

Liposomal vaccines—targeting the delivery of antigen

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Abstract

Vaccines that can prime the adaptive immune system for a quick and effective response against a pathogen or tumor cells, require the generation of antigen (Ag)-specific memory T and B cells. The unique ability of dendritic cells (DCs) to activate naïve T cells, implies a key role for DCs in this process. The generation of tumor-specific CD8⁺ cytotoxic T cells (CTLs) is dependent on both T cell stimulation with Ag (peptide–MHC-complexes) and costimulation. Interestingly, tumor cells that lack expression of T cell costimulatory molecules become highly immunogenic when transfected to express such molecules on their surface. Adoptive immunotherapy with Ag-pulsed DCs also is a strategy showing promise as a treatment for cancer. The use of such cell-based vaccines, however, is cumbersome and expensive to use clinically, and/or may carry risks due to genetic manipulations. Liposomes are particulate vesicular lipid structures that can incorporate Ag, immunomodulatory factors and targeting molecules, and hence can serve as potent vaccines. Similarly, Ag-containing plasma membrane vesicles (PMV) derived from tumor cells can be modified to incorporate a T cell costimulatory molecule to provide both TCR stimulation, and costimulation. PMVs also can be modified to contain IFN- γ and molecules for targeting DCs, permitting delivery of both Ag and a DC maturation signal for initiating an effective immune response. Our results show that use of such agents as vaccines can induce potent anti-tumor immune responses and immunotherapeutic effects in tumor models, and provide a strategy for the development of effective vaccines and immunotherapies for cancer and infectious diseases.

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1. Introduction

An effective vaccine is empowered with the ability to “prime” the immune system in a way that enables it to respond quickly and effectively to prevent or limit infection by a specific pathogen, or to destroy cells of an emerging tumor, in the vaccinated host. Evidence suggests that live attenuated pathogens and viral vectors can induce protective effects when used in mucosal immunisations [1–4], but these can often induce unwanted side-effects or raise safety concerns [5]. To overcome potential difficulties recent efforts in vaccine development have focused on the use of killed vaccines and vaccines that employ subunit features

such as peptide epitopes and protein Ags [6–11]. These alternate approaches, however, often result in a vaccine that exhibits poor immunogenicity and efficacy [12]. Also, while the use of adjuvants such as alum and MF59 can augment responses to certain Ags, such agents can sometimes lead to the induction of an inappropriate response, e.g., a humoral response, instead of a cell-mediated response [13–15].

Recent work also has seen the development of cell-based vaccines that show promise in immunotherapeutic strategies against cancer. One approach employs vaccination with tumor cells that have been transfected to express one or more T cell costimulatory molecules and/or adhesion molecules on their surface, a strategy that has been shown to have significant preventative and/or immunotherapeutic effects in various tumor models [16–19]. Another approach has been the use of Ag-pulsed dendritic cells (DCs) as an

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autologous vaccine [20–24]. This latter strategy involves the isolation of DCs or their precursors from a patient, growing and differentiating the cells in culture, before pulsing them with Ag, maturing them in culture, and then introducing the cells back into the patient [20–25]. While these strategies show promise, they can be cumbersome and expensive to perform in a clinical situation. An approach that permits the targeting of tumor and/or other Ags directly to immune cells *in vivo*, in a way that can elicit the induction of an appropriate immune response could arguably circumvent these difficulties. However, evidence suggests that the targeting of soluble protein or peptide Ag to immune cells such as DCs *in vivo*, can often lead to the induction of tolerance to the Ag, rather than to immunity [26–28]. An ability to design vaccines that can induce an effective priming of the immune system with minimal side-effects or risk to the patient, and yet induce protection against pathogens, or show efficacy as an immunotherapeutic for cancer, could provide enormous benefit for the prevention/treatment of human disease.

Liposomes are particulate structures composed largely of natural or synthetic phospholipids which can encapsulate both Ag and immunomodulatory agents [29–32], thus allowing targeted delivery to specific immune cells *in vivo*. Depending on their lipid composition, liposomes also may exhibit potent adjuvant-like properties [33,34]. Similarly, membranous vesicular structures, such as plasma membrane vesicles (PMVs) derived from tumor cells, potentially contain numerous Ags, and additionally can be modified to contain immunomodulatory factors for targeted delivery to immune cells such as T cells [35] and DCs [36]. Liposomal structures will no doubt have much to contribute in the quest for the development of more effective vaccines. This review will begin with a short background on key elements of the immune system considered to be important for the action of liposomal vaccines, and then will proceed to dis-

cuss how such vaccines can be constructed to induce potent immune responses against tumors in animal models. The potential of this approach for the future development of vaccines and immunotherapies against cancer and infectious diseases in humans also will be discussed.

2. A role for DCs in vaccine action

Crucial to the mechanism by which vaccines can elicit protective immunity, is the role played by the various immune cells such as T cells, B cells, NK cells, macrophages, and DCs in eliciting the “priming” or protective response [37–39]. Following initial exposure to Ag, immune protection is likely to be mediated by Ag-specific memory T and B cells, which become “primed” in readiness for activation and effector function [40,41]. The two main subpopulations of T cells, namely the CD4⁺ and CD8⁺ (or cytotoxic) T lymphocytes are known to play an important role in mediating Ag-specific responses. Naive CD4⁺ T cells can differentiate into either the Th1 or Th2 phenotype, which, respectively, can provide help to either CD8⁺ T cells (leading to cell-mediated immunity) or to B cells (leading to the induction of humoral or antibody-mediated responses) [42]. Importantly, the effector function of the T and B cells is known to be regulated primarily through an interaction of naive T cells with professional Ag-presenting cells, namely the DCs [43–45].

DCs constitute a small population of professional Ag-presenting cells (APCs) present in the blood and other body tissues and play a key role in initiating immune responses to infectious diseases and tumors [46–49]. Subtypes of DCs that display a different cell surface phenotype and function are known to exist [38,50–53]. Immature DCs encounter most Ags and vaccine immunogens as extracellular proteins which are taken up by the cells, processed, and presented on MHC class II (and sometimes class I)

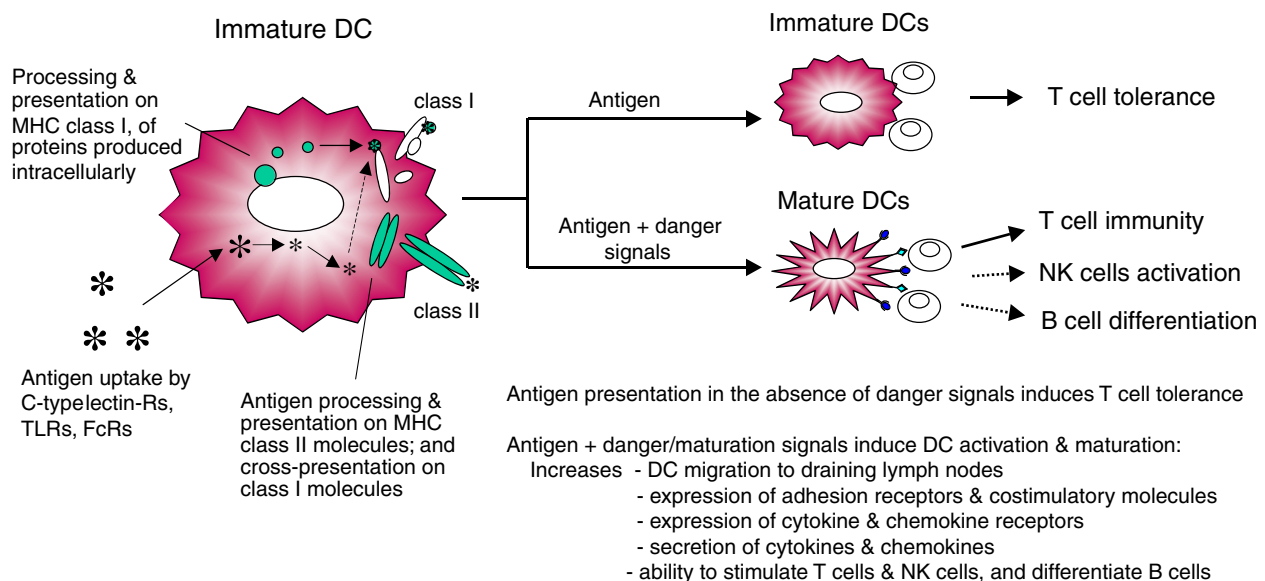


Fig. 1. Summary of the role of DCs in the induction of immunity. [Adapted from Fig. 1, Altin et al., *Expert Opin. Biol. Ther.* 4(11) (2004) 1735–1747.]

molecules, leading to the activation of T cells (Fig. 1). Immature DCs can capture and internalise Ag via a number of surface receptors, including Fc receptors, the complement receptor CD11c/CD18 [54,55], C-type lectins (e.g., DEC-205 and DC-SIGN) [56–58], and numerous Toll-like receptors (TLRs) [59,60]. Upon Ag uptake, immature DCs also can receive “danger” signals [61], as a result of the recognition of conserved pathogen-associated molecular patterns (PAMPs), by TLRs and other receptors [62–64]. Danger signals also can be delivered by heat shock proteins and inflammatory stimuli and/or cytokines such as interferon- γ (IFN- γ) [65,66], and members of the tumor necrosis factor (TNF) family generated as a consequence of the infection [67,68]. In the presence of a danger/inflammatory signal the uptake and processing of Ag by immature DCs can initiate a cascade of events, leading to migration of the DCs to secondary lymphoid organs and their maturation to become competent APCs [69–72]. Mature DCs are the major cells thought to activate naïve Ag-specific T cells [43–50]; they are potent initiators of immune responses to Ag, and exert a determining factor in the action of vaccines.

3. Tumor immunotherapy and cell-based vaccines

3.1. Tumor immunology

Many tumors are known to express tumor-specific Ags [73–75], and evidence suggests that effective cell-mediated immune responses to these Ags is crucial for tumor elimination [75,76], presumably through the activation of CTLs and other cell-mediated responses [37,75–77]. The elimination of tumors is thought to require the activation of Ag-specific CTLs, namely CD8⁺ T cells (Fig. 2) [78–82], a process dependent on Ag presentation via the class I pathway. DCs can present Ag in the context of MHC

class II and class I [83], and the processing of exogenous Ag for presentation via the MHC class I pathway may occur through Ag cross-presentation [38]. Importantly, evidence suggests that activation of both Ag-specific CD8⁺ and CD4⁺ Th (helper) T cells may be required to develop coordinated responses that lead to tumor regression [40,84].

3.2. T cell costimulation

While many tumor cells may express tumor specific Ags that are recognisable by TCRs, evidence suggests that tumor cells may escape elimination by the immune system because they often lack expression of T cell costimulatory molecules required for effective T cell activation. Thus, in the absence of an appropriate costimulatory signal being delivered through T cell surface molecules-like CD28, CD40L, and 4-1BBL (CD137L) [85], stimulation of the TCR by Ag-class I MHC complexes on tumor cells can lead to the Ag-specific T cells becoming anergic [86] and hence unresponsive to subsequent TCR stimulation by the tumor (Fig. 3). The induction of T cell anergy thus provides a mechanism enabling tumors to escape destruction by Ag-specific CTLs. Consistent with this notion, protective immunity has been reported following vaccination of animals with tumor cells that have been transfected to express different T cell costimulatory and/or adhesion molecules such as B7.1 (CD80), B7.2 (CD86), CD40, 4-1BB (CD137), and ICAM-1 [16–19,87], and different immunostimulatory cytokines such as GM-CSF, IL-2, and IL-12 [88–91]. These strategies thus enable tumor-specific T cells to become activated, presumably by overcoming Ag-specific T cell anergy (see Refs. [92–94]), hence leading to the induction of an effective anti-tumor response [86,95]. Whilst most studies using transfected tumor cells as vaccines have been carried

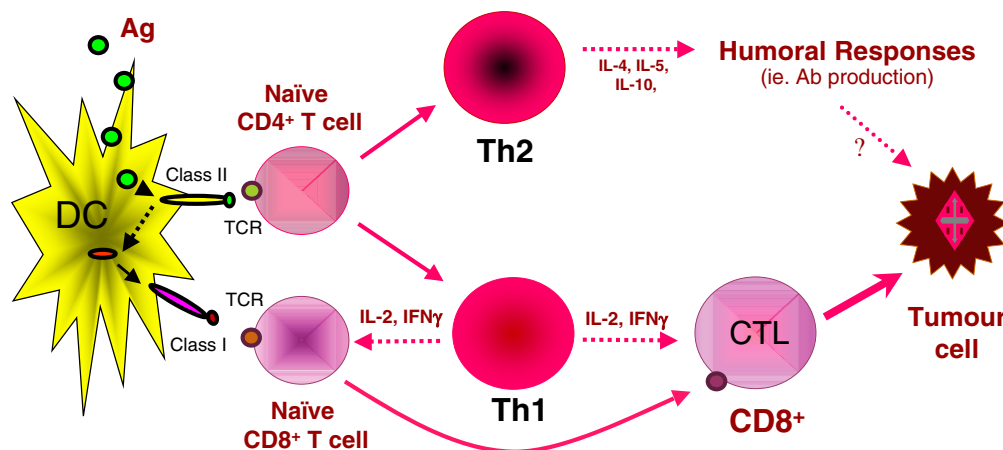


Fig. 2. A role for dendritic cells (DCs) in tumor immunity. The simplified diagram shows DCs taking up and presenting exogenous Ags (e.g., tumor Ags from dying tumor cells) to CD4⁺ T cells. The DCs can efficiently activate naïve CD4⁺ T cells which can differentiate primarily into the Th1 and Th2 phenotypes. The Th1 cells produce IL-2 and interferon- γ which can provide help to activate naïve CD8⁺ T cells and/or cytotoxic T lymphocytes (CTLs) which can mediate killing of tumor cells and virus-infected cells in an Ag-specific manner. The CD8⁺ T cells are activated by Ag presented on MHC class I by cells including DCs, which can occur directly by presentation of endogenous Ags on MHC class I, or indirectly by cross-presentation of exogenous Ags (Fig. 1). The Th2 CD4⁺ T cells produce cytokines which can provide help to B cells to produce antibody (Ab) and mediate humoral responses against Ag bearing target cells.

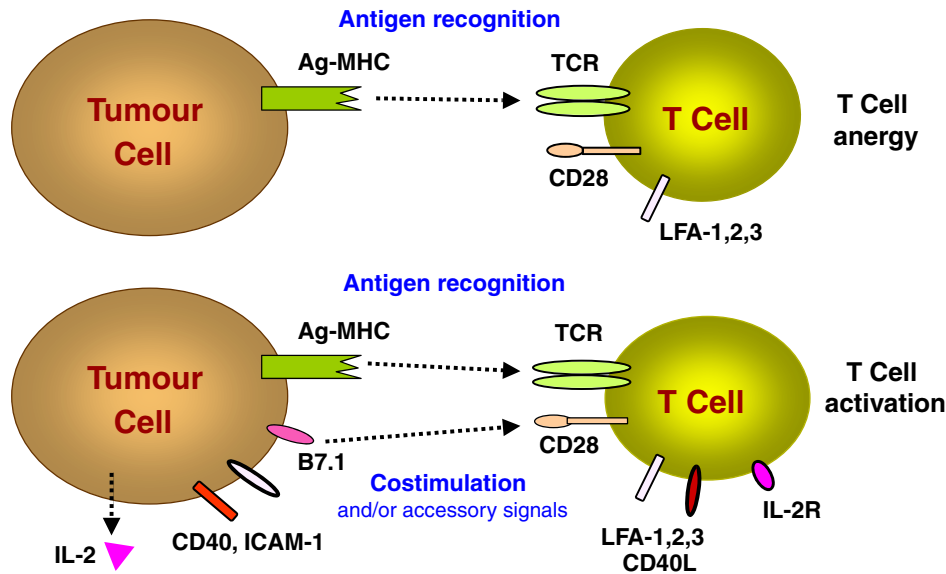


Fig. 3. T cell costimulation and tumor immunity. The simplified diagram depicts tumor cells (upper diagram) expressing tumor-associated antigens (Ag-MHC) but failing to express T cell costimulatory molecules (e.g., B7.1). In the absence of costimulation the T cells may become anergic. Tumor cells that have been transfected to express costimulatory and/or adhesion molecules like B7.1, CD40, and ICAM-1 and to secrete cytokines like IL-2 can activate T cells and induce anti-tumor responses when used as cell-based vaccines (lower diagram). Evidence suggests that the use of cell-based vaccines such as tumor cells modified to express T cell costimulatory molecules can lead to T cell activation and the induction of anti-tumor responses. However, while this approach has potential for the immunotherapy of cancer, the approach is not straightforward, and may present safety concerns.

out in animal models, it appears the approach also has relevance to human cancers [95].

3.3. DC-based immunotherapies

The unique ability of DCs to take up, process, and present Ag and to activate naïve T cells, makes them attractive for use in adoptive immunotherapies for the control of infections [43], autoimmune diseases [96–98], and cancer [20,48,49]. In the quest for effective DC-based cancer vaccines and immunotherapies, most clinical studies use monocyte- or CD34⁺ cell-derived DCs obtained from peripheral blood, which have developed into immature DCs following culture in the presence of GM-CSF and IL-4 for a period of 3–5 days. The cells are then either loaded with peptides or pulsed *ex vivo* with tumor Ags such as tumor cell extracts, protein(s) and/or peptide Ag(s), or perhaps tumor-derived RNA or DNA vectors encoding for tumor Ags [99,100], and then activated with stimuli such as CD40 ligand or TLR ligands for 1–2 days, before administering the cells to the patient [25]. As the immune system in cancer patients is often biased towards the Th2-type of T cell response, the goal of many DC-based vaccines and immunotherapies is to induce in patients a switch from a Th2- to a Th1-type of response, and a strong induction of tumor-specific CTLs [79,84]. Clinical trials using Ag-primed DCs have been conducted and are underway in patients with many different types of cancers including malignant melanoma, renal cell carcinoma, non-Hodgkins lymphoma, multiple myeloma, prostate cancer, and colorectal and lung cancer [21,22]. Evidence so far indicates that vaccination with Ag-primed

DCs can induce anti-tumor responses [21,43,49] and often benefit patients by delaying tumor progression and improving patient survival [21–23].

3.4. Difficulties with cell-based vaccines and immunotherapies

Whilst the use of cell-based vaccines shows promise for the immunotherapy of cancer, the wide-spread use of this approach is hampered by the complexity and associated cost of these techniques. Thus, the stable transfection of primary tumor cells with one or more costimulatory and/or adhesion molecule(s) (or cytokines) to produce a vaccine that can enhance Ag-specific T cell activation and avoid/overcome anergy can be difficult to achieve, making the approach impractical to use in a clinical setting [95]. Also, strategies employing Ag-pulsed DCs as vaccines and immunotherapies are cumbersome and expensive to use clinically, especially since multiple vaccinations may be required to elicit an effective response [99,100].

A cell-free system that has the benefits of a cell-based vaccine, but which is safe, simple and relatively inexpensive, could have enormous potential for simplifying the development and use of cancer vaccines and immunotherapies. Thus, a cell-free system that can enhance immunity by providing T cell costimulatory and/or other signals to avoid/overcome T cell anergy, without the need to use tumor cells that have been transfected with foreign DNA vectors, would be of significant benefit in this area. Similarly, a cell-free system that can target exogenous tumor Ags directly to DCs *in vivo*, could circumvent current diffi-

culties with the use of DC-based vaccines and immunotherapies. The delivery of exogenous tumor Ags directly to DCs *in vivo*, however, can often fail to deliver the expected outcome, possibly due to the inability to target the Ag in conjunction with an appropriate DC maturation signal [26–28,86].

Below we describe an alternative employing stealth liposomes (SLs) and liposomal agents, namely modified plasma membrane vesicles (PMVs) from tumor cells, that are modified to contain cytokines, and molecules that enable targeting of these structures to immune cells [35,36]. Thus, modified PMV can be engrafted with either T cell costimulatory molecules, for targeting and costimulating T cells (Fig. 4A), or molecules that bind surface markers on DCs to permit targeted delivery of Ag and a DC maturation signal(s) directly to DCs *in vivo* (Fig. 4C). Similarly, SLs-containing Ag(s) and cytokine(s) can be engrafted with a DC-targeting protein for delivery of both Ag(s) and a DC maturation signal(s) directly to DCs *in vivo* (Fig. 4B). Importantly, our recent results demonstrate that use of such liposomal agents as vaccines can induce potent anti-tumor responses in animal models (see below), thereby suggesting potential efficacy also in the prevention and immunotherapy of human cancers.

4. Liposomes and liposomal agents—alternatives to cell-based vaccines?

4.1. Liposomes—conventional vs stealth

Liposomes are small particulate structures which can be produced by suspending a mixture of either natural or synthetic lipids/phospholipid in aqueous media. The nature of the phospholipid component(s) can vary, with neutral liposomes containing predominantly a neutral phospholipid (e.g., phosphatidylcholine), and cationic and anionic liposomes containing lipids that are either positively or negatively charged, respectively [101]. Liposomes of most relevance in the present context are neutral and have a spherical bilayer ~100 nm in diameter with a central aqueous core, which can be used to encapsulate any compatible payload, such as drugs, protein or peptide Ags, or DNA [101,29,30]. Such liposomes initially were hailed as being highly useful as carriers of therapeutic agents, but were found to be cleared rapidly (within minutes) from blood, primarily through their elimination by phagocytic cells of the reticulo-endothelial system, particularly hepatic Kupffer cells, and by macrophages [102,103]. Despite their potential to deliver large amounts of encapsulated Ag, such “conventional” liposomes are of limited use for targeted delivery.

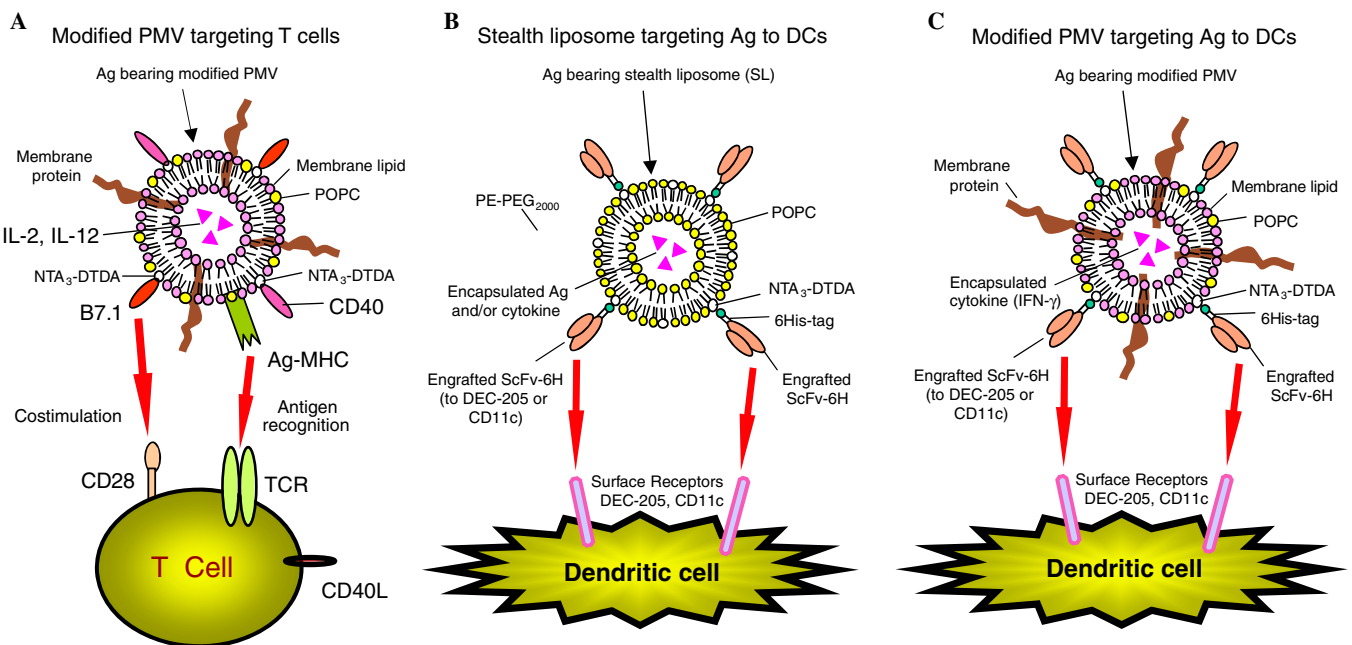


Fig. 4. Liposomal vaccines could provide a simpler and safer alternative to cell-based cancer vaccines and immunotherapies. The simplified diagram in (A) depicts how plasma membrane vesicle (PMV, derived from tumor cells) can be modified to contain an incorporated chelator lipid (e.g., NTA₃-DTDA), encapsulated cytokines (e.g., IL-2 and IL-12) and engrafted with His-tagged forms of T cell costimulatory molecules such as B7.1 and CD40. These agents can target T cells when administered *in vivo*, providing both antigenic (TCR) stimulation and costimulatory signals (through CD28 and CD40L) inducing effective T cell activation, with the cytokines also promoting this process. The diagram in (B) depicts how chelator lipid-containing stealth liposomes (SLs) once made to incorporate Ag(s) and cytokine (e.g., IFN-γ) and engrafted with His-tagged forms of ScFv that target CD11c and DEC-205 on DCs can be used to target both Ag and a maturation signal directly to DC *in vivo*, thereby inducing Ag-specific immunity and potentially circumventing the need for adoptive immunotherapy with Ag-pulsed DCs. Similarly, (C) shows how Ag-containing PMV once modified to incorporate cytokine (IFN-γ) and engrafted with a ScFv targeting DCs, also can be targeted directly to DCs *in vivo*. The disadvantage of approach (A) when used as a vaccine is that there must be MHC compatibility between the cells from which the PMVs are derived and host T cells; MHC compatibility, however, is not required for approaches (B) and (C).

The finding that liposomes can be made “stealth-like” by the inclusion of a small proportion (~5%) of a sterically stabilising lipid [104–106], has been a major advance in the use of liposomes for therapeutic applications. Stealth liposomes have a longer lifetime in the blood circulation, with their half-life being ~2 days, rather than just ~1–2 h for conventional liposomes [107]. Sterically stabilising lipids that promote “stealth-like” properties include the natural lipid ganglioside GM1, and synthetic derivatives of phosphatidylethanolamine containing a polyethylene glycol chain in its headgroup, e.g., phosphatidylethanolamine-(polyethylene glycol)-2000 (PE-PEG₂₀₀₀) [104–107]. The “stealth” lipid increases liposome stability and lifetime in serum by reducing non-specific interactions with blood components, and avoiding elimination by the reticulo-endothelial system [107,108]. Such liposomes, therefore, can be developed as targeted delivery vehicles for targeting Ags to immune cells *in vivo* [29,30,109]. This requires the anchoring of receptor or ligand molecules onto the liposome surface, to permit specific interaction of the liposomes with cells expressing the appropriate cognate-binding partner.

4.2. Liposomes as adjuvants

Considerable evidence suggests that liposomes, or suspensions of lipids and/or phospholipids, can exert immunomodulatory effects when introduced into the body as a vaccine adjuvant [33,34]. Commonly used adjuvants such as aluminium hydroxide (Alum) and Freund’s complete adjuvant (FCA) can augment the non-specific immune response to Ag, and influence the balance between cell-mediated and humoral immunity [31,109–111]. Interestingly, liposomes also are purported to exhibit adjuvant-like properties when used with many bacterial, viral, protozoan, tumor, and other Ags [29,112,113]. The mechanism by which liposomes elicit their adjuvant effect is not well understood, but passive targeting by virtue of their particulate nature and tendency to interact with macrophages of the reticulo-endothelial system is likely to be an important factor, particularly for non-targeted conventional liposomes. Depending on their lipid composition, liposomes also may interact with macrophages and DCs via cell surface lipid receptors, such as CD1a. The immunogenicity of liposomes also is influenced by their physicochemical properties such as their charge and fluidity (i.e., phase transition temperature of lipid components) [101,109]. For example, liposomes containing phosphatidylserine are more effective than neutral phospholipids at inducing IFN- γ secretion by murine spleen cells [114]. The lipid composition of the liposomes, therefore, may need to be considered to achieve the desired adjuvant effect.

4.3. Targeted liposomes as Ag delivery vehicles

4.3.1. Facilitated delivery

The delivery of Ag-containing liposomes to immune cells can be facilitated by agents that bind selectively

towards molecular structures on the surface of the cells to be targeted. For example, it is reported that monocytes express surface receptors for phosphatidylserine, that charged lipids bind selectively to DCs, and that anionic liposomes composed of phosphatidylcholine, cholesterol, and phosphatidylglycerol or phosphatidylserine interact with a subset of the total population of monocyte-derived human DCs and murine bone marrow-derived DCs *in vitro* [115]. Liposomes could thus potentially be designed to promote the interaction of Ag-containing liposomes with these respective cells. Also, DCs express lectin-like receptors including the mannose receptor, DEC-205 and DC-SIGN, which are thought to be involved in the phagocytosis of pathogens [56–58,116,117]. These receptors can facilitate binding and endocytosis of ligands that have a terminal sugar such as mannose, fucose and *N*-acetylglucosamine, and hence could be utilised to direct delivery of liposome-associated Ag to DCs [118–120]. Selectivity towards DCs has been reported with liposomes-containing phosphoethanolamine conjugated to one or several mannan moieties [30,121]. Since the mannose receptor is expressed on a number of different cell types including monocytes, macrophages, and DCs, however, it is unclear whether the targeting of liposomal Ag via this approach can be used to elicit the desired immune response after *in vivo* administration.

4.3.2. Attachment of targeting molecules

Antibodies and recombinant proteins such as soluble forms of cell surface receptors or their ligands, also can be used to target liposomes to receptors on cells, but this requires attachment of the targeting protein onto the liposomes surface. Liposomes with surface-attached monoclonal antibodies (mAb) that can target-specific cells expressing molecules bound by the mAb are known as immunoliposomes [122,123]. Attachment of the protein can be achieved by chemical crosslinking between reactive groups on the protein and those on a lipid such as phosphatidylethanolamine incorporated in the liposomal membrane. Different methods for the chemical coupling of targeting proteins to functionalised lipids used with liposomes have already been described [124]. However, coupling procedures are not always straightforward to perform and usually do not allow anchoring of the protein in the correct orientation onto the liposome surface thereby reducing the efficiency of interaction with the targeted receptor.

4.3.3. Engraftment of targeting molecules with chelator lipids

We recently developed a novel approach employing metal chelator lipids to anchor targeting molecules onto the surface of liposomes. Thus, our studies show that the incorporation of a metal chelator lipid like nitrilotriacetic acid ditetradecylamine (NTA-DTDA) and NTA₃-DTDA into the liposome membrane allows the convenient anchoring or “engraftment” of His-tagged proteins onto the liposomes, enabling convenient targeting to cells [125,126].

This approach was used to engraft liposomes with His-tagged forms of the T cell costimulatory molecules B7.1 and CD40 [35]. Interestingly, the chelator lipid also could be incorporated into biological membranes such as tumor cell-derived plasma membrane vesicles (PMV) (see below). The engraftment of NTA-DTDA-containing liposomes or PMV with recombinant His-tagged forms of B7.1 and CD40, enabled the liposomal membranes to bind T cells in a receptor-ligand specific manner *in vitro* and *in vivo*, enhancing their immunogenicity [35]. Of particular interest was the finding that such modified PMV when used as vaccines in syngeneic mice challenged with the native tumor-induced potent anti-tumor responses and prolonged survival [35]. Other studies [36] showed that SLs and PMV engrafted with ScFv specific for CD11c and DEC-205 on DCs, could target Ag and the cytokine IFN- γ to DCs *in vivo*, inducing potent anti-tumor immune responses and immunotherapeutic effects against the highly metastatic murine melanoma B16-OVA [this line, derived from B16 melanoma, has been transfected to secrete low levels of ovalbumin (OVA) as a surrogate secreted tumor-specific Ag]. The engraftment of targeting molecules onto liposomes and modified PMV in this way is convenient, and permits attachment of any appropriately tagged protein(s) for targeting to different immune cells. The procedure used for producing these liposomal constructs is outlined below.

5. Preparing liposomes for the targeted delivery of Ag and cytokines

5.1. Preparation of liposomes

Liposomes can be prepared by suspending a mixture of desiccated lipids in an aqueous buffer. The lipid mixture should usually contain a large proportion of a neutral lipid such as phosphatidylcholine, and lipids used as either tracers or protein anchors, and the suspension is made by either sonication or extrusion through polycarbonate filters of defined pore size, to produce liposomes of uniform size (usually 50–100 nm in diameter). The membrane extrusion method is likely to produce liposomes of more uniform size distribution compared to sonication, but is somewhat more cumbersome to use requiring the setting up of syringes, filters, etc., followed by a cleaning of apparatus when the task is completed. The advantage of sonication is that it is a quick procedure which can be used to prepare small sample volumes (usually 100–1000 μ l). The sonication time required depends on the sonicator and the membrane transition temperature (T_m) of the lipids being used, but for lipids-like POPC with low T_m (-2°C) can vary from ~ 1 min when using a probe sonicator (e.g., TOSCO 100W ultrasonic disintegrator, Measuring and Scientific Ltd., London, UK) at maximum amplitude, to 5–10 min when using cup or bath sonicators which are usually less energetic. Longer sonication times also are required when suspending lipids like dipalmitoylphosphatidylcholine (DPPC) and distearylphosphatidylcholine (DSPC) which have a higher T_m

(~ 41 and $\sim 55^\circ\text{C}$, respectively). A typical protocol for preparing SLs would require the preparation of an ethanolic solution of lipids such as POPC, PE-PEG₂₀₀₀ (for stealth liposomes), NTA₃-DTDA (for protein engraftment), and PC-BODIPY (lipid tracer) in an appropriate molar ratio (e.g., 94:3:1:1:1). The lipids can be mixed in a 1.5 ml Eppendorf tube if the stock is dissolved in ethanol (but a glass tube must be used if the stock is dissolved in chloroform), and then dried under a stream of N₂. After drying, the lipids are hydrated by adding an aqueous buffer such as PBS and sonicating or subjecting the solution to extrusion to produce the liposomes. If the lipid mixture contains a chelator lipid such as NTA₃-DTDA an appropriate amount of Ni²⁺ (usually in the form of NiSO₄) also needs to be included in the buffer. The liposome suspension typically is made to a final concentration of 1 mM total lipid, before then engrafting with an appropriate His-tagged protein.

It should be noted that stealth liposomes generally contain $\sim 5\%$ PEG₂₀₀₀ and that only relatively small amounts of tracer and protein anchor lipids are required ($\sim 1\%$ each). Also, lipid mixtures containing phosphatidylcholines with longer and/or saturated carbon chains such as DPPC (C16:0) and DSPC (C18:0) as well as cholesterol ($\sim 45\%$), can be included or used instead of POPC to increase the rigidity of the liposomes and their stability in serum.

5.2. Encapsulation of Ag and cytokines

An important attribute of liposomes is that soluble protein or peptide Ag and immuno-modulatory factors such as cytokines, can be conveniently encapsulated within the aqueous core, and/or associated with the liposomal membrane [127,128]. Encapsulated agent(s) are protected from degradation by proteases and neutralisation by interacting proteins in plasma, with the liposome structure providing a vehicle for targeting the encapsulated payload directly to immune cells *in vivo*. The ability to co-deliver Ag and cytokines simultaneously to the same immune cell provides a powerful approach for manipulating Ag-specific immune function, while minimising the potential adverse effects that such cytokine(s) may have if administered systemically. Liposome-encapsulated cytokines also have been reported to have an increased potency and longer half-life compared to free cytokines [35,129,130]. Different strategies can be used to encapsulate protein or peptide Ag and cytokines for targeted delivery to immune cells.

5.2.1. Encapsulation by passive entrapment

The advantage of this approach is that proteins and cytokines can be used in their native form. For example, unmodified soluble protein and/or peptide Ag and cytokines can be encapsulated simply by dissolving these agents in an aqueous buffer (e.g., PBS) and then using the solution to suspend the lipid mixture for liposome production. Suspension of the lipids (e.g., by sonication) will result in entrapment of the agents within the aqueous core and also, in some cases (e.g., IL-2), incorporation in the lipid bilayer

of the liposomes being produced. The efficiency of encapsulation will depend on a number of factors: notably the concentration of the agent to be incorporated, the final concentration of the lipid suspension, and the physiochemical properties (i.e., electric charge and hydrophobicity) of both the agent(s) to be incorporated and the lipids. Although exceptions are likely to exist, there is no doubt that some protein Ags and cytokines can be encapsulated in liposomes with high efficiency.

In recent studies we encapsulated the xenogenic Ag OVA and various cytokines into SLs and PMV with high efficiency by passive entrapment. Encapsulation was achieved by rehydrating the lipid mixture consisting of POPC, NTA₃-DTDA, and PE-PEG₂₀₀₀ in PBS containing 0.1 mg OVA (1 mg/ml) (or 50 ng of cytokine in 100 μ l), followed by brief sonication. OVA protein was readily encapsulated within SL, with SDS-PAGE and densitometric analysis revealing an encapsulation efficiency of 85% (Fig. 5). Similarly, cytokines such as IL-2, GM-CSF, and IFN- γ , also were encapsulated into liposomes by this procedure with incorporation efficiency varying from 70% to 90% [35,36,131]. Importantly, functional studies indicate that the liposome- and PMV-incorporated IL-2, IL-12, GM-CSF, and IFN- γ are active in functional assays. Thus, this approach should be applicable to the encapsulation of other proteins and/or cytokines.

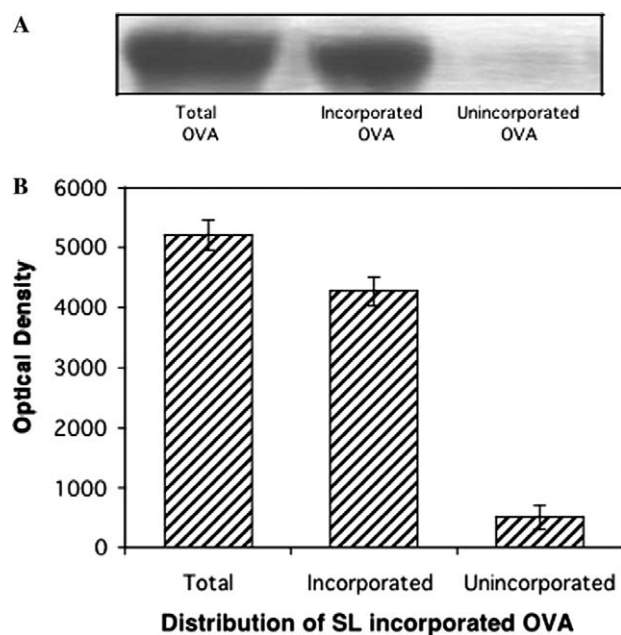


Fig. 5. Efficient encapsulation of OVA into stealth liposomes. A desiccated mixture of POPC, NTA₃-DTDA, PE-PEG₂₀₀₀, and LPS (molar ratio 97:1:1:1) was rehydrated in PBS containing OVA (1 mg/ml) and sonicated to form liposomes. The SL were purified by size-exclusion chromatography on a G200 Sephadex column, with the liposome-incorporated and -unincorporated OVA fractions being collected separately. Each fraction was then concentrated, and analysed by reduced SDS-PAGE, the gel was stained with Coomassie blue and the result is shown in (A). The gel was scanned and a densitometric analysis of the intensity of the OVA band for each condition was carried out using NIH Image 1.61 software shown in (B). [Data are from a Ph.D. thesis by Christina van Broekhoven (ANU, 2003).]

5.2.2. Active loading or association of protein and peptide Ags with liposomes

A high efficiency of encapsulation of proteins/peptides and cytokines can sometimes be achieved by passive entrapment. Encapsulation efficiency is usually poor, however, when the agent to be encapsulated is used at low concentrations during suspension of the lipids—perhaps due to poor solubility. In these situations, active incorporation of proteins or peptides with liposomes can be promoted by engineering the protein/peptide to have an affinity tag that permits active-binding to the liposomes. Thus, protein/peptide Ags bearing a 6His-tag can be produced and active incorporation of the His-tagged protein/peptide with the liposomes can be promoted by inclusion of a small proportion (1–5%) of chelator lipid in the liposome lipid mixture to allow protein-binding via metal-chelating linkage.

Our initial efforts to encapsulate into liposomes the peptide SIINFEKL, the MHC class I immunodominant epitope of OVA in H-2^b mice, were unsuccessful probably because this peptide has poor solubility at physiological pH. Upon production of SIINFEKL with a 6His tag at the COOH terminal (SIINFEKL-6H), however, we observed that the fusion peptide is very soluble, permitting convenient incorporation into liposomes. Since our studies also utilised a targeting strategy that employed proteins engrafted onto the liposomes by metal chelating linkage, it was essential to ensure that incorporation of the His-tagged peptide did not substantially compete or interfere with the engraftment of the targeting protein onto the liposomes. This was assessed by examining the effect of including the SIINFEKL-6H peptide on the ability of the liposomes to be targeted via targeting proteins engrafted onto the liposome surface.

Lipid mixtures for producing NTA₃-DTDA-containing SLs were suspended in the presence of different concentrations of SIINFEKL-6H, before incubating the liposomes with a DC-targeting protein, namely a ScFv targeting CD11c or DEC-205, for 1 h at room temperature. The engrafted SLs were then incubated with the cell line murine fetal skin DC (FSDC) *in vitro* (1 h, 4 °C), and the cells then washed and analysed for liposome-binding by flow cytometry. The results indicate that up to 2 μ M SIINFEKL-6H can be included in the liposome lipid mixture with only a slight reduction (~10–20%) in the targeting of the liposomes to the DCs (Fig. 6). Interestingly, the addition of the 6H-tag did not significantly affect the ability of the SIINFEKL-6H to be processed and presented correctly on the DCs, since strong OVA-specific CTL responses could still be generated (see later—Fig. 9). Other immunological experiments using liposomes that had incorporated the optimum concentration of SIINFEKL-6H (2 μ M) and were targeted to DEC-205 on DCs *in vivo*, demonstrated that the association of the peptide with liposomes in this way is effective at inducing OVA-specific immunity in mice immunised with the liposomes and challenged with B16-OVA melanoma cells [36].

Our observation that the fusion of SIINFEKL with 6H-tag (SIINFEKL-6H) is a highly effective method for associ-

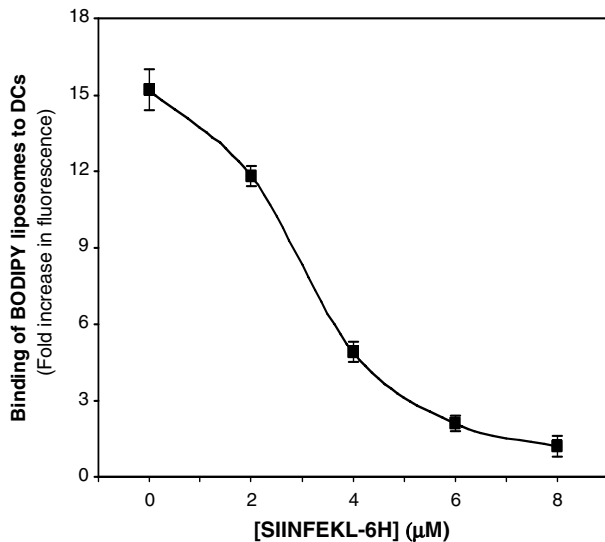


Fig. 6. The effect of SIINFEKL-6H on the binding of fluorescently labelled stealth liposomes targeting CD11c on mouse fetal skin DCs (FSDC). Stealth liposomes (SL) composed of POPC, NTA₃-DTDA, PE-PEG₂₀₀₀, and PC-BODIPY were incubated with CD11c-ScFv (SL-CD11c) in the presence of different concentrations of the peptide SIINFEKL-6H (0, 2, 4, 6, and 8 μM), before incubating the engrafted liposomes separately with FSDCs (for 1 h at 4 °C). The cells were then washed and analysed for BODIPY-fluorescence by flow cytometry. Each point represents the fold increase in the binding of the engrafted SLs to the cells (as reflected by the relative increase in fluorescence intensity) plotted against the concentration of SIINFEKL-6H. Each of the data points represents the result from three separate experiments performed in duplicate $\pm$ SEM. [Data are from a Ph.D. thesis by Christina van Broekhoven (ANU, 2003).]

ating the peptide with chelator-lipid-containing liposomes, suggests that the same approach can be applied to other peptide Ags for active incorporation into liposomes, and targeted delivery to cells *in vivo*. Moreover, the solubility of peptides may be increased by fusing with a 6H-tag, as seen with SIINFEKL. Since the His-peptide is included in the lipid mixture *before* production of the liposomes, in addition to potential encapsulation of the peptide, the peptide can bind to chelator lipids on both sides of the liposome bilayer, increasing liposome payload. It is conceivable, however, that the approach is best suited for use with peptides and small proteins that do not sterically interfere with either the anchoring of the targeting protein on the liposomal surface, or with the ability of this protein to interact with its binding partner on target cells.

6. Ag-containing membrane vesicles as vaccines

6.1. Advantages of membrane vesicles

Considerable evidence suggests that many immunologically relevant Ags reside on the membranous surface of cells and pathogens. Similar to the immunisation with a live or killed pathogen as vaccine, therefore, the ability to deliver the membrane (or membranous vesicles derived from the membrane) of cells and pathogens, together with

their associated Ags, to immune cells such as DCs, could constitute a powerful approach to triggering an effective immune response and the protective effects of a vaccine. The advantages of using cell or pathogen-derived membranous vesicles as a component of a vaccine are: (1) the membranes may contain numerous unknown or as yet uncharacterised Ags that could be immunologically important; (2) potentially all Ags associated with the membrane structure could be targeted to immune cells such as DCs under condition known to be optimal for their activation—hence promoting immunity, rather than tolerance; and (3) such membranous material, that does not contain foreign DNA, is likely to have fewer safety concerns compared to live pathogens or cells that have been genetically modified to express Ags or costimulatory molecules. Various studies using modified membrane vesicles as cancer vaccines and immunotherapies are described below; the findings constitute “a proof of principle” for use of this approach for developing vaccines against cancer and infectious diseases.

6.2. Modified plasma membrane vesicles as cancer vaccines and immunotherapeutics

The potential for using cell-derived liposomal membranes to deliver tumor Ags to DCs recently has been explored using tumor cell-derived plasma membrane vesicles (PMVs). Tumor cell-derived PMVs are known to retain the antigenic properties of the original tumor cells, and can be prepared from isolated tumors and established tumor cell lines, and can be stored frozen until required. They can be prepared by brief sonication of the cells, followed by separation of the membrane fraction by high-speed centrifugation on sucrose [35,36,132]. The membrane fraction (PMVs) is then collected, washed, and used for vaccine production. A liposome mixture can then be used to modify the PMVs to incorporate cytokines and a chelator lipid. Thus, ethanolic solutions of POPC, NTA₃-DTDA, and the tracer lipid PC-BODIPY (molar ratio 96:2:2) are mixed, dried under a stream of N₂, and then rehydrated in 100 μl PBS containing 60 μM Ni²⁺. One or more cytokines such as IL-2, IL-12, IFN-γ, or GM-CSF (50 ng) can be included in the rehydration buffer, for incorporation into the liposomes. Following hydration the mixture is sonicated (three times, 15 s bursts) using a probe sonicator (e.g., TOSCO 100W ultrasonic disintegrator) at maximum amplitude. A longer sonication time (2–5× longer) may be required when using either a cup or bath sonicator which is less energetic. To preserve the activity of the cytokines however, it is essential to ensure that sonication is kept to a minimum (while still allowing dispersion) and that the temperature is maintained close to 4 °C throughout the procedure. The cytokine and NTA₃-DTDA-containing liposomes (100 μl) are then mixed with 100 μl of the appropriate PMV (1 × 10⁷ cell equivalents), and fusion is promoted by adding 15% PEG₄₀₀ and diluting 10 times with PBS. The NTA₃-DTDA- and cytokine-containing PMV can be purified by size-exclusion chromatography (e.g., on a Sephadex G-50

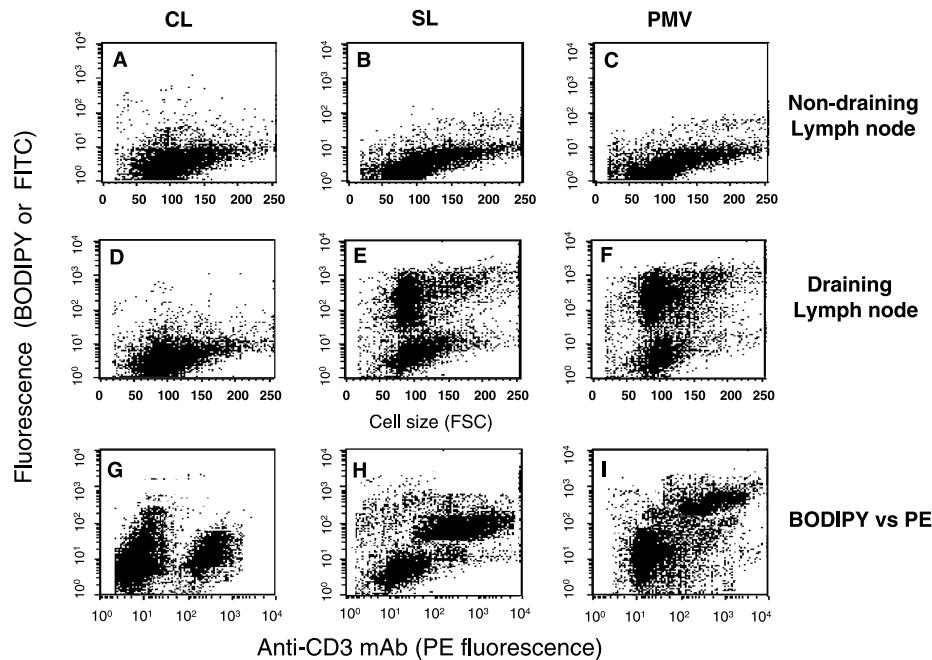


Fig. 7. Targeting of engrafted NTA₃-DTDA-containing liposomes and PMV to T cells *in vivo*. NTA₃-DTDA and PC-BODIPY (each at 1% molar ratio) were incorporated into conventional liposomes (CL) lacking PE-PEG₂₀₀₀, and stealth liposomes (SL) containing PE-PEG₂₀₀₀. Also, NTA₃-DTDA was incorporated into FITC-labelled P815 tumor cell-derived plasma membrane vesicles (PMV). Each liposome/membrane preparation was then engrafted with either a control protein (not shown) or B7.1-6H plus CD40-6H, before being injected subcutaneously (hind footpad) into mice. After 14 h the draining and non-draining popliteal lymph nodes were removed, and lymphocytes isolated and stained with biotinylated-CD3 ϵ mAb followed by streptavidin-PE. The cells were then analysed for BODIPY/FITC- and PE-fluorescence by flow cytometry, to detect binding of the liposomes and PMVs, and staining with streptavidin-PE (to identify CD3⁺ cells). No fluorescent cells could be detected after injection of liposome/membrane preparations engrafted with a control protein (not shown). (A–C) Show dot plots of fluorescence vs FSC for lymphocytes-derived from the non-draining lymph nodes of mice injected with B7.1-CD40-engrafted CL, SL, or PMV, respectively. Results for draining lymph nodes are shown in (D–F). The corresponding BODIPY/FITC vs PE-fluorescence for CD3⁺ lymphocytes from draining lymph nodes are shown in (G–I), respectively. Each plot is representative of two experiments. [Reproduced with permission from van Broekhoven and Altin (2005). *Biochim. Biophys. Acta* 1716, 104–116].

mini-column or similar) [35,36], before engraftment of an appropriate targeting molecule to complete production of the vaccine. The vaccine is best used within a few hours of preparation. If storage is required it is best to store the modified PMVs frozen and then to re-sonicate these briefly when required and before engraftment of the targeting protein. This ensures that the PMVs used for vaccination are not aggregated, and avoids a potential deterioration in the binding activity of the targeting protein due to sonication.

Various tumor vaccine combinations recently have been prepared from tumor-cell derived PMVs that have been modified to contain cytokines (e.g., IL-2, IL-12 GM-CSF, and IFN- γ) and engrafted with either T cell costimulatory molecules (B7.1 and CD40) (Fig. 4A), or with ScFv that target the surface markers CD11c and DEC-205 on murine DCs (Fig. 4C). Such liposomal preparations are targeted *in vivo* to cells expressing the appropriate ligands. For example, the injection of SLs and PMVs engrafted with a ScFv to CD11c or DEC-205 into the hind footpad of mice results in the targeting of the liposomes to DCs in the draining lymph node [36]. Similarly, the injection of B7.1-CD40-engrafted SLs and PMVs results in them being targeting to T cells in the draining lymph node, but no targeting of conventional liposomes could be detected (Fig. 7). Interestingly, unlike conventional liposomes which

require a sterically stabilising lipid to make them “stealth”, therefore, the modified PMVs appear already to possess stealth-like activity (Fig. 7). Importantly, immunological studies showed that PMV preparations containing costimulatory molecules and cytokines can exert potent anti-tumor responses, including the inhibition of tumor growth and regression of established tumors (Fig. 8 and Refs. [35,131]). Similarly, modified PMV-containing IFN- γ and engrafted with ScFv to target CD11c and DEC-205 on DC-induced dramatic anti-tumor responses when used as a vaccine in the B16-OVA tumor model (Fig. 9 and Ref. [36]). The use of PMVs that have been modified to contain cytokines and that can be targeted to specific immune cells in this way, therefore, could provide a powerful approach for future development of cancer vaccines and cancer immunotherapies.

7. Conclusions and future directions

The ability of a vaccine to prime the immune system for an effective response upon Ag exposure is dependent on the Ag-specific activation of effector T and B cells of the adaptive immune system. Due to their ability to activate naive T cells, DCs play a key role in initiating an immune defence against infectious diseases and cancer, and hence their role

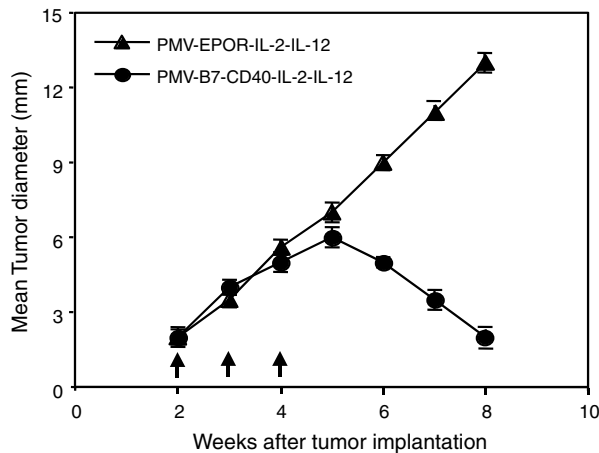


Fig. 8. Immunisation with PMVs containing encapsulated IL-2 and IL-12 and engrafted with recombinant costimulatory molecules induces regression of established tumors. Separate groups of syngeneic DBA/2J mice were injected subcutaneously with 1×10^5 P815 tumor cells. Two weeks after injection the mice were immunised subcutaneously (arrows) by administering 3 injections (at one week interval) with P815 cell-derived: control PMVs engrafted with the erythropoietin receptor and containing IL-2 plus IL-12 (PMV-EPOR-IL-2-IL-12), or PMV engrafted with B7.1 and CD40 and containing IL-2 and IL-12 (PMV-B7-CD40-IL-2-IL-12), as indicated. The mice in each group were then monitored for tumor growth. The data show the mean tumor diameter of each group of mice as a function of time for the first 8 weeks. [Reproduced with permission from van Broekhoven and Altin (2005). *Biochim. Biophys. Acta* 1716, 104–116.]

is paramount for vaccine action. Since the use of live vaccines can have safety concerns, current strategies often focus on the use of subunit vaccines such as protein or peptide Ags, with and without adjuvants. However, this approach, can result in the production of vaccines that have poor immunogenicity and efficacy, or result in the induction of an inappropriate response—such as tolerance, rather than immunity. Liposomes are particulate lipid structures that can incorporate both Ag and immunomodulatory factors, permitting their targeted delivery to specific immune cells. A convenient approach for targeting liposomes is to incorporate a metal chelator lipid such as NTA₃-DTDA enabling engraftment of a His-tagged targeting protein via metal-chelating linkage. Interestingly, Ag-containing membrane vesicles (e.g., PMVs derived from tumor cells), also can be modified to incorporate cytokines and chelator lipids for engraftment of targeting molecules [35,36,131]. The advantage of targeted liposomal membranes as vaccines is that they can be used to deliver many Ags and immunomodulatory factors to immune cells simultaneously, allowing the vaccines to be readily “tailored” to induce the most appropriate type of immune response.

The use of cell-based vaccines, such as tumor cells transfected to express T cell costimulatory molecules and Ag-pulsed DCs, show promise for the immunotherapy of cancer, but the approach is cumbersome and expensive to use clinically. Modified PMV that concomitantly target tumor Ags and cytokines to immune cells *in vivo* could circumvent this difficulty. Our studies using P815 tumor cell-derived PMV modified to contain cytokines (IL-2 and IL-12) and engrafted with

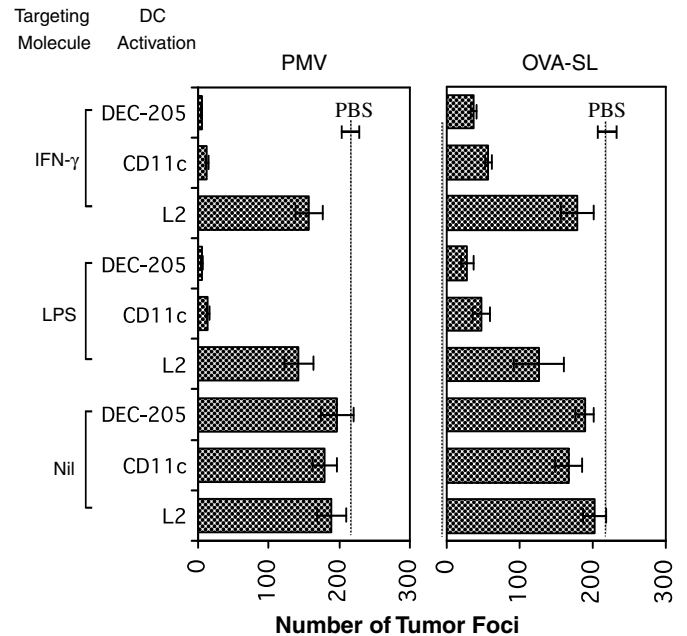


Fig. 9. Vaccination with modified PMVs and Ag-containing stealth liposomes targeting DCs elicits protective anti-tumor immunity. (A) Separate groups of six syngeneic C57BL/6 mice were immunised (three times at weekly intervals) with PMV engrafted with L2 (a control peptide), CD11c-ScFv, or DEC-205-ScFv; and OVA-containing SL engrafted with L2, CD11c-ScFv, or DEC-205-ScFv, with each vaccine preparation being injected alone or in combination with LPS or IFN-γ, as indicated. Mice were challenged with 3×10^5 B16-OVA melanoma cells administered i.v., and after 16 days the lungs were removed and examined for lung metastases. Results show the mean number of tumor foci for each group of mice $\pm$ SEM. The dotted line refers to the number of tumor metastases in control mice that were injected with PBS. [Data are derived from Broekhoven et al. (2004). *Cancer Res.* 64, 4357–4365.]

T cell costimulatory molecules (B7.1 and CD40), show that these can induce potent anti-tumor responses and tumor regression when used as vaccines in syngeneic animals [35,131]. Also, PMV derived from the B16-OVA melanoma could be modified to contain a DC maturation signal (e.g., IFN-γ) and engraftment of a ScFv targeting either CD11c or DEC-205 on DCs. The targeting of both Ag and a DC maturation signal to DCs *in vivo* was found to induce dramatic anti-tumor responses and immunotherapeutic effects in the B16-OVA tumor model [36]. These findings are consistent with observations [133], and indicate that liposomal vaccines could potentially circumvent the need for more complex cell-based vaccines for cancer immunotherapy. Clearly the next step is to demonstrate that such targeted liposomal vaccines also are effective against human cancers.

The same approach outlined above for use of membrane vesicles to develop vaccines and immunotherapies against cancer could be used to develop vaccines against infectious agent/pathogens. The use of liposomal agents enables the delivery of multiple Ags and/or immunomodulatory agents to immune cells such as DCs, and could be a particularly effective strategy for the development of vaccines against complex pathogens with immune evasion strategies, such as TB and malaria.

In future studies, the potential of liposomal vaccines for the prevention and immunotherapy of cancer could be further explored by examining the effect of engrafting Ag-containing PMVs with different T cell costimulatory molecules, and/or the incorporation of different cytokines targeting different immune cells. Also, as multiple subsets of immune cells such as DCs are known to exist, it is conceivable that the targeting of Ags or immunomodulatory factors to different DCs subtypes (e.g., plasmacytoid DCs vs other DCs) may be a crucial factor in eliciting the optimum response. Different antigen-capture receptors such as TLRs on DCs also may have different capacities to internalise and/or to direct processing of internalised antigen, and to induce DC maturation. Thus, Ag-containing liposomes that target different TLRs, or perhaps that contain iNKT ligands [134,135], also could be harnessed to elicit the most appropriate and effective response. Since both tumors and pathogens are known to escape immune detection by actively suppressing different aspects of immune function, including the function of T cells and DCs [136–138], future studies also should be aimed at exploring ways of employing targeted liposomes to overcome such immunosuppressive effects. In addition to Ag and immunomodulatory factors, liposomal agents can potentially also deliver nucleic acids directly to immune cells, perhaps enabling the use of targeted nucleic acids as vaccines [139–143]. In conclusion, the ability to target both Ag and immunomodulatory factors directly to immune cells could have enormous potential for the development of novel and more effective vaccines for the prevention and immunotherapy of infectious diseases and cancer.

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