoplasm [25]. It was also noted in these studies that the induction of IFN-α release by intracellular microbes was TLR independent, suggesting activation of cytoplasmic nucleic acid receptors [24].

8. The biodistribution of liposome–nucleic acid complexes

The biodistribution of liposome–nucleic acid complexes used as immunotherapeutics has not been formally investigated. However, information regarding the distribution of these compounds *in vivo* can be inferred from previous studies in which nearly identical complexes were used for systemic gene delivery. For example, following the intravenous injection of CLDC formulated with the cationic liposome DOTIM (octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium chloride) and cholesterol and plasmid DNA, the DNA was found to be widely distributed through the body, with the highest levels of plasmid DNA accumulation occurring in the lung, liver and spleen [39,65]. Within 5 min of intravenous injection, the majority of plasmid DNA was found in the lung, and by 2 h, > 95% of injected DNA was found in the liver [65]. By 24 h postinjection, intact plasmid DNA could be found in nearly all organs, although not in the bloodstream. Others have found that the lung is the major site of accumulation of plasmid DNA when complexes prepared with a net positive charge are intravenously injected [66,67]. Plasmid DNA in the lung has been found to be rapidly degraded, and free plasmid DNA was excreted in the urine [68]. The half-life in circulation and the biodistribution of liposome–DNA complexes was also found to be dependent on the physicochemical properties of the complexes, including net charge and particle size [69]. Thus, it appears that the lung and liver are the major early sites of DNA accumulation following intravenous delivery of liposome–plasmid DNA complexes, with later distribution to most major organs, followed by slow degradation of intact plasmid DNA molecules. Much less is known about the biodistribution of liposome–nucleic acid complexes following other routes of delivery, or when complexes are prepared with nucleic acids other than DNA.

9. The use of cationic liposome–nucleic acid complexes for cancer immunotherapy

A great deal of attention has been recently focused on the therapeutic uses of PRR agonists for cancer immunotherapy [4,6,27,28]. The majority of studies have been perfomred using TLR9 agonists – particularly CpG ODNs – and it is clear that CpG ODNs are potent inducers of antitumor immunity [6,28,70]. A number of early phase trials of CpG immunotherapy have been initiated or recently completed, and the results of these studies are anticipated soon [71].

Although a great deal of attention has focused on the use of CpG ODNs for cancer immunotherapy, liposome–nucleic acid complexes may offer certain unique advantages for this application. These advantages include increased potency, particularly with regard to the induction of interferon responses, and the ability to elicit systemic immune activation and control tumor metastases following intravenous as well as subcutaneous administration. Both plasmid DNA and CpG ODNs combined with cationic liposomes have been shown to elicit significant antitumor activity. For example, the intravenous delivery of CLDC or liposomal CpG ODN complexes has been shown to elicit significant inhibition of tumor growth in experimental tumor models in mice, including established lung tumor metastases, cutaneous tumors and peritoneal tumors [40-43,72]. The adminstration of CLDC elicited rapid systemic activation of innate immunity, characterized by systemic

release of high concentrations of cytokines with antitumor activity, including TNF, IL-12, IFN-γ, and IFN-α [40,41]. In addition, CLDC injection has also been shown to elicit rapid cellular activation, including spontaneous NK cell infiltration and cytotoxicity and upregulation of co-stimulatory molecules on macrophages and dendritic cells [40].

The antitumor activity elicited by CLDC immunotherapy has been shown to be critically dependent on NK cell activation [40]. For example, tumors from mice treated with CLDC were often heavily infiltrated with NK cells, and NK cell depletion prior to CLDC injection significantly abolished antitumor activity [40,72]. Other studies have suggested an important role for $CD8^+$ T cells in mediating the antitumor activity induced by CLDC [43]. IFN-γ has been identified as a key cytokine responsible for inducing antitumor activity in solid tumor models following CLDC immunotherapy [40]. However, in a lymphoma tumor model, a key role for type I interferons in mediating tumor rejection following CpG immunotherapy was also noted [73]. Thus, it seems likely that synergistic interactions between type I and type II interferons, plus the effects of NK cells and $CD8^+$ T cells, may account for the majority of the antitumor activity elicited by liposome–DNA complexes. The cellular targets for cytokine and NK and $CD8^+$ T cell-mediated effects may include tumor cells themselves, the tumor-associated vasculature, and possibly tumor infiltrating macrophages and dendritic cells.

10. Route of delivery and immune activation

The route of delivery of liposome–nucleic acid complexes has a significant impact on the magnitude of the immune responses elicited. Studies in mice have established a route-dependent hierarchy for the degree of systemic immune activation, with the intravenous route being most potent, followed by the intraperitoneal route and then the subcutaneous and intramuscular routes (S Dow; unpublished data). The local delivery of CLDC to the airways by inhalation or intranasal delivery also elicits strong local activation of innate immunity, although not necessarily systemic immune activation [74,75].

Because liposome–nucleic acid complexes are such potent immune activators, they have also been associated with toxicity following the administration of high doses systemically or by inhalation [75-78]. The toxicity elicited by intravenous administration of high doses of CLDC appears to be critically dependent on the induction of IFN-γ production, inasmuch as IFN-$\gamma^{-/-}$ mice are almost completely protected from CLDC-induced toxicity (S Dow; unpublished data). However, the side effects and toxicity associated with CLDC immunotherapy are clearly both dose and route dependent, as are measures of treatment efficacy. Thus, dosage adjustment is generally sufficient to eliminate the toxicity induced by liposome–nucleic acid immunotherapeutics.

Studies in animals reveal that there is a substantial therapeutic window between effective and toxic doses of CLDC. This is particularly true when CLDCs are administered by routes other than the intravenous route, as for example in the case of parenteral or mucosal vaccination using CLDCs as vaccine adjuvants. Moreover, preclinical studies of liposome–nucleic acid immunotherapeutics administered by a variety of different routes, including the intravenous route, to non-human primates as well as rabbits and rodents have all demonstrated a high margin of safety (D Liggitt; pers. commun.).

11. Cancer immunotherapy using spontaneous canine tumor models

Some of the more compelling evidence for the effectiveness of liposome–nucleic acid immunotherapy for cancer has been obtained from studies in pet dogs. Dogs are outbred animals that spontaneously develop many of the same cancers as humans, and also live in the same environment as humans. Therefore, dogs can serve as a valuable animal model for evaluation of new therapeutics [79,80]. A Phase I study has been conducted to assess the safety and efficacy of intravenous delivery of CLDC immunotherapy in dogs with advanced, chemotherapy-resistant bone cancer metastasis to the lungs [81]. In that study, the CLDC contained plasmid DNA encoding the *IL-2* gene, but the extremely low levels of *IL-2* gene expression that were achieved *in vivo* in that study rendered the effects of IL-2 negligible. The CLDC dose required to elicit significant immune activation in dogs was found to be ~ 1/50th the dose used for immunotherapy and gene delivery in mice. Notably, CLDC infusion in dogs was associated with significant activation of innate immunity, including NK cell activation, fever and upregulation of co-stimulatory molecules. Importantly, a significant increase in survival was observed in treated dogs in that study, compared with historical control animals. Repeated intravenous treatments were well-tolerated, and in other studies dogs with cancer have been treated continuously for up to 5 years with intravenously administered CLDC without serious or cumulative side effects (S Dow; unpublished data).

A second study of CLDC immunotherapy has also been conducted in dogs with soft tissue sarcomas [82]. That study found that intravenous infusion of CLDC elicited significant inhibition of tumor angiogenesis, as well as tumor growth inhibition or regression in dogs with large, established cutaneous tumors [82]. Although both of the preceding studies in dogs were designed originally as a gene therapy studies, infusion of CLDC with non-coding DNA was found to elicit equivalent immune activation to that elicited by cytokine gene encoding CLDC [81]. Moreover, in mouse studies it was noted that the effectiveness of intravenous cytokine gene delivery using CLDC was only marginally greater than injection of CLDC formulated

with non-coding plasmid DNA [72]. Thus, these results indicate that systemic CLDC immunotherapy can be successfully scaled up in a relevant large animal model of cancer.

12. Antiviral immunotherapy with cationic liposome–DNA complexes

The ability of liposome–nucleic acid complexes to elicit high levels of both type I and II interferons suggests that they may also be well suited to antiviral immunotherapy. To assess the antiviral activity of CLDC, mice treated with CLDC immunotherapy were found to be significantly protected from lethal infection with Punta Tora virus [52]. Significant protection was also achieved when the CLDCs were administered within 24 h of infection. It was also found that the intravenous route of delivery was more effective than the intraperitoneal route and the increase in protection was associated with higher peak levels of interferon production. In recent ongoing collaborative studies at Colorado State University, the present author's group have been able to achieve significant protection of mice from three different lethal viral encephalitis infections, using CLDC immunotherapy (S Dow; unpublished data).

Significant protection from experimental influenza virus infection in mice has also been achieved using cationic liposomes complexed to a synthetic double-strand RNA mimic (poly ICLC) [48]. Indeed, in those studies, durable and complete protection from lethal virus challenge could be achieved by intransal administration of the liposome–poly ICLC complexes for up to 3 weeks prior to virus challenge. Moreover, those studies also demonstrated that liposome-encapsulated poly ICLC was more effective and less toxic than uncomplexed poly ICLC. A significant reduction in the levels of hepatitis B viral transcripts in a mouse transgenic model has also been achieved recently by CLDC immunotherapy (J Fairman; pers. commun.). Therefore, there is good reason to believe that liposome–nucleic acid immunotherapy may be an effective means of eliciting endogenous interferon-mediated antiviral immunity.

Cationic liposome–nucleic acid immunotherapy has also been found to be effective in reducing the clinical signs associated with chronic upper airway infection in pet cats [83]. Chronic rhinitis and sinusitis in cats is a poorly understood but common clinical condition of pet cats that is thought to be possibly associated with chronic feline herpesvirus or calicivirus infections [84]. Cats with chronic rhinitis treated with CLDC immunotherapy have been shown to experience a significant reduction in clinical signs, including frequency of sneezing and nasal discharge, when compared with placebo-treated animals. Cats treated with CLDC experienced transient fever and elevations in white blood cell counts, along with persistent increases in $CD8^+$ T-cell counts after prolonged treatment. Future studies of liposome–nucleic acid immunotherapy for viral infections are likely to focus on more efficient means of delivering the complexes to the lungs and other mucosal surfaces for local suppression of viral replication.

13. Cationic liposome–nucleic acid complexes as vaccine adjuvants

A number of factors, including charge, particle size and degree and duration of induction of innate immunity all play important roles in determining the efficiency of vaccine adjuvants [85]. Cationic liposome–nucleic acid complexes possess two properties that make them particularly well suited for use as vaccine adjuvants. Firstly, they are potent activators of innate immunity and Th1 cytokine responses. Secondly, the net positive charge on complexes also makes it possible to directly bind antigens such as proteins or peptides, which targets all three components of the vaccine to antigen-presenting cells such as dendritic cells. Recent studies have demonstrated that CLDC have potent vaccine adjuvant properties when formulated with protein or peptide antigens [47,86]. These CLDC-formulated vaccines were particularly effective in inducing both $CD4^+$ and $CD8^+$ T-cell responses, particularly when compared with existing conventional vaccine adjuvants [47]. CpG ODNs formulated with liposomes have also demonstrated impressive adjuvant activity when used to deliver peptide antigens [87,88]. Liposome–nucleic acid adjuvants are also very effective in eliciting antibody responses, with equivalent or superior potency to aluminum hydroxide adjuvant or Freund's complete adjuvant. In all of these studies, the liposome and nucleic acid and antigen must be in physical contact in order to elicit effective T-cell or antibody responses [47,64,86,89].

Cationic liposomes by themselves have important immunological properties that contribute to their effectiveness as vaccine adjuvants [90,91]. Some adjuvant activity can be accounted for by the fact that positively charged liposomes can directly activate dendritic cells [64]. Cationic liposomes also affect the route of antigen uptake by antigen-presenting cells, inducing efficient uptake via endocytosis [92,93]. The liposome composition also has an important influence on vaccine efficacy. As a general rule, cationic liposomes are more effective vaccine adjuvants than neutral or anionic liposomes [94-97].

However, it should also be noted that within the broad category of cationic liposomes, important differences in immune stimulatory potency have been observed [97]. For example, cationic liposomes bearing phosphocholine head groups were more potent immune stimulants than those bearing trimethylammonium head groups. According to the experience of the present author's group, important differences between between the cationic liposomes DOTIM (octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium chloride) and DOTAP (dioleoyl-trimethylmmonium-propane) have not been

observed, in terms of their ability to elicit innate immune activation or to function as vaccine adjuvants [40]. In part, the increased effectiveness of cationic liposomes results from more efficient uptake of cationic liposomes by dendritic cells, relative to neutral or anionic liposomes [98,99]. In addition, the composition of the neutral or helper lipid incorporated with the cationic liposome also influences vaccine efficacy [100]. The targeting of liposomes to dendritic cells by the addition of mannose targeting groups to the liposome can also improve vaccine efficacy [101].

The effectiveness of liposome–nucleic acid vaccines is largely dependent on the activation of innate immunity by the nucleic acid component of the adjuvant [47]. However, cationic liposomes themselves also elicit substantial cellular necrosis [46,102] As a result, certain endogenous activators of innate immunity, such as uric acid, are also released by cationic liposome adjuvants, and these too can help promote activation of innate immunity [103,104]. Thus, the inflammatory responses elicited by cationic liposomes may also contribute to their adjuvant activity.

Charged liposomes can also serve another important function when used as adjuvants in vaccines intended for immunization against cancer or viral infections, where $CD8^+$ T cells are critically important. Liposomes promote the entry of proteins into the cytoplasm of antigen-presenting cells, which results in cross-priming, or the induction of $CD8^+$ T-cell responses against exogenous protein antigens [105,106]. The ability to elicit extremely efficient cross-priming is one of the more important properties of liposome–nucleic acid adjuvants [47].

CpG ODNs have been extensively evaluated as vaccine adjuvants [6,107-111]. The adjuvant properties of CpG ODNs are significantly improved when the ODNs are directly conjugated antigens. However, the conjugation process is often technically challenging and expensive. As an alternative, investigators have found that the adjuvant properties of CpG ODNs can be substantially enhanced by the incorporation of CpG ODNs with a liposome or lipid emulsion [111]. This process is technically simpler and more efficient than direct conjugation of CpG ODNs or other nucleic acid agonists to antigens. For example, complexes of CpG ODNs with sterically stabilized liposomes significantly have been shown to increase immune activation and the induction of antibody responses against protein antigens in mice [45]. In another study, antibody responses to a hepatitis vaccine were significantly increased when animals were immunized with antigen in CpG ODNs plus liposomes as adjuvant, as opposed to immunization with antigen and CpG ODNs alone [112]. It was also shown that the effectiveness of polyI:C as a vaccine adjuvant could be substantially improved by the incorporation of liposome complexes in the vaccine [113]. Finally, it was also shown that complexes of cationic liposomes and polyI:C were effective for tumor immunotherapy and the induction of tumor-specific $CD8^+$ T-cell responses [114].

Several conclusions regarding cationic liposomes and nucleic acid vaccine adjuvants can be drawn from these studies. Cationic liposomes themselves have desirable properties as vaccine adjuvants, including uptake by dendritic cells, entry into the endosomal pathway of antigen processing, and introduction of antigens into the cytosol. However, it is also clear that the effectiveness of cationic liposomes as vaccine adjuvants can be greatly increased by combining the liposomes with nucleic acids, particularly nucleic acids such as DNA or RNA that serve as ligands for intracellular PRRs. The ability of charged liposomes to spontaneously complex with negatively charged nucleic acid and antigens simplifies vaccine formulation. Many liposome–nucleic acid complexes can also be readily lyophilized, thereby greatly increasing their shelf life and stability [115,116]. Future improvements in the design of vaccine adjuvants based on liposome–nucleic acid complexes will require an improved understanding of the effects of size, charge and liposome composition on interaction with PRRs and induction of specific innate immune responses.

14. Conclusions

Immunotherapy will become an increasingly important option for the treatment of cancer and infectious diseases. As newer innate immune system receptors and their ligands and agonists are discovered and used therapeutically, drug delivery will become an increasingly important issue. For certain PRR agonists, particularly those whose receptors are located intracellulary, liposomal delivery offers attractive advantages in terms of efficiency and selectivity of targeting. The ability of cationic liposomes to target nucleic acids to the endosomal compartment of dendritic cells and other antigen-presenting cells for increased immune stimulation is the best example of this principle so far. For the formulation of vaccine adjuvants, liposome–nucleic acid complexes also offer simplified formulation, with greater targeting of antigens to antigen-presenting cells and more efficient induction of T-cell responses to recombinant protein and peptide antigens. Thus, it is likely that liposomes will play an important role in the development of the next generation of immunotherapeutics for cancer and infectious diseases.

15. Expert opinion

The field of immunotherapy has re-emerged in terms of both research and commercial interest. This growing embrace of immunotherapy is driven largely by a greatly improved understanding of the immunologic principles that underlie innate immunity. Also fueling the new enthusiasm is the discovery of a number of key innate immune system receptors, as well as their cognate ligands and signaling pathways, all of which are potential drug targets. Moreover, it is also becoming clear that carefully targeted modulation of innate immunity can yield significant clinical benefits,

while also avoiding the unpredictable toxicity associated with previous forms of immunotherapy.

15.1 What molecules are currently being evaluated for immunotherapy?

The current major targets for therapeutic immune modulation include several Toll-like receptors (TLRs) and their ligands. In addition to TLR4, a great deal of attention has also focused on the endsomally located TLRs (TLR3, TLR7/8, and TLR9) and their ligands. Ligands and/or agonists for each of these receptors have been evaluated for immunotherapeutic applications and one (the TLR7/8 agonist imiquimod [Aldara™]) is now approved for the topical treatment of early basal cell carcinoma, actinic keratosis, and genital warts in humans. A TLR4-based vaccine adjuvant (MPL®; Corixa and GlaxoSmithKline) has been widely evaluated in a number of different vaccine applications. Drugs designed to activate TLR9, especially CpG ODN, have been extensively investigated for immunotherapy of cancer, allergy, asthma, and as vaccine adjuvants.

At present, TLR agonists used for immunotherapy have been delivered by injection, inhalation, or topical application. For example, the current TLR7/8 agonist drugs such as Aldara™ are typically delivered topically as creams. In contrast, TLR9 agonists such as CpG ODNs are usually delivered by subcutaneous injection, which results in activation of innate immune responses primarily in draining lymph nodes. The CpG ODNs are not particularly effective in eliciting immune activation following intravenous infusion. CpG ODNs have also been administered by inhalation for immunotherapy of asthma and allergic rhinitis. When CpG ODNs or polyI:C are used as vaccine adjuvants, they are usually administered by parenteral injection.

15.2 What advantages do liposome–nucleic acid compounds offer over current TLR-based immunotherapeutics?

There currently are two major advantages to using liposome–nucleic acid complexes for immunotherapy, which are increased potency and increased vaccine adjuvant activity. Liposomal delivery of nucleic acids, such as DNA and RNA molecules or their homologs, demonstrates clearly the potency effect. Combining cationic liposomes with immune stimulatory nucleic acids typically generates a 2 to 10-fold increase in the degree of immune activation. Cationic liposomes also render even weakly stimulatory or non-stimulatory DNA molecules highly stimulatory, including even those devoid of CpG sequences.

Formulating cationic liposomes with nucleic acids (and other innate immune receptor agonists) also induces immune responses that are qualitatively different from those elicited by the original ligand/agonist. The best recent example of this is ability of cationic liposome delivery of DNA molecules to trigger activation of the cytosolic DNA receptor DAI, whereas treatment with DNA alone fails to activate this receptor. Thus, liposomal delivery may fundamentally alter the quality as well as the magnitude of immune responses. It may therefore be more appropriate to consider liposome-ligand/agonist complexes as new drug entities distinct from the agonist being delivered.

15.3 Challenges facing the development of liposome–nucleic acid immunotherapeutics

Several challenges face the translation of liposome–nucleic acid immunotherapeutics into large scale clinical trials. The extreme potency of these immune activators will necessitate careful attention to both dose and route of administration. However, the degree of immune activation elicited by liposome–nucleic acid compounds appears to be clearly dose and route dependent and therefore largely predictable, thus facilitating the design of Phase I trials. Moreover, preclinical toxicology studies in rabbits and non-human primates have all shown a high margin of safety for liposome–nucleic acid complexes when administered by multiple routes, including the intravenous route (D Liggitt; pers. commun.). Liposome–nucleic acid complexes used for gene therapy have also previously shown a high margin of safety following cutaneous administration in humans with cancer. The cutaneous, intramuscular or mucosal routes, which are associated with minimal reactogenicity in animal studies, are the most likely routes to be used for vaccination of humans. Cancer patients or patients with chronic viral infections are likely to be the only patients where intravenous administration of liposome–nucleic acid complexes would be considered, and in those patients a certain degree of self-limiting reactogenicity (such as fever) is generally acceptable.

15.4 Potential commercial opportunities

Presently, the major commercial opportunities for immunotherapeutics based on the liposome–nucleic acid platform include use in cancer immunotherapy, immunotherapy of chronic viral and fungal infections, and as vaccine adjuvants. There remains a strong need for effective cancer immunotherapeutics that can be combined with either chemotherapy or radiation therapy. The CpG ODN-based immunotherapeutics are presently being evaluated as combination therapeutics for lung cancer and melanoma, and there are a number of other cancer applications where liposome–nucleic acid immunotherapeutics may also prove beneficial. Because of their potent ability to induce interferon responses, liposome–nucleic acid immunotherapeutics are very attractive for the treatment of chronic viral infections such as hepatitis B and hepatitis C virus infection, where the induction of interferons can be used to non-specifically suppress viral replication.

Liposome–nucleic acid complexes are also very effective vaccine adjuvants. Here, a major advantage of the cationic liposome platform is the ability to easily couple the antigen to the liposome and nucleic acid adjuvant. This interaction

occurs spontaneously, primarily through charge–charge interactions, thereby eliminating the need for complicated or expensive chemical coupling of the antigen to the nucleic acid immunotherapeutic. Liposome–nucleic acid complexes are presently among the most effective non-viral vaccine adjuvants for generating $CD8^+$ T-cell responses to protein antigens. Liposome–nucleic acid adjuvants also elicit strong $CD4^+$ T-cell responses and high-titered antibody responses with a strong Th1 bias.

Interestingly because of the large amount of preclinical efficacy and safety data generated with liposome–nucleic acid complexes in companion animals (dogs and cats), the first products to reach the market using the liposome–nucleic acid platform technology may well be intended for the growing veterinary market. This market, although still relatively small, is expanding much more rapidly than the market for human products, and the regulatory hurdles are easier to overcome, particularly for biologicals such as immunotherapeutics. For example, a cancer vaccine for dogs with melanoma has recently received conditional approval by the United States Department of Agriculture, and is being marketed by Merial. Possible veterinary applications of the liposome–nucleic acid technology would include cancer immunotherapy, immunotherapy of allergy and infectious diseases and as adjuvants for therapeutic vaccines.

15.5 What new liposome–nucleic acid immunotherapeutics will be developed in the future?

Future applications of the liposome–nucleic acid technology will likely include the use of cationic liposomes to deliver additional pattern recognition receptor ligands or agonists. Indeed, the most promising candidates for liposomal delivery are likely to be those new ligands or agonists with intracytoplasmic receptors in antigen-presenting cells. Although ligands or agonists with net negative charges are presently much more easily formulated with cationic liposomes than uncharged or cationic ligands, in the future it may also be possible to deliver cationic agonists using anionic liposomes. Strategies designed to allow the formulation of cationic liposomes with relatively uncharged ligands, such as flagellin, or with lipophilic ligands such as lipopolysaccharide derivatives, may also expand the available repertoire of liposome–TLR agonist compounds. Another major development will likely be the increased use of combinations of ligands and agonists delivered by liposomes, as combinations of TLR–TLR and TLR–NLR agonists show significant synergistic activation of innate immunity *in vitro*. Finally, considerable attention will need to be devoted to optimizing the formulation of liposome-based immunotherapeutics, including physical variables such as particle size and charge, each of which exert important influences on the speed, magnitude and duration of innate immune responses.

Declaration of interest

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The author is named as an inventor on several patents that cover aspects of the liposome–nucleic acid technology described here. In addition, the author serves on the Scientific Advisery Board of Juvaris Biotherapeutics, a privately held company which seeks to commercialize the liposome–nucleic acid technology. The author also holds shares in the company.

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