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Liposome-Nucleic Acid Immunotherapeutics

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Abstract

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 markedly potentiate activation of the innate immune system by certain Toll-like receptor (TLR) agonists. The immune enhancing properties of cationic liposomes are most obvious when they are combined with nucleic acid agonists for endosomally-located TLRs, including TLR3, TLR7/8, and TLR9. How this immune potentiation by cationic liposomes is mediated is not completely understood, but is thought to reflect the combined effects of more efficient endosomal targeting, protection from extracellular degradation, and signaling through newly identified cytoplasmic receptors for nucleic acids. The potent innate immune stimulatory properties of liposome-nucleic acid complexes make them particularly effective as immunotherapeutics or vaccine adjuvants. As stand-alone immunotherapeutics, liposome-nucleic acid complexes have demonstrated impressive anti-cancer activity in a number of different animal tumor models. Moreover, liposome-nucleic acid complexes also show promise for immunotherapy of acute viral and bacterial infections and 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 T cell responses. Thus, cationic liposomes combined with nucleic acids form the basis for a potent and versatile immunotherapeutic platform.

Keywords

Liposome; DNA; immunotherapy; cancer

Introduction

Our understanding of the innate immune system has advanced remarkably over the past decade. Among the more important development has been identification of a number of pattern recognition receptors (PRR) including particularly the Toll-like receptor (TLR) family of PRRs. Concurrently, ligands for many PRRs have been identified while synthetic agonists have also been developed. Another major advance in the field has been a greater understanding how just how interconnected the innate and adaptive immune systems are and how 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, I will discuss

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Conflict of interest disclosure: 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 Advisory Board of Juvaris Biotherapeutics, a privately held company which seeks to commercialize the liposome-nucleic acid technology, and also holds shares in the company.

briefly the components of the innate immune system that have evolved to recognize nucleic acids and related molecules, including the TLRs and NOD-like receptors (NLRs). The mechanisms by which cationic liposomes are thought to potentiate the immune stimulatory properties of certain nucleic acid ligands for TLRs will also be discussed. Finally, the potential therapeutic uses for cationic liposome-nucleic acid complexes will be discussed, including their application to cancer immunotherapy and vaccine adjuvants.

Recognition of pathogens by the innate immune system depends on a series of innate immune receptors known as pattern recognition receptors (PRRs), which 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 (CLRs) and the triggering receptors expressed on myeloid cells (TREMs) (2, 5, 6). All of the TLRs are membrane bound, as are the CLRs (eg, DC-SIGN) and the TREMs (eg, macrophage scavenger receptor and the macrophage mannose receptor). In contrast members of the NLR family are expressed primarily in the cytoplasm of cells (7–10). All of these receptors are believed to have evolved primarily to recognize and help eradicate pathogens. 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.

TLR and NLR families of pattern recognition receptors

The TLR family of receptors and their agonists have received the greatest research attention to date in part because of their potential for use as immunotherapeutics. 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 discovered for certain TLRs(11). Cells of the innate immune system, including macrophages, monocytes, and dendritic cells, typically express the greatest variety and highest density of TLRs, though a variety of other cell types may also express TLRs.

Ligand or agonist binding to the antigen recognition domain (leucine-rich region; LRR) of a TLR triggers a conformational change in the receptor that results in recruitment of a series of adaptor proteins which interact with the Toll/interleukin-1 homology region of the TLR (10, 11). Most TLRs signal via an NF-kB mediated pathway, which ultimately results in release of pro-inflammatory cytokines such as TNF, IL-1b, IL-12 and IL-6. TLR activation can also signal via an IFN regulatory factor-dependent pathway, which results in production of type I IFNs. 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, including adaptive immune responses(13, 14).

The NOD family of receptors (NOD-like receptors; NLRs) also plays an important role in regulation of innate immunity(7, 9). Diseases associated with dysregulation of NLR-dependent immune responses are currently 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 {Rescigno, 2007 #8}(15). The primary agonists identified to date for NLRs consist primarily of degradation products of bacterial peptidoglycans (9). For example, muramyl dipeptide and IAP 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 activate cells. Activation of NLRs results in triggering in many of the same NF-kB-dependent pathways that are activated by TLR signaling (7). It is

also likely that there are qualitative as well as quantitative differences will be found in the immune responses elicited by the TLR and NLR family of receptors, though as yet little has been done to investigate this issue. Undoubtedly these differences will have important implications for the development of immunotherapeutics based on the TLR and NLR pathways.

TLR and NLR agonists as immunotherapeutics

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 developed for immunotherapy and used to target activation of specific receptors (4, 6, 16, 17). To date, most attention has focused on development of 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.

Toll-like receptor 9-based immunotherapeutics have progressed the furthest in development (6, 17, 18). Current applications for TLR9-based agonists include cancer immunotherapy, allergy and asthma immunotherapy, and use as vaccine adjuvants (18). Synthetic oligodeoxynucleotides (ODN) that contain unmethylated CpG dinucleotide repeats are widely used as TLR9 agonists (6, 18). Plasmid DNA is also enriched in CpG sequences relative to mammalian DNA and stimulates innate immune responses, though less potently than CpG ODN. Toll-like receptor 9 is primarily expressed within the endosomal compartment of dendritic cells (DC) and B cells and these cells can be efficiently activated by CpG ODN. Administration of CpG ODN results in rapid immune activation, primarily in draining lymph nodes and the spleen, with release of pro-inflammatory cytokines including TNF, IL-1b, IL-6, IL-12, IFN-γ, IFN-β and IFN-α (18, 19). Immunotherapy with TLR9 agonists elicits Th1-type immune responses, which promotes the development of cell-mediated immune responses necessary to control intracellular pathogens and control tumor growth (17). Toll-like receptor 3 agonists such as polyinosine-polycytidylic acid (polyI:C) also promote development of Th1 immune responses, as do small molecule agonists of TLR7/8 such as the imidazoquinoline compounds (11).

Earlier studies have also demonstrated that liposomes can be used to enhance the immune stimulatory properties of the NOD 2 agonists muramyl dipeptide (MDP) and muramyl tripeptide (MTP) (20–22). In fact, complexes of phosphatidylethanolamine liposomes and MTP (MTP-PE) were among the first liposomal immunotherapeutics developed and are still being evaluated today for adjuvant therapy of melanoma and sarcoma in humans and also in dogs (21, 23, 24). The immunologic mechanism of action of MTP-PE appears to involve primarily macrophage activation and release of pro-inflammatory cytokines such as TNF (25).

Liposomes enhance innate immune activation by nucleic acids

Shortly after the immune stimulatory properties of bacterial DNA were described, several groups discovered that cationic liposomes markedly enhanced the immunogenicity of bacterial DNA and CpG ODN. This discovery was largely a by-product of research on systemic non-viral gene delivery. Cationic liposomes had been used extensively for in vivo non-viral gene delivery using plasmid-based vectors and this method of gene therapy was generally considered safer than viral-vectored gene therapy (26, 27) However, when cationic liposome-DNA complexes (CLDC) were administered intrevenously, significant and unexpected toxicity developed, which was found to be independent of the transgene being expressed. Our group investigated this phenomenon and reported that the toxicity of CLDC

was mediated by marked activation of innate immunity (28). Many of these findings were subsequently confirmed by other groups (29–32).

Complexes of cationic liposomes with bacterial DNA are extremely potent activators of innate immune responses. For example, on a per weight basis CLDC elicited much greater immune stimulation than other conventional activators of innate immunity, including LPS and polyI:C (28, 29). The ability of cationic liposomes to potentiate activation of innate immunity was not restricted to plasmid DNA and was could also elicited using CpG ODN (30, 33). In fact, even mammalian DNA becomes a potent immune stimulant when combined with cationic liposomes, as demonstrated recently in a murine tumor model (34). Moreover, cationic liposomes can be used to potentiate immune activation by other TLR agonists, including the TLR3 agonist polyI:C and TLR7/8 agonists such as single-stranded RNA (35–38).

The interaction of cationic liposomes with nucleic acid agonists appears to elicit both qualitative as well as quantitative changes in immune responses. Liposome-nucleic acid complexes are clearly more potent when overall levels of cytokine release are compared. For example, the addition of cationic liposomes to CpG ODN 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 (IFN) (28, 39). But it also appears that there are important qualitative differences in immune responses elicited by liposome-complexed agonists as well. Notably, liposomal delivery of CpG ODN was shown to result in activation of several TLR9 independent immune activation pathways when compared to CpG ODN alone (40–42). In part, these differences may be ascribed to the interaction of DNA with a newly described cytosolic receptor (DNA-dependent activator or interferon regulatory factors; DAI) that occurs when liposome-complexed DNA gains entry into the cytoplasm of cells (42). One of the major differences noted is the significant increase in induction of type I IFN production following liposomal delivery of CpG ODN, due in part to activation of the IRF-7 and now the DAI pathways (41–43). Thus, combining cationic liposomes with nucleic acid TLR agonists fundamentally alters their immune stimulatory properties, largely as a consequence of altering their intracellular trafficking.

Other positively charged molecules, in addition to cationic liposomes, can be used to alter the intracellular trafficking of nucleic acids and augment the innate immune stimulatory effects of plasmid DNA or CpG ODN. Thus, Cui et al (44) demonstrated increased immune activation as a result of complexing nucleic acids with protamine sulfate. This phenomenon was also illustrated recently in bacterial and viral infection models. Bacteria and viruses that introduced DNA into the cytoplasm of infected cells were found to induce significantly greater innate immune activation than bacteria or viruses that failed to enter the cell cytoplasm (40). These studies also showed that induction of IFN-α release by intracellular DNA was TLR independent, suggestive again of the alternative DAI-dependent pathway of immune activation(42).

Influence of cationic liposomes on the intracellular trafficking of nucleic acid molecules

Cationic liposomes complexed with plasmid DNA enter cells via endosomal uptake (45–47). After endocytosis, the liposome-nucleic acid complexes are localized initially to the early endosomal compartment. Importantly, uptake of liposome complexes is an active process and does not occur 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 (48). Localization of the complexes in the early endosomal compartment probably accounts for most of the potentiation of immune activation that occurs following liposomal delivery of DNA, inasmuch as TLR9 is also located primarily in this same compartment ((10, 11, 49). Interestingly, release of DNA from the liposome complex, which is required in

order for the DNA to interact with TLR9 and probably DAI, appears to be mediated primarily by interactions with anionic liposomes released from the endosomal membrane (50). Thus, this sequence of events probably explains why helper or neutral liposomes are often required along with cationic liposomes for efficient introduction of DNA into cells.

Physical properties (eg, charge, higher order molecular structure) of liposome-nucleic acid complexes also affect the nature of the immune response elicited. For example, Guiducci et al (51) investigated the interaction of CpG ODN with TLR9 in plasmacytoid DC and demonstrated that production of IFN-α was strongly upregulated when CpG ODN complexes were localized to the early endosome. In contrast, localization of the liposome-CpG ODN complexes to the late endosome or lysosome resulted in DC maturation, but without the production of IFN-α. Thus, 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 pattern recognition receptor agonists. For example, complexes of cationic liposomes with ligands and agonists for TLRs expressed on the cell surface (eg,. TLR2 and TLR4) do not appear to induce increased immune activationn (35). In fact, in some cases combining TLR agonists with cationic liposomes may actually suppress immune activation by the agonist, which has been observed with lipophilic agonists such as LPS. Sequestration of the TLR agonist within intracellular compartments, which blocks access to the extracellularly expressed TLR, probably accounts for these observations. Thus, as a general rule, cationic liposomes appear to be most effective in augmenting immune activation when combined with agonists whose receptors are expressed in intracellular compartments, such as the endosomally expressed TLRs (TLR3, TLR7/8, and TLR9) and the cytosolically expressed NLRs.

Use of cationic liposome-nucleic acid complexes for cancer immunotherapy

A great deal of attention has been focused recently on the therapeutic uses of PRR agonists (4, 6, 16, 17). The majority of studies have been done using TLR9 agonists, particularly CpG ODN, and it is clear that CpG ODN are potent inducers of antitumor immunity (6, 17, 52). A number of early phase trials of CpG immunotherapy have been initiated or recently completed and the results of these studies are anticipated soon (53).

While a great deal of attention has focused on the use of CpG ODN for cancer immunotherapy, it is also apparent that cationic liposome-nucleic acid complexes may offer certain unique advantages for use in cancer immunotherapy. Among these advantages include increased potency, greater selective induction of interferon responses, and the ability to elicit immune activation following intravenous as well as subcutaneous administration. Both plasmid DNA and CpG ODN combined with cationic liposomes have been shown to elicit significant antitumor activity. Intravenous delivery of CLDC or liposome CpG ODN complexes elicited significant inhibition of tumor growth in experimental established tumor models, including lung tumor metastases, cutaneous tumors, and peritoneal tumors (28–31, 54). Injection of the cationic liposome and DNA complexes elicited rapid systemic activation of innate immunity, characterized by systemic release of high concentrations of cytokines with antitumor activity, including TNF, IL-12, IFN-g, and IFN-a (28, 29). In addition, CLDC injection also elicited rapid cellular activation, including spontaneous NK cell infiltration and cytotoxicity and upregulation of co-stimulatory molecules on macrophages and DC (28). The factors common to these studies that may have accounted for the strong antitumor activity included systemic administration of the complexes and the use of cationic liposome and DNA complexes with relatively high charge ratios.

The antitumor activity elicited by CLDC immunotherapy is critically dependent on NK cell activation (28). For example, tumors from mice treated with CLDC were often heavily infiltrated with NK cells, and NK cell depletion prior to CLDC injection significantly diminished anti-tumor activity (28, 54). Other studies have suggested an important role for $CD8^+$ T cells in mediating the antitumor activity induced by CLDC (31). Interferon-γ was identified as a key cytokine responsible for inducing antitumor activity in solid tumor models following CLDC immunotherapy (28). However, in a lymphoma tumor model, a key role for type I interferons in mediating tumor rejection following CpG immunotherapy was also noted (55). Thus, it seems likely that synergistic interactions between type I and type II IFNs, plus the effects of NK cells and $CD8^+$ T cells, could account for the majority of the antitumor activity elicited by liposome and plasmid DNA or CpG ODN complexes. The therapeutic targets for liposome-nucleic acid induced interferons are likely to include tumor cells themselves, tumor-associated vasculature, and possibly tumor infiltrating macrophages and DC.

Route of delivery of liposome-nucleic acid complexes has a significant impact on the strength of innate immune activation. Studies in mice have established a route hierarchy for systemic immune activation potency, with the i.v. route being most potent, followed by the i.p. route and then the s.c. and i.m. routes (Dow S; unpublished data). Local delivery of CLDC to the airways by inhalation or intranasal delivery has been shown to elicit local activation of innate immunity, though not necessarily systemic activation (56, 57). Such

Because they are such potent immune activators, liposome-nucleic acid complexes can also elicit toxicity after either systemic or local administration(57–60). Not surprisingly, the degree of systemic toxicity induced by CLDC immunotherapy appears to be directly related to the route of delivery, with i.v. delivery eliciting the greatest systemic toxicity (28, 29, 39). Inhalation of CLDC can also elicit local pulmonary cytokine release and inflammatory responses (57). The toxicity of CLDC immunotherapy is largely cytokine mediated and is critically dependent on production of IFN-γ, inasmuch as IFN-γ −/− mice are almost completely protected from CLDC-induced toxicity (Dow, S; unpublished data). The side-effects elicited by CLDC administration can be reduced by concurrent treatments designed to suppress cytokine production, such as concurrent administration of corticosteroids and inhibitors of NF-kB signaling (59–61). However, studies have not yet been conducted to determine how selective suppression of cytokine production may impact the overall efficacy of CLDC immunotherapy for cancer.

Convincing evidence of the effectiveness of CLDC immunotherapy for cancer has also been obtained in studies in pet dogs, which serve as a realistic large animal model of spontaneous neoplasia. Dogs are outbred animals which spontaneously develop many of the same cancers as humans, while also living in the same environment, and therefore can serve as a valuable model for evaluation of new therapeutics intended for ultimate use in humans (62, 63). A phase I study was conducted to assess the safety and efficacy of i.v. delivery of CLDC in dogs with advanced, chemotherapy-resistant bone cancer metastasis to the lungs (64). In that study, the CLDC contained plasmid DNA encoding the IL-2 gene, but the extremely low level of IL-2 gene expression that were achieved in vivo rendered the effects of IL-2 negligible. The CLDC dose required to elicit significant immune activation in dogs was found to be approximately $1/50^{th}$ 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, and a significant increase in survival was observed in treated dogs compared to historical control animals with lung metastases.

Though the preceding study in dogs was designed originally as a gene therapy study, infusion of CLDC with non-coding DNA was found to elicit equivalent immune activation

to that elicited by IL-2 encoding CLDC(64). Moreover, in mouse studies that the effectiveness of i.v. cytokine gene delivery using CLDC was only marginally more effective than injection of CLDC with non-coding plasmid DNA(54). A second study of CLDC infusion in dogs with cancer was also conducted in dogs with soft tissue sarcomas (65). This study found that i.v. infusion of CLDC elicited significant inhibition of tumor angiogenesis as well as growth inhibition or regression of tumors in animals with large, established cutaneous tumors (65). Thus, it appears that systemic CLDC immunotherapy can be successfully scaled up in a highly relevant large animal species.

Antiviral immunotherapy with CLDC

The ability of liposome-nucleic acid complexes to elicit efficient production of both type I and II IFNs suggests this immunotherapeutic may be particularly well-suited to antiviral immunotherapy. This potential was confirmed recently in a mouse arbovirus infection model (66)l. Treatment of mice with CLDC elicited strong induction of IFN-a and IFN-γ production and the treated mice were significantly protected from lethal infection with Punta Tora virus (PTV) (66). Significant protection was also achieved when the CLDC were administered within 24 hours of infection. As predicted, the i.v. route of delivery was more effective than the i.p. route and the increase in protection was associated with higher levels of IFN production.

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) (36). In that model, durable, complete protection from lethal virus challenge could be achieved by intransal administration of the liposome-poly ICLC complexes up to 3 weeks prior to virus challenge. Moreover, that study demonstrated that liposome-encapsulated poly ICLC was more effective and less toxic than free poly ICLC. In recent ongoing collaborative studies at Colorado State University, we have been able to achieve significant protection of mice from 3 different lethal viral encephalitis infections, using CLDC immunotherapy (Dow, S; unpublished data). Significant reduction in the levels of hepatitis B viral transcripts in a mouse transgenic model has also been achieved recently by CLDC immunotherapy (Fairman, J; unpublished data). 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 was also found to be effective in reducing the clinical signs associated with chronic upper airway infection in pet cats (67). Chronic rhinitis and sinusitis in cats is a poorly understood but common clinical condition thought to be possibly be associated with chronic feline herpesvirus infection (68). Cats with chronic rhinitis treated with CLDC immunotherapy experienced a significant reduction in clinical signs, including frequency of sneezing and nasal discharge, when compared to 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.

Cationic liposomes-nucleic acid complexes as vaccine adjuvants

A number of factors, including charge, particle size, and degree of induction of innate immunity all play important roles in determining the efficiency of vaccine adjuvants (69). Cationic liposomes-nucleic acid complexes possess two properties that make them particularly effective as vaccine adjuvants. One, they are potent activators of innate immunity and Th1 cytokine responses. Second, the net positive charge on complexes also

makes it possible to directly bind antigens such as proteins or peptides to the complexes, resulting in trafficking and update of all three components of the vaccine by antigen presenting cells such as dendritic cells (DC). Recent studies have demonstrated that CLDC have potent vaccine adjuvant properties when formulated with protein or peptide antigens (35, 70). These vaccines appear to be particularly effective in inducing both CD4 and CD8 T cell responses, particularly when compared to existing conventional vaccine adjuvants (35). The 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. These studies have also all noted that the three vaccine components (liposome, antigen, TLR agonist) must be physically associated within a single complex in order to elicit efficient immunization(35, 44, 70, 71).

Cationic liposomes by themselves have important immunological properties that render them effective vaccine adjuvants(72, 73). Some of this adjuvant activity can be accounted for by the fact that cationic liposomes can activate DC directly (44). Cationic liposomes also strongly influence the route of antigen uptake by antigen presenting cells, inducing uptake via endocytosis (74, 75). 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 (76–78). In part, this increased effectiveness is a result of greater interaction of cationic liposomes with DC than occurs when neutral or anionic liposomes are used (79, 80). In addition, the composition of the neutral or helper lipid incorporated with the cationic liposome also influences vaccine efficacy (81). Targeting of liposomes to DC, by for example addition of mannose groups to the liposomal membrane, can also improve vaccine efficacy (82).

The adjuvant activity of liposome-nucleic acid vaccines clearly depends to a large degree on activation of innate immunity by microbial TLR mimics, such as CpG ODN or polyI:C (35). However, cationic liposomes complexed to nucleic acids such as DNA or RNA also elicit significant direct cellular toxicity and cell death (34, 83) As a consequence, certain endogenous compounds such as uric acid are released during cell necrosis and these can function as endogenous ligands for activation of innate immunity (84, 85). Thus, the inflammatory process elicited by liposome-nucleic acid vaccine adjuvants 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. In vaccines formulated with these adjuvants, the cationic liposomes promote cross-priming, or induction of $CD8^+$ T cell responses against protein antigens. Cross-priming is accomplished by the ability of the liposomes to introduce protein antigens into the MHC class I antigen processing pathway for presentation to $CD8^+$ T cells (86, 87). The ability to elicit efficient cross-priming is one of the most notable properties of vaccine adjuvants based on the cationic liposome-nucleic acid platform (35).

CpG ODN have been evaluated extensively as vaccine adjuvants (6, 88–92). The adjuvant properties of CpG ODN are significantly improved when the ODN are directly conjugated antigens. But the conjugation process is often technically challenging and expensive. As an alternative, investigators have found that the adjuvant properties of CpG ODN can be substantially enhanced by incorporation of the CpG ODN with a liposome or lipid emulsion (92). This process is technically much simpler and more efficient than direct conjugation to antigens. For example, complexes of CpG ODN with sterically stabilized liposomes significantly increased immune activation and induction of antibody responses against protein antigens in mice (33). In another study, antibody responses to a hepatitis vaccine were significantly increased when animals were immunized with antigen in CpG ODN plus

liposomes as adjuvant, as opposed to immunization with antigen and CpG ODN alone (93). It was also shown that the effectiveness of poly I:C as a vaccine adjuvant could be substantially improved by incorporation of liposome complexes in the vaccine(94). Finally, it was also shown that complexes of cationic liposomes and poly I:C were effective for tumor immunotherapy and induction of tumor-specific $CD8^+$ T cell responses (95).

Several important conclusions regarding cationic liposomes and nucleic acid vaccine adjuvants can be drawn from these studies. Cationic liposomes themselves have very desirable properties as vaccine adjuvants, including uptake by DC, 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 or agonists for intracellular TLRs. The ability of charged liposomes to spontaneously complex with negatively charged nucleic acid and antigens greatly simplifies vaccine formulation. Most of the liposome-nucleic acid compounds can also be readily lyophilized, thereby greatly increasing their shelf life and stability (96, 97). 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.

Conclusions

Immunotherapy is likely to become an increasingly important option for treatment of certain cancers and infectious diseases. As newer ligands and agonists for innate immune system receptors are discovered and applied therapeutically, drug delivery is likely to become an increasingly important issue. For certain agonists, particularly those whose receptors are located intracellulary, liposomal delivery offers a number of important advantages over treatment with the innate immune receptor agonist alone. The advantages for delivery of immunotherapeutics have been most clearly demonstrated with cationic liposome-nucleic acid complexes designed for stimulation of endosomal TLRs. These advantages include increased potency, increased breadth of immune activation, and more efficient targeting to specific cell types. For formulation of vaccine adjuvants, liposome-nucleic acid complexes also offer simplified formulation, with more specific targeting of antigens to antigen presenting cells and greater induction of T cell responses to recombinant protein and peptide antigens. Thus, in the future it is likely that liposomal formulations will be employed increasingly to specifically and efficiently target innate immune agonists to their intracellular receptors.

Expert Opinion

The field of immunotherapy is undergoing a resurgence in terms of both research and commercial interest. This growing embrace of immunotherapy is being driven in large part by a greatly improved understanding of the immunological 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.

The major current targets for therapeutic immune modulation include several Toll-like receptors (TLRs) and their ligands. In particular, most attention has focused on a subset of these receptors, the endsomally located TLRs (TLR3, TLR7/8, and TLR9). 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. Drugs designed to activate TLR9, especially CpG ODN, are being extensively investigated for immunotherapy of cancer, allergy, asthma, and as vaccine adjuvants.

At present, TLR agonist as therapeutics in clinical studies in humans have been delivered by parenteral injection, inhalation, or topical application. For example, most TLR7/8 agonist drugs are currently delivered topically, typically in slow-release gels. On the other hand, TLR9 agonists such as CpG ODN intended for systemic immunotherapy are usually delivered by s.c. injection, which results in activation of innate immune responses primarily in draining lymph nodes. In contrast, CpG ODN are not particularly effective in eliciting immune activation following i.v. infusion. CpG ODN have also been administered by inhalation for immunotherapy of asthma and allergic rhinitis. When CpG ODN and polyI:C are used as vaccine adjuvants, they are usually administered by parenteral injection.

So where do liposome-nucleic acid complexes fit in the growing list of immunotherapeutics? Specifically, what advantages do these compounds offer over current TLR-based immunotherapeutics? There currently appear to be two major advantages to the use of liposome-nucleic acid complexes for immunotherapy: increased potency and increased vaccine adjuvant activity. The advantages of liposomal delivery for immunotherapy have been most clearly demonstrated with immunotherapeutics based on nucleic acids, either DNA and RNA molecules or their homologues. Increased potency of immune stimulation is the most obvious effect of combining cationic liposomes with immune stimulatory nucleic acids, with 2 to 10-fold increases in the degree of immune activation being typical. Another important effect of liposomal delivery is to render relatively even weakly stimulatory or non-stimulatory DNA molecules, including those that are devoid of CpG sequences, highly immunostimulatory. These augmented immune responses often reflect the effects of re-directed intracellular trafficking and uptake of ligands and agonists after they are complexed with cationic liposomes.

However, it is also important to note that cationic liposomes combined with nucleic acids (and other innate immune receptor agonists) also induce 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 to trigger activation of the cytosolic DNA receptor, an effect that does not occur when DNA molecules alone are added to cells. Thus, liposomal delivery may fundamentally alter the character 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.

Currently, the major commercial opportunities for immunotherapeutics based on the liposome-nucleic acid platform include cancer immunotherapy, immunotherapy of chronic viral infections, and vaccine adjuvants. There remains a strong need for an effective cancer immunotherapeutic that can be combined with either chemotherapy or radiation therapy. The CpG ODN based immunotherapeutics are currently being evaluated as combination therapy for lung cancer and melanoma and there are a number of other cancer applications where liposome-nucleic acid immunotherapeutics may prove effective. Because of their potent ability to induce interferon responses, liposome-nucleic acid immunotherapeutics are also being considered for the treatment of chronic viral infections, especially hepatitis B and hepatitis C virus infections.

Liposomes complexed with nucleic acids can also be used to produce very effective vaccine adjuvants. Here, one of the major advantages provided by cationic liposomes is the ability to physically 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. The resulting three-part complex results in very efficient delivery of the antigen to the same antigen presenting cell that is being activated by the liposome-nucleic acid complex. Adjuvants based on liposome-nucleic acid complexes are currently the most effective non-replicating vaccine adjuvants identified to date for eliciting $CD8^+$ T cell responses to protein antigens. Liposome-nucleic acid adjuvants also elicit broad-based immune responses, including high-titered antibody responses.

Where does the field of liposome-nucleic acid immunotherapeutics go from here? The simplest applications will undoubtedly occur when cationic liposomes are used to deliver newly discovered pattern recognition receptor ligands or agonists. The most promising candidates for liposomal delivery are also likely to be those ligands/agonists whose receptors reside within the endosomal compartment or within intracytoplasmic locations. Ligands or agonists with net negative charges will be much more easily formulated with cationic liposomes than uncharged or cationic ligands. In the future, strategies designed to allow formulation of cationic liposomes with relatively uncharged ligands, such as flagellin, or with lipophilic ligands such as LPS derivatives, may expand the available repertoire of liposome-TLR agonist compounds. It is also likely that we will see increased use of combinations of ligands and agonists being delivered by cationic liposomes, since combinations TLR and TLR and NLR agonists have shown significant synergistic activation of innate immunity in in vitro studies. Finally, considerable attention will also be devoted to formulating liposome-based immunotherapeutics, as physical factors such as particle size and charge all play an important role in regulating innate immune responses.

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