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Effective delivery of tumor antigens to APCs is one of the key steps for eliciting a strong and durable immune response to tumors. Several cancer vaccines have been evaluated in clinical trials, based on soluble peptides, but results have not been fully satisfactory. To improve immunogenicity particles provide a valid strategy to display and/or incorporate epitopes which can be efficiently targeted to APCs for effective induction of adaptive immunity. In the present review, we report some leading technologies for developing particulate vaccines employed in cancer immunotherapy, highlighting the key parameters for a rational design to elicit both humoral and cellular responses.

KEYWORDS: antigen presenting cells • cancer vaccines • HBV • HPV • ISCOMs • liposomes • nanoparticles • polymers • virus-like particles

General considerations

Particles are designed to mimic the structure and size of microbes that are themselves nanoparticles (virus) and microparticles (bacteria). In this perspective, particles offer a great opportunity to design advanced platforms to stimulate APCs, especially dendritic cells (DCs), by properly combining and delivering the target antigen and, if needed, an immunomodulator. As compared to soluble antigens, engineered particulate carriers can: display a dense array of linear and/or conformational epitopes; protect the antigen from proteolytic enzymes; target immunocompetent sites (NALT, GALT in mucosal vaccination); guide antigen internalization process and presentation in DCs; and allow safe access of antigen to lymphatic tissues and organs.

Indeed, antigen-bearing particles are preferentially taken up via endocytosis or phagocytosis by immature DCs and Mφ in the peripheral tissues and then transported to lymph nodes for recognition by immune cells and induction of an adaptive immune response.

Particles smaller than 200 nm (e.g., VLPs) are internalized in DCs via receptor-mediated endocytosis, whereas larger particles (>0.5 μm) enter via phagocytosis or macropinocytosis. Opsonization of particles by interaction with receptors located on APC surface such as albumin, immunoglobulin or other serum proteins is a key step for effective DCs and Mφ entry.

Depending on internalization pathway, antigen is directed into distinct populations of early phagosome/endosomes resulting in its release from the carrier, intracellular processing and presentation by MHC class I and/or MHC class II pathways. Endocytosis is recognized to promote antigen presentation through MHC class II pathway whereas particulate antigens can activate cross-presentation. Role of size, particle shape, surface and mechanical properties on particle internalization has been reviewed recently [1,2].

Fine-tuning of particle properties is needed to attain specific requirements and effective immune response. Particles can be prepared on nanoscale or microscale using different materials (lipids, polymers and inorganic materials) and surface-engineered to attain specific functions. An antigen can be loaded on the surface (adsorbed or chemically modified) or embedded in a particulate system, depending on its stability in physiological environment. Particles can act as adjuvant themselves or need a specific adjuvant to elicit proper immune response whereas targeting an antigen to DCs via surface modification of particulate carriers can be beneficial to facilitate both particulate uptake and immune response [3]. Indeed, it has been demonstrated that particles, upon uptake by DCs, lysosomal acidification and cathepsin B activity, promote activation of the NALP3 inflammasome and secretion of IL-1β, IL-18 and IL-1α [4,5]. This

Figure 1. Virus-like particle structure.
TAA: Tumor-associated antigen.

is required for the effective priming of IFN- γ -producing tumor antigen-specific CD8⁺ T cells [6]. The ability of different particles to activate the immune response through the inflammatory pathway has been recently reviewed [7].

A large array of different particulate systems for antigen delivery to DCs has been studied so far. On the basis of the overall properties driving interaction with DCs, particles can be classified as biological (e.g., virus-like particles-VLPs) and chemical particles, with the latter sub-divided in: passively targeted, actively targeted and smart particles. Furthermore, advanced particle design can be useful to track their biological fate in cells as well as in the whole body by different techniques (fluorescence, MRI).

With the exception of few strategies already in clinical trial or licensed for human use, most of the immunological results described in the present review are generated in animal models and need to be confirmed in humans.

Virus-like particles

VLPs closely resemble the native viruses, allowing the display and delivery of repetitive arrays of antigens, with improved induction of antibodies and immune response in its whole. VLPs are made of viral capsid proteins which self-assemble into particulate structures and are replication as well as infection incompetent [8,9]. Antigenic structures as well as DNA molecules are efficiently delivered by VLPs to APCs [10–13], and antigens are cross-presented in association with both MHC class I and class II [14,15] to elicit both CD4⁺ T helper and CD8⁺ cytotoxic T cells [12].

VLPs have been produced from a broad spectrum of enveloped and non-enveloped viruses [16]. In particular, the particle structure of non-enveloped VLPs can be based on single or multiple capsid proteins without a surrounding cell membrane [17–20].

Alternatively, enveloped VLPs are made of assembled capsid proteins surrounded by cell membrane and have been

developed for enveloped viruses such as HBV and HCV, influenza A and retroviruses, including HIV-1 [21–25].

According to their structure, VLPs have distinct properties for displaying antigens. Enveloped VLPs, indeed, can display full-length monomeric or multimeric conformational proteins anchored through trans-membrane domains (FIGURE 1) [24,26]. Non-enveloped VLPs, on the contrary, may display mainly short peptides, either generating chimeric capsid proteins expressing foreign epitope in frame [27,28], or chemically linking the foreign epitope to the assembled capsid protein [29]. Large antigens have also been shown to be efficiently linked to non-enveloped VLPs based on bacteriophage Q β protein [30].

Immunogenicity of VLPs

VLPs, similar to native viruses, display an array of pathogen-associated molecular patterns (PAMPs) to the innate immunity, leading to activation of adaptive and inflammatory responses [31,32]. Morphological properties of VLPs (size and shape) represent the key for efficient up take by the APCs, particularly DC, resulting in: i) processing and presentation by MHC class II molecules and ii) induction of DC maturation and migration to lymph nodes to activate T cells [31,33–36]. Moreover, unlike other exogenous antigens which cannot reach the MHC class I pathway [37,38], VLPs undergo to cross-presentation within APCs and efficiently reach the MHC class I pathway in the absence of infection or intracellular replication [14,15]. As consequence of this biological property, VLPs have also been shown to be highly effective at stimulating CD4 proliferative and cytotoxic T lymphocyte (CTL) responses [39–42].

In addition, VLPs have a size suitable for reaching draining lymphoid organs, upon subcutaneous injection, where they are taken up by lymph node resident cells. In contrast, larger particles fail to directly reach lymphoid organs but need to be transported by skin resident dendritic cells, which do not express TLR9 [43].

VLPs are generally more immunogenic than subunit or recombinant protein immunogens. In particular, VLPs display conformational epitopes and can activate B-cell receptors and T-cell-independent IgM responses [44,45]. This is particularly relevant for surface proteins of enveloped viruses displayed on the surface of enveloped VLPs, rather than the soluble ectodomains alone.

In this context, our group has extensively shown that HIV-1 Pr55gag VLPs induce maturation and activation of *in vitro* cultured myeloid-derived dendritic cells (MDDCs) partly through TLR-3 and -9 signaling [33,34,46–48].

In order to elicit a more potent CTL response, co-administration of VLPs with stimuli of innate immunity system has been proposed. VLPs have been developed to co-deliver TLR ligands and T-cell epitopes to DCs and numerous ligands for TLRs have been tested [49–51]. DNA containing CpG motifs, a ligand for TLR-9, were by far the most potent adjuvants inducing robust T-cell responses [49,52]. In particular, the immune potentiators packaged into VLPs, are protected from

degradation and their non-specific binding to serum proteins is significantly reduced. Furthermore, the specific targeting of APCs reduces side effects, such as splenomegaly [49] and autoimmune disorders [53].

VLP-based cancer vaccines

VLP-based preventive and therapeutic cancer vaccines are in different stages of development. In particular, the anti-HBV and anti-HPV vaccines are the only two preventive cancer vaccines licensed for clinical use. Indeed, they prevent the HBV-associated hepatocellular carcinoma and the HPV-associated cervical carcinoma, respectively (TABLE 3). On the contrary, therapeutic cancer vaccines are in a much earlier stage of development.

HBV

The currently marketed form of the HBV vaccine is based on HBsAg recombinantly expressed in yeast from which immunogenic VLPs composed of HBsAg and yeast phospholipids are harvested by gradient centrifugation and subsequently properly disulfide linked *in vitro* [22,54,55]. Since its introduction over 1 billion doses of HBV, have been used worldwide with a 95% effectiveness in preventing both HBV infection and its chronic consequences. In many countries, upon introduction of universal vaccination, the percentage of HBV chronic infections in children drastically dropped from 8–15% to <1% [301]. Consequently, the HBV vaccination program has resulted in the dramatic reduction of incidence of primary hepatocellular carcinoma [56–58].

More recently, plants-derived VLPs have been described as alternative strategies of HBV-VLP production [59,60]. They are still in a research and pre-clinical stage but confirm the continuous interest in the VLP technology applied to HBV.

Papillomavirus

VLPs derived from papillomaviruses (animal and human) represent the most studied example as preventive vaccines, among non-enveloped VLPs. They have been produced by overexpressing the major capsid L1 protein in different cellular systems [17,61–64].

VLPs produced in both insect cells and yeast (*Saccharomyces cerevisiae*) were found to be both safe and immunogenic in humans [65–67]. Based on such results, they have been approved for clinical use by regulatory agencies, and two HPV vaccine formulations are currently on the market for human vaccination. Both of them are specifically targeted to HPV-16 and HPV-18, which are the high risk types responsible for >70% of invasive cervical cancers. However, one of the two formulations contains only those two types (bivalent vaccine), the other formulation contains low-risk HPV-6 and HPV-11 types (quadrivalent vaccine) also, and they both are effective in eliciting high titers of neutralizing antibodies in vaccinated subjects within 1 month after completion of the vaccination protocol [68–72]. More important, both vaccines show an efficacy >98% in preventing development of HPV16/18 – associated CIN 2/3 – *in situ* adenocarcinoma or invasive cervical cancer [73–75].

However, L1 VLP-based vaccines are ineffective against all but the most closely related HPV types [68,69]. On the contrary, HPV minor capsid protein L2 peptides have been reported to elicit cross-neutralizing antibodies in animal models [76–79]. However, a vaccine solely based on L2 peptide is less immunogenic than L1 VLP [80,81] and chimeric HPV-VLPs have been generated either by co-expressing L1 and L2 proteins [82] or presenting cross-neutralizing L2 epitopes on L1 HPV-VLPs [79,83].

Alternative strategies to display HPV epitopes have been developed, generating chimeric VLPs in which the hepatitis B core antigen (HBcAg) or the hepatitis surface antigen (HBsAg) are modified to express HPV 16 E7 oncoprotein [84].

Therapeutic strategies

VLPs generated based on HBV core protein carrying MAGE-3 epitopes have been described [85,86]. Such chimeric HBC-VLPs have been used to pulse DCs which induce stronger CTL activity compared with peptide-pulsed DCs and significantly inhibits the growth of established tumors in a mouse model [86].

CTL epitopes from human mucin 1 (MUC1) have been inserted in VLPs derived from hamster polyomavirus (HaPyV) VP1 major capsid protein [87] as well as from bovine papillomavirus (BPV) [88]. MUC1-expressing HaPyV-based VLPs have been used to generate MUC1-specific monoclonal antibodies and *ex vivo* primed hDCs were able to activate MUC1 epitope-specific CD8(+) T cells [87].

Murine polyomavirus (MPyV)-VLPs have been generated to display human HER2/neu and have been shown to prevent the outgrowth of a human HER2/neu expressing tumor in a transplantable tumor model as well as outgrowth of spontaneous rat HER2/neu carcinomas in BALB-neuT mice [89]. In parallel, the same tumor antigen has been expressed on murine pneumotropic virus (MPtV)-VLPs, showing preventive as well as therapeutic effects in mice [90].

MPyV-VLPs have also been used to carry the entire human prostate specific antigen (PSA) that is PSA-MPyVLPs [91]. Immunization of mice with PSA-MPyVLPs alone was able to induce partial protection against outgrowth of a PSA-expressing tumor. Improved protection was obtained by immunizing animals with PSA-MPyVLPs in the presence of adjuvant CpG and loaded onto murine DCs, correlating to strong elicitation of PSA-specific CD4(+) and CD8(+) cells [91].

Bacteriophage Q β -VLPs have been covalently linked to a long peptide derived from the melanoma self-antigen Melan-A (MelQ β G10). Such VLPs have been evaluated in melanoma patients showing elicitation of strong specific antibody responses (IgG1 and IgG3 isotypes) correlating with a Th1-based CD4⁺T-cell responses. Although the response was much stronger against the carrier VLP than the Melan-A cargo peptide, the data in human patients demonstrate the high potential of VLPs for inducing both humoral and cellular immune responses [92].

Table 1. Passively targeted particles with potential in the development of cancer vaccines.

Type	Composition	Administration route	Pros	Cons	Development stage
Liposome					
Conventional liposomes	Cholesterol phospholipids	<ul style="list-style-type: none"> Intravenous administration targets APCs of spleen and liver macrophages Readily cleared by MPS after intravenous administration 	<ul style="list-style-type: none"> Biodegradable/biocompatible Higher numbers of antigen-specific T cells compared to vaccination 	<ul style="list-style-type: none"> Poor encapsulation of hydrophilic uncharged antigens Expensive to manufacture Poor storage stability 	Phase III discontinued (StimuVax®)
ISCOMs	Cholesterol Phospholipids Quill A fraction	<ul style="list-style-type: none"> Parenteral Oral Intranasal 	<ul style="list-style-type: none"> Long-term stability 	<ul style="list-style-type: none"> CD4⁺T cell mediated immunity MHC I class response 	Marketed for veterinary use (ISCOMATRIX®)
Archaeosomes	Polar lipids of <i>Methanobrevibacter smithii</i>	<ul style="list-style-type: none"> Fast clearance after intravenous or oral administration 	<ul style="list-style-type: none"> Biocompatible Intrinsic capacity to recruit NK cells to the tumor site Increased cross-presentation Intrinsic adjuvant properties Good storage stability 	<ul style="list-style-type: none"> Poor encapsulation of hydrophilic antigens 	Prophylactic and therapeutic studies in animal models of melanoma after id. injection
Fusogenic liposomes	Cholesterol egg phosphatidylcholine L-α-dimyristoyl phosphatidic acid	<ul style="list-style-type: none"> Intradermal Intranasal 	<ul style="list-style-type: none"> Increased uptake by APCs and improved cross-presentation after id. administration 		Prophylactic studies in animal models of melanoma after id. injection
Positively charged particles					
Cationic lipids	DOTAP cholesterol	<ul style="list-style-type: none"> Intradermal 	<ul style="list-style-type: none"> Active DC stimulator resulting in the induction of chemokines, cytokines and co-stimulatory molecules 	<ul style="list-style-type: none"> An overdose of DOTAP-mediated massive ROS production produces apoptosis of DCs in draining lymph node 	Therapeutic studies in HPV positive TC-1 tumor
Cationic lipids/DNA complexes	DOTAP Cholesterol DNA	<ul style="list-style-type: none"> Intradermal Intraperitoneal 	<ul style="list-style-type: none"> Internalized within DCs and macrophages Induction of CTL responses <i>in vivo</i> Increases immunopotency of CpG 	<ul style="list-style-type: none"> Dose-dependent toxicity Triggering undesired macrophage-mediated pro-inflammatory cytokine production 	Phase II study in patients with metastatic melanoma (Allovectin-7®) Prophylactic and anticancer activity in a mice model of cervical cancer Prophylactic studies in AB12 mesothelioma model

CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; DOTAP: 1,2-dioleoyl-3-trimethylammoniumpropane; EphA2: Ephrin type-A receptor 2; EPR: Enhanced permeability and retention; HPV: Human papilloma virus; ip: Intraperitoneal; ISCOMs: Immune stimulating complexes; iv: Intravenous; MPLA: Multiplex ligation probe assay; MPLA: Monophosphoryl lipid A; PLGA: Poly(lactic-co-glycolic acid); ROS: Reactive oxygen species; sc: Subcutaneous; TRP2: Tyrosinase-related protein2.

Table 1. Passively targeted particles with potential in the development of cancer vaccines (cont.).

Type	Composition	Administration route	Pros	Cons	Development stage
Lipid-polycation-DNA	DOTAP Protamine DNA	<ul style="list-style-type: none"> Intradermal Subcutaneous 	<ul style="list-style-type: none"> Protects the DNA from enzymatic degradation Uptake in DCs, NK cells and macrophages in popliteal lymph nodes Antigen specific CTL responses 	<ul style="list-style-type: none"> Potential systemic toxicity due to high level of TNF-α 	Children with Canavan's disease
Chitosan	Chitosan N-trymethyl-chitosan	<ul style="list-style-type: none"> Mucosal (nasal, pulmonary) Intradermal/subcutaneous Less frequently oral and intraperitoneal 	<ul style="list-style-type: none"> Activation of macrophages and NK cells to express cytokines Induction of CTL related cytokines after parenteral administration Mixed Th1/Th2 response after nasal administration Strong Th1 response after pulmonary administration 	<ul style="list-style-type: none"> No immunostimulating effect as such Restricted local immune response in humans after nasal immunization 	Intranasal immunization with influenza vaccine (Phase I)
<i>Biodegradable polymeric particles</i>					
Polyesters	PLGA PLA PCL	<ul style="list-style-type: none"> Intradermal Mucosal 	<ul style="list-style-type: none"> Biodegradable/biocompatible Approved for parenteral use Phagocytosed by APCs Protect antigen from enzymatic degradation Prolong antigen presentation by DCs Induction of a humoral and cytotoxic T-cell response Manufacturing in GMP is feasible Antigens are presented by both MHC class I and II pathways 	<ul style="list-style-type: none"> Negligible adjuvant properties Possible protein antigen degradation during manufacturing and <i>in vivo</i> degradation Potential expensive system 	Therapeutic antitumor (B16) effect in mice of particles encapsulating TRP2 and MLPA
Polyaminoacids	γ -PGA	Intratumoral	<ul style="list-style-type: none"> Biodegradable/non toxic Intrinsic adjuvant properties 		Antitumor effect of Eph2A in mice

CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; DOTAP: 1,2-dioleoyl-3-trimethylammoniumpropane; EphA2: Ephrin type-A receptor 2; EPR: Enhanced permeability and retention; HPV: Human papilloma virus; ip.: Intraperitoneal; ISCOMs: Immune stimulating complexes; iv.: Intravenous; MLPA: Multiplex ligation probe assay; MPLA: Monophosphoryl lipid A; PLGA: Poly(lactic-co-glycolic acid); ROS: Reactive oxygen species; sc.: Subcutaneously; TRP2: Tyrosinase-related protein2.

Figure 2. How different functional components can be combined. (A) Passively targeted and **(B)** actively-targeted particles for cancer vaccines. DC: Dendritic cell.

Particles for passive targeting to DCs

Passively targeted particles deliver antigens to DCs by virtue of their capacity to phagocytose particulate matter without specific recognition. Many different particles with more or less complicated design have been developed so far and there has been intense effort for understanding how their properties can be properly manipulated for shaping immune response. General features of each particulate system and corresponding achievements are summarized below with special emphasis on advantages, disadvantages and specific design rules for their rational design (TABLE 1, FIGURE 2A).

Liposomes

Liposomes are supramolecular vesicles made from one or more phospholipids associated to cholesterol which provides suitable rigidity to the bilayer as well as stability in biological environment [93]. Some phospholipids commonly employed in cancer vaccines include dimyristoyl phosphatidylglycerol (DMPG), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylserine (DOPS) and dilauroyl phosphatidylethanolamine (DLPE). Liposomes provide the great advantage of design versatility since single lipid blocks can be assembled, resulting in a structure with desired properties.

However, due to prompt recognition in MPS after intravenous injection, conventional liposomes suffer for a very brief half-life *in vivo* and accumulation in liver and spleen. Nevertheless, they can act as a Trojan horse in MPS as in the case of liposome harboring a muramyl dipeptide derivative (ImmTher[®]), which has shown an antitumor-activity in Phase I clinical trial against lung and liver colorectal metastasis [94]. One of the most advanced liposome carrier is BLP25 (Stimuvax[®]) containing lipid A adjuvant

and a 25-mer MUC1 peptide, which mimics the exposed core of the glycoprotein highly overexpressed in adenocarcinomas [95]. However, after promising Phase I–II results [96], the vaccine failed to improve overall survival in patients with stage IIIB non-small-cell lung cancer (START study). Interesting results have been obtained also with conventional liposomes encapsulating a peptide derived from tyrosinase-related protein2 (TRP2) (artificial virus envelope 3) in combination with adjuvant CpG-oligodeoxynucleotides. Formulation induced higher numbers of antigen-specific T cells compared to vaccination with free TRP2 after intravenous injection [97]. Optimal antigen dose, the relevance of vaccine injection site and T-cell stimulatory synergism of liposome/adjuvant combinations are currently under investigation [98].

Lipid vesicles containing natural components

The use of elements derived from plants, nonpathogenic microbes or inactivated viruses, represents another strategy to design immunogenic lipid vesicles. The main systems described in the literature are ISCOMs, archaeosomes and fusogenic liposomes.

ISCOMs are spherical cage-like particles (40 nm in diameter) made up of fractions from soapbark tree (*Quillaja saponaria* and Quill A) formulated with phospholipids, cholesterol and the desired antigen. ISCOMs are negatively charged and have a long stability (*viz.* 2 years at 2–8°C) [99]. ISCOMs are interesting candidates for antigen delivery to DCs as they can induce broad range of immunity, encompassing CD4⁺T cell-mediated immunity, antibody production and, most relevant, a very strong MHC class I-restricted response [100]. ISCOMs have been evaluated upon parenteral as well as mucosal administrations (i.e., oral [101] and intranasal [102]). The capability of ISCOMs to induce CTL responses is due to an improved antigen entry into the APCs cytosol, probably resulting from an interaction between ISCOMs and the lipid membranes of endosomes or phagosomes. The question whether the saponin component or the lipids is responsible for this phenomenon remains to be elucidated [103]. However, despite their potential and good performance in clinical trials [104], ISCOM-based vaccines are thus far approved for veterinary use only [105].

Archaeosomes are produced as liposomes but comprise the unique glycerolipids of the nonpathogenic microbe Archaea, which engenders membrane stability and promotes potent immune memory. Additionally, the variable head domains of these glycerolipids have potent and unique APC-stimulating properties [106]. On a pharmacokinetic level, archaeosomes behave as conventional liposomes. The type of immune

response appears to be dependent upon the strain of Archaea used as the source of lipids. In particular, the *Methanobrevibacter smithii* strain has gained the most attention for cancer immunotherapy [107–109]. In murine tumor models, *M. smithii*-derived archaeosomes were found to be potent TLR-independent activators of CTLs, likely due to the presence of cardarchaeol and capable of inducing both prophylactic and therapeutic effects. Furthermore, archaeosomes harboring TAAs have been shown to induce cross-presentation, which is not generally observed with conventional liposomes. More recently, to further improve the adjuvant properties intrinsic to archaeosomes, a series of novel synthetic archaeal glycolipids were generated and evaluated in a murine model, demonstrating potent CTL activity [110]. Studies using archaeosomes in clinical settings have not been attempted although they appear to have the necessary credentials for further evaluation.

Fusogenic liposomes are conventional liposomes engineered with elements of the UV inactivated Sendai virus, in particular its fusion protein [111]. These liposomes can fuse with a variety of mammalian cells and have been tested in animal models as cancer vaccines. Prophylactic studies on the tumor lysate of B16 melanoma cells have shown that the formulation slowed down the rate of tumor progression as compared to conventional liposomes and tumor cell lysate with complete Freund's adjuvant [112].

Positively charged particles

As underlined above, cationic particles are efficiently taken up by APCs due to electrostatic interactions which also help internalization of particles >1 μ m and proper maturation of DCs. Nevertheless, cationic particles show a natural tendency to interact with anionic proteins and to aggregate at site of administration. In general, extensive phagocytosis overrules any attempt to actively drive particles to DCs [3]. Cationic particles can be prepared from cationic lipids or polymers and represent an effective strategy to deliver polyanions such as DNA and nucleic acid fragments.

Lipid-based cationic particles (also referred as cationic liposomes) are the most widely studied approach in the field of cancer immunotherapy [3]. They are composed of cationic groups covalently linked to a lipophilic tail and the most common derivatives include 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), dimethyldioctadecylammonium (DDAB) and N,N-dioleoyl-N,N-dimethyl ammonium chloride (DODAC). This system has been greatly used to condense and deliver intracellularly negatively charged DNA and short nucleic acids. DOTAP helping the antigen presentation to MHC I *in vivo* had been demonstrated in the early 1990s [113,114]. As compared to anionic or neutral liposomes, cationic liposomes are promptly internalized within DCs and macrophages and induce CTL responses *in vivo* [115]. Nevertheless, some examples of cationic lipid-based particles designed for cancer vaccines have been reported. When tested in animal models, cationic liposomes containing antigens such as OVA, tetanus toxoid, glioma cell extract and CpG

oligonucleotides show enhanced antitumor effect as compared to free antigen [116–118].

Complexes of DNA encoding immunostimulatory proteins and cationic liposomes comprising CpG also as adjuvant have shown antitumor activity in several mouse tumor models by inducing innate and adaptive immune response [119,120].

Another system reported by Huang and collaborators is based on lipid-polycation-DNA (LPD) particles [121,122]. Upon administration of LPD, levels of TNF- α , IL-12 and IFN- γ increase rapidly [123]. E7 peptide-loaded LPD (LPD/E7) generated antigen specific CTL responses along with complete tumor regression in the treated mice (tumor injected 6 days before treatment) [121,124].

The most advanced system reported at the moment in clinical trials is Allovectin-7[®], a cationic liposome combined with plasmid DNA encoding HLA-B7 and β -2 microglobulin. When given intratumorally to patients with late stage melanoma, Allovectin-7[®] has induced a 9% overall response rate (complete and partial remissions) in a Phase II clinical trial [125]. When a dose increase was evaluated in a subsequent Phase II clinical trial, an 11.8% overall response rate was observed. A Phase III clinical trial is ongoing in subjects with recurrent metastatic melanoma to evaluate the safety and efficacy of intralesional treatment with Allovectin-7[®] [302].

Some drawbacks of cationic liposomes are related to potential dose-dependent cellular toxicity [126] and, when complexed with DNA, macrophage-mediated pro-inflammatory cytokine production [127]. ROS play a central role in both activity and toxicity of cationic liposome-based cancer vaccine [128]. Nevertheless, recent evidences suggest that positively charged liposomes can be toxic whereas negatively ones are not [126].

Polymer-based cationic particles are generated through cationic polymers or by coating preformed particles with a cationic polymer. Given their excellent mucoadhesive and absorption-enhancing properties, particles based on chitosan and chitosan derivatives (N-trimethyl-chitosan, TMC) have been extensively studied for delivery of therapeutic vaccines via mucosal routes demonstrating induction of both systemic and local immune responses [129]. Despite promising properties, the number of studies where chitosan particles were used as delivery system in cancer vaccines is relatively few. In a very recent example, a legumain-based DNA oral vaccine to protect against breast cancer was tested [130].

Amid cationic polymers, polyethyleneimine (PEI) and cationic dextran (C-dextran) were recently found to specifically stimulate macrophages to secrete IL-12 production via TLR-4 signaling [131]. In a recent study, it was found that both copolymers could reverse TAMs polarization and promote IL-12 expression both *in vitro* and *in vivo* [132]. Additionally, these cationic polymers exerted direct tumoricidal activity by promoting Th 1 and NK cell infiltration, suppressing tumor angiogenesis and prolonging the survival of sarcoma-bearing wild-type mice. These results highlight that cationic polymers could revert the tolerogenic phenotype of TAMs through TLR-4 signaling, promoting a therapeutic anti-tumor immunity.

Biodegradable polymeric particles

Amongst synthetic biodegradable polymers, thermoplastic aliphatic poly(ester)s like poly(lactic acid)s (PLAs) and their derivatives poly(lactic-co-glycolic acid) (PLGAs) have generated tremendous interest due to excellent biocompatibility as well as the possibility to tailor their biodegradability. This allows to control release rate of the entrapped drug by varying composition (lactide/glycolide ratio), molecular weight and chemical structure (i.e., capped and uncapped end-groups). Polymer nano- and micro-particles with a scale size from few nanometers to several microns have been employed to deliver effectively small drugs, peptides and proteins as well as DNA [133].

PLGA particles with different formulation designs have been developed for the delivery of antigens by parenteral and mucosal administration [134]. They have been shown to be efficiently phagocytosed by APCs *in vivo* [135], to prolong antigen presentation by DCs [136], to deliver their antigen cargo to DCs and macrophages for induction of a humoral and cytotoxic T-cell response after systemic and mucosal administration [137–141]. Moreover, PLGA particles are taken up by DCs after intradermal administration in mice even in the absence of immunomodulators, demonstrating their adjuvant activity [142]. All of these considerations emphasize PLGA particles as a promising delivery system for cancer vaccines.

Association of adjuvants to PLGA nanoparticles can offer *a priori* several advantages over soluble adjuvants. In case of TLR ligands, persistent signaling can allow to overcome tumor suppression mediated by Treg cells [143] and to sustain TLR signaling in DCs, avoiding the need of repeated administrations or high doses of free adjuvant [134]. Mice vaccinated subcutaneously (with two boosts) with PLGA particles encapsulating both a TAA peptide (TRP-2180-188) 7-acyl lipid A could control tumor growth through the induction of TRP-2-specific CTLs [144]. On the other hand, PLGA particles entrapping tumor lysate proteins (from the B16 murine melanoma) and CpG were capable of protecting against subsequent tumor challenge after intraperitoneal vaccination only if Tregs were concomitantly diminished [145].

Another polymer that is receiving attention in tumor immunotherapy is poly(γ -glutamic acid) (γ -PGA) and its amphiphilic derivatives. γ -PGA is produced by several *Bacillus* species as an extracellular polymer and is entirely biodegradable and non-toxic to humans. Preliminary studies indicated that γ -PGA particles are efficiently phagocytosed by DCs *in vitro*, inducing their maturation by a MyD88-dependent signaling pathway involving NF- κ B activation. Studies in models using OVA-expressing tumors demonstrated that this system could be used to deliver antigen vaccines that target APCs and promote the MHC class I presentation pathway, ultimately conferring significant tumor protection [146,147]. Most recently, γ -PGA nanoparticles loaded with a known TAA, EphA2, were found to be safe and effective in protecting mice challenged directly in the liver with EphA2-expressing tumor cells in the liver thus simulating metastasis [148]. Enhanced EphA2-specific CD8⁺ T lymphocyte activation, target cell lysis and decreased liver size were observed to indicate tumor protection.

Ligand-decorated particles for active targeting to DCs

Recent strategies for developing prophylactic and therapeutic cancer vaccines have focused on carriers able to deliver antigen to DCs in a targeted manner through anchoring receptor-ligands on particle surface (FIGURE 2B).

A variety of ligands for DC receptors, including TLRs or C-type lectin receptors (CLRs), have been used *in vitro* and *in vivo* to enhance antigen-presentation to DCs. Particle decoration can occur on both lipid- and polymer-based systems. It is generally recognized that chemical modification of base material with poly(ethylene glycol) (PEG) chains cannot give rise to 'stealth' systems with improved extravasation at tumor level through EPR effect but also ameliorate surface exposition of a targeting ligand promoting effective recognition at cell level. Excellent reviews have summarized recently design opportunities and resulting *in vitro* and in mice humoral and cellular responses [3,149–151]. The most promising systems have been described in the following paragraphs (TABLE 2).

Particles targeting TLRs

TLR ligands, such as MPLA or CpG DNA, may act as very potent immune modulators and vaccine adjuvants and could have a huge impact on targeted immunotherapy of cancer, given the preferential Th-1 profile involved. An open question is how TLR ligands should be employed in vaccine particles, since they are commonly co-delivered with TAA [151] other than actually employed to 'decorate' the surface of the carrier to achieve an active targeting. There are only a few examples exploiting the potential of surface decoration with TLR ligands to actively target nanocarriers to DCs. A proof of principle of the potential in DNA vaccination of TLR-targeted nanoparticles was recently achieved on chitosan or chitosan derivatives (i.e., TMC) [152,153]. Decoration with a TLR-2 agonist coupled through a PEG spacer of DNA-loaded chitosan nanocarriers allowed protection of plasmid DNA against enzymatic degradation and a 10-fold increase of IL-8 secretion by human THP-1 macrophages as compared to non-decorated carriers [154]. Similar results were very recently observed when functionalizing chitosan-DNA nanoparticles with a TLR-7 agonist [153], whereas engrafting of liposomes with two flagellin-related peptides (TLR5 agonists) promoted strong liposome binding to murine bone marrow-derived DCs and functional maturation of DCs *in vivo*, which resulted in potent inhibition of tumor growth/metastasis and complete tumor regression in mice [155].

Particles actively targeting CLRs

DCs express several C-type lectin receptors, including the mannose receptor (MR/CD206), DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN/CD209), and DEC-205/CD205, which have been implicated as promising targets due to their physiological roles in antigen uptake [156]. Depending on the CLR targeted, either CD4⁺ or CD8⁺ T-cell responses can be specifically induced [157].

Targeting MR/CD206 by vaccine delivery systems has been the focus of many recent studies to boost immune response in

Table 2. Ligand-decorated particles for active targeting to DCs of cancer vaccines.

Type	Targeting moiety	Development stage	Administration route	Key findings	Ref.
<i>TLR-targeted particles</i>					
DNA/chitosan derivatives nanoparticles	TLR-2 agonist TLR-7 agonist	Pre-formulation studies <i>In vitro</i> cytotoxicity and activation studies on human macrophages	No <i>in vivo</i> assay	10-fold increase of IL-8 secretion by human macrophages as compared to non-decorated carriers Dose-dependent cytotoxicity against macrophage-like THP-1 cells	[152,154,153]
Stealth liposomes	Flagellin-related peptides (TLR-5 ligands)	Prophylactic and therapeutic studies in mice bearing B16-OVA or P815 tumor cells	Intravenous	Potent inhibition of tumor growth/metastasis and complete tumor regression	[155]
<i>CLR-targeted particles</i>					
Liposomes	Mannose-modified cholesterol	Biodistribution and therapeutic studies in an experimental mouse model of liver metastasis	Intravenous	Massive hepatic uptake by liver non-parenchymal cells Significant reduction of the number of liver metastasis as compared to free antigen	[159]
	Lipid-mannose conjugate	Therapeutic studies in mice bearing ErbB2 protein-expressing tumor cells	Intravenous	Higher therapeutic efficacy as compared to non-mannosylated liposomes at low doses of TLR ligands and ErbB2 epitopes	[162]
Cationic liposomes	Mannose-modified cholesterol	Prophylactic and therapeutic studies in mice Therapeutic studies in an experimental mouse model of peritoneally disseminated tumor	Intraperitoneally Intramuscularly	Administration route-dependent CTL activity (higher upon intraperitoneal injection as compared to subcutaneously/intramuscularly) Increased survival time and more efficient inhibition of the tumor cell proliferation as compared to conventional lipoplexes	[163,160]
		Therapeutic studies in an experimental mouse model of liver metastasis	Intravenous	Comparable reduction of the number of liver metastasis with both conventional and mannosylated cationic liposomes/CpG DNA complexes	[161]
Chitosan/DNA cationic nanoparticles	Mannosylated chitosan	Therapeutic studies in mice bearing CT-26 carcinoma cells	Intratumoral	Suppression of tumor growth and angiogenesis, and significant induction of cell cycle arrest and apoptosis	[165]

CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; DC-SIGN: Specific intracellular adhesion molecule-3 grabbing non-integrin; im: Intramuscularly; ip: Intraperitoneally; iv: Intravenously; LN: Lymph nodes; TLR: Toll-like receptor; MR: Mannose receptor; PLGA: Poly(lactic-co-glycolic acid); PEG: Poly(ethylene glycol); OVA: Ovalbumin; sc: Subcutaneously.

Table 2. Ligand-decorated particles for active targeting to DCs of cancer vaccines (cont.).

Type	Targeting moiety	Development stage	Administration route	Key findings	Ref.
PLGA nanoparticles	Mannan-conjugated PLGA (MR ligand)	Immunization studies in C57BL/6 mice	Subcutaneously	Enhancement of antigen-specific CD4 ⁺ and CD8 ⁺ T-cell responses compared to non-decorated nanoparticles	[134]
PEGylated PLGA particles	Anti-DC-SIGN monoclonal antibody	<i>In vitro</i> binding/uptake studies in human DCs Immunization studies in C57BL/6 mice	Intravenous	Size-, PEG chain length- and antibody type-dependent <i>in vitro</i> binding and uptake of particles by human DCs Induction of cellular immune responses at very low doses of TLR ligands (100-fold lower as compared to undecorated particles) associated to reduced serum cytokine storm	[171,202,172]
PAMAM dendrimer	Mannopyranosyl(phenyl) isothiocyanate (MR ligand)	Prophylactic studies in mice challenged with B16-OVA tumor cells	Intradermal	Delayed or prevented growth of B16-OVA melanoma Specific targeting to APC in the LN and induction of <i>in vivo</i> cross-presentation in LN derived from injected mice	[166]
pH-responsive polymeric microparticles	Anti-DEC-205 monoclonal antibody	Immunization studies in C57BL/6 mice	Subcutaneously	Increased receptor-mediated uptake by DCs as well as migration of particle-carrying DCs to LNs Stimulation of naive T cells leading to enhanced cellular immune response as confirmed by specific cell lysis and IFN- γ secretion.	[203]
FcR targeted particles					
Liposomes	Different IgG isotypes	Immunization studies in transgenic mice Therapeutic studies in mice with established OVA-expressing tumors	Subcutaneously	1000–10,000-fold augmentation of antigen presentation by DCs as compared to free antigen or non-targeted liposomes PEG- and size-dependent endocytosis by human DCs Complete prevention of tumor growth upon administration of human monocyte-derived DCs that endocytosed tetanus toxoid-containing IgG liposomes	[173,174]

CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; DC-SIGN: Specific intracellular adhesion molecule-3 grabbing non-integrin; ip.: Intraperitoneally; iv.: Intravenously; LN: Lymph nodes; TLR: Toll-like receptor; MR: Mannose receptor; PLGA: Poly(lactic-co-glycolic acid); PEG: Poly(ethylene glycol); OVA: Ovalbumin; sc.: Subcutaneously.

Table 2. Ligand-decorated particles for active targeting to DCs of cancer vaccines (cont.).

Type	Targeting moiety	Development stage	Administration route	Key findings	Ref.
Stealth liposomes	Rat IgG2a Armenian hamster IgG	Biodistribution studies and therapeutic studies in mice intravenous challenged with B16-OVA melanoma cells	Subcutaneously (hind footpad of mice)	Strong B16-OVA-specific CTL responses in splenic T cells and marked protection against tumor growth Dramatic immunotherapeutic effect and prolonged disease-free survival	[175]
Other targeted particles					
Liposome	HMGB1-related peptides CD11c/CD18-interacting peptides	Biodistribution and therapeutic studies in syngeneic mice challenged with highly metastatic B16-OVA melanoma	Intravenous	Specific targeting to murine CD11c(+) and CD11b(+) cells <i>in vitro</i> and <i>in vivo</i> Inhibition of tumor growth and metastasis	[204,178]
γ -PGA nanoparticles	Anti-CD40 monoclonal antibodies	Therapeutic studies in mice bearing bladder cancer MB49 cells	Peritumoral	Enhanced immunostimulatory effect and minimized toxicity associated with systemic cytokine release	[179]

CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; DC-SIGN: Specific intracellular adhesion molecule-3 grabbing non-integrin; im: Intramuscularly; ip: Intraperitoneally; iv: Intravenously; LN: Lymph nodes; TLR: Toll-like receptor; MR: Mannose receptor; PLGA: Poly(lactic-co-glycolic acid); PEG: Poly(ethylene glycol); OVA: Ovalbumin; sc: Subcutaneously.

cancer and some products are already in clinical trials [158]. Since first attempts at the end of 80's, many efforts have been devoted to the development of mannoseylated lipid vesicles also, for cancer immunotherapy through the incorporation of either mannose-modified cholesterol (Man-C4-Chol) [159–162] or lipid-mannosyl conjugates [163], both showing great *in vivo* potential.

Mannan-decorated antigen-loaded PLGA nanoparticles were demonstrated to simultaneously enhance *in vitro* antigen-specific CD4⁺ and CD8⁺ T-cell responses [164]. Upon intratumoral injection, cationic mannoseylated chitosan nanoassemblies with a plasmid encoding murine IL-12 complex clearly suppressed tumor growth and angiogenesis as compared to non-targeted particles [165]. An enhanced antigen presentation and DC maturation was achieved by novel mannose-based antigen delivery system that utilizes a polyamidoamine (PAMAM) dendrimer [166]. Mice immunized with mannose dendrimer ovalbumin (MDO) generated strong OVA-specific CD4⁺/CD8⁺ T-cell and antibody responses. Moreover, upon challenge with B16-OVA tumor cells, tumors in mice pre-immunized with MDO either did not grow or displayed a much more delayed onset, and had slower kinetics of growth than those of OVA-immunized mice (i.e., prophylactic and therapeutic effect). MR/CD206-targeted endosomolytic nanoparticles have been recently proposed for siRNA delivery [167].

Specific targeting of particles towards DCs can also be accomplished by grafting of a DC-specific antibody directed against the C-type lectin, DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and DEC-205 [168–172].

Although numerous lines of evidence in different experimental models support the efficacy of CLR targeted vaccine strategies, it is becoming increasingly clear that additional signals, such as those afforded by adjuvants, may be critical to elicit sustained immunity. A specific and potent anti-tumor cellular immune response against ErbB2 protein-expressing tumor cells was achieved when mice were injected with mannoseylated liposomes co-delivering peptide epitopes in combination with TLR agonists [162]. Similarly, targeted PLGA nanoparticles surface-coated with lipid-PEG as well as DC-SIGN-specific humanized antibodies were loaded with a combination of the TLR3 ligand polyinosinic:polycytidylic acid (poly I:C) and the TLR7/8 ligand resiquimod (R848) as adjuvants, to synergize their effect on Th1-polarizing program in DCs [172].

Particles actively targeting FcR

Fc receptors (FcR), which bind the constant domains of antibodies and serve as a linker between humoral and cellular immune responses, have also been employed as a target for particle-mediated cancer immunotherapy. Most of the results have been achieved with targeted liposomes, paving the way to the application of these targeted vesicles for tumor immunotherapy. First attempts were made with Ig-G coated liposomes containing T-cell specific hen egg lysozyme, allowing a 1000–10,000-fold increase of antigen presentation by DCs compared with free antigen or non-targeted liposomes [173]. In a more

recent study, the influence of liposome physicochemical properties was elucidated, pointing to the importance of liposome size and composition on DC uptake, T-cell induction and antitumor efficacy [174].

If Ig-G coated liposomes represent the first FcR-targeted particles, novel synthetic ligands are under investigation to specifically deliver antigens through FcR receptor-mediated DC endocytosis. A novel chelator-lipid, 3(nitrilotriacetic acid)-ditecylamine (NTA3-DTDA) was recently used to anchor histidine-tagged forms of single chain full-length variable antibody fragments onto either tumor-derived plasma membrane vesicles (PMV) or antigen-containing stealth liposomes [175]. A strong antitumor response was elicited by providing an effective alternative to the *ex vivo* manipulation of DCs for use in cancer immunotherapy. A variety of peptide-based ligands for FcR, have been more recently synthesized to develop novel FcR targeted liposomes and gold nanoparticles for the treatment of androgen-responsive prostate cancer [171].

Other actively-targeted particles

Apart from receptors mainly involved in particle uptake from surrounding DC (i.e., TLRs, CLRs, and FcRs), DCs also express a wide array of internalizing receptors that mediate DC adhesion, activation and migration, including integrin and cytokine receptors, such as CD11c and CD40.

CD11c, a type I transmembrane protein found at high levels on most human DCs, was demonstrated to be an effective immunotarget for the generation of both CD4⁺/CD8⁺ T-cell responses *in vivo*, providing a rationale for further development of this approach in cancer immunotherapy [176,177]. CD11c/CD18-interacting peptides were used as targeting moieties of antigen-loaded stealth liposomes and demonstrated to elicit dramatic antitumor responses *in vivo*, inhibiting tumor growth/metastasis in the subcutaneous tumor model and the lung, respectively [178].

Agonistic monoclonal antibodies directed against CD40, a DC surface protein that triggers DC maturation and activation, also holds a great potential for cancer immunotherapy via targeted particles. In very recent studies, local treatment with anti-CD40 γ -PGA nanoparticles resulted in a significant reduction in serum levels of IL-6, IL-10, IL-12 and TNF- α in a bladder cancer model as compared to soluble CD40 agonist [179]. Another safe and effective tumor immunotherapy approach was recently reported using PLA microparticles presenting antibodies that independently recognized CD40 and RNEU [180].

Administration route of particulate vaccines

Administration route of a particulate vaccine drives the ultimate fate of the antigen and presentation process. The presence of physiological barriers imposed by the administration site as well as local microenvironment should be properly taken into account while designing a particulate carrier. In a simplistic view, a stronger immune response may be expected, the closer a vaccine is administered either to the tumor site, to local LNs or to afferent lymphatic vessels. In fact, the vast differences

between properties of particles currently under investigation for vaccination provide a powerful mean for optimizing immune response as a function of the administration route [2,181].

Parenteral

One of the most typical routes of vaccine administration is subcutaneous injection. Upon administration, vaccine particles are placed in the interstitial tissue beneath the dermis, where a superficial layer of initial lymphatics has been described. In lymphatic capillaries, numerous endothelial cells overlap extensively at their margins and, following interstitial injection, may be separated, thus providing passageways between the interstitium and lymphatic lumen. If adequately engineered, particles are conveyed by lymph to the LNs. Generally, smaller the particle size, faster the lymphatic uptake. Nevertheless, depending on their surface properties (e.g., surface hydrophobicity/hydrophilicity), small sized particles may also aggregate at the injection site and/or interact with the ground substance of interstitium. Similarly, larger particles (<100 nm) are believed to be trapped in the interstitial compartment, where they can slowly release their vaccine cargo. The retained particles are also susceptible to phagocytosis or macropinocytosis by interstitial phagocytes [182]. Several studies with polymer nanoparticles and liposomes have demonstrated that surface characteristics, such as charge, hydrophilicity and surface decoration can affect particle drainage from the subcutaneous injection site and distribution within the lymphatic system [183–186]. In particular, decoration of particle surface with PEG, an approach known as PEGylation, was shown not only to enhance passive LN targeting of antigen-loaded cationic liposomes but also to regulate their biodistribution, both of which contributing to enhanced immune responses [186].

Intramuscular injections are widely employed in vaccination schedule, providing potent humoral and cellular responses similarly to intradermal and subcutaneous routes. Nevertheless, the effects seen after intramuscular injections are much longer lasting as compared to intradermal injection, likely due to efficient antigen presentation by myoblasts in the muscle tissue. Cell response is Th2-dominated unless booster injections are applied.

Systemic injection of targeted particles may be the choice to block tumor cell function directly (e.g., via antibodies), although major challenges include overcoming clearance in the body, increasing cellular uptake into target tissue/cells. Upon intravenous injection, a particulate vaccine should resist to natural elimination processes including both renal clearance and mononuclear phagocytic system (MPS) uptake. MPS uptake proceeds quickly and must be avoided in order to have an acceptable circulation time for intravenous particulate vaccine to attain increased particle ability to reach its target cells. Recognition by the MPS is aided by opsonization, inducing its phagocytosis and accumulation in the liver (Kupffer cells) [187]. Strategies to avoid or limit this effect include design and generation of particles of 100 nm or less in diameter with a hydrophilic non-interacting surface (i.e., PEGylated particles) [187]. Although paradoxically detrimental for cell uptake at tumor

level, PEGylation should allow the nanoparticles to extravasate more effectively through their dysfunctional and highly permeable capillary bed and to remain in tumor matrix due to poor lymphatic drainage, an effect known as enhanced permeability and retention (EPR) effect [188]. PEGylated nanomedicines are already approved for clinical use in cancer therapy (Doxil®/Caelyx, Genexol-PM®) whereas several others are currently in clinical trials, and in early/late-stage preclinical development.

Oral

Whilst oral vaccination is a potentially preferred route in terms of patient adherence and mass vaccination, the ability to formulate effective vaccine particles for oral administration is still challenging. To be effective as oral vaccine, antigens need to be protected from acidic and enzymatic denaturation before reaching their target site (i.e., epithelial M cells), where their uptake should be enhanced to increase immunogenicity [189,190]. A promising strategy to surmount these hurdles is the encapsulation of cancer vaccines in biodegradable particulate delivery systems. Nevertheless, particle uptake by the intestinal epithelium is rather poor. Although modification of their size, surface charge or hydrophobicity can increase the efficiency of epithelial uptake, surface decoration of the antigen-loaded particles with targeting ligands, specific for epithelial receptors, could further enhance the uptake and transepithelial transport of antigens [191]. To mimic the 'gold standard' intracolorectal immunization while circumventing the limitations of oral delivery, microparticles based on pH-sensitive methacrylate-based polymers were used to deliver PLGA-based vaccine nanoparticles, which resulted in an efficient protection against rectal and vaginal viral challenges [192].

Mucosal

The intrinsic limitations of oral administration route have prompted research interest in drug delivery platforms that can deliver vaccines to accessible mucosal tissues, such as nose, lung and vaginal. Although promising in the development of novel needle-free vaccines, the viscous and adhesive mucus layer that lines mucosal surfaces can efficiently trap and rapidly clear foreign molecules and particles, limiting access of particle vaccines to the underlying tissue. Concerning the VLP strategy, according to the mucosal site to be targeted (e.g., genital, respiratory) scientists have taken advantage of the broad spectrum of viruses, generating VLPs derived from the corresponding mucosal-tropic viruses (e.g., HPV, influenza). Concerning particles, recent efforts have focused on developing delivery systems with adequate surface properties and size consisting of a lipophilic core and a hydrophilic shell (mucous penetrating particles: MPP) that can penetrate mucus layers and deliver cargo materials to the underlying epithelium [193]. Moreover, it is also possible to employ specifically designed materials able to interact with mucus or to enter cells via receptor-mediated endocytosis [194]. Engineered nanoparticles that, upon delivery to the lung, target pulmonary DCs, enhancing antigen uptake and transport to the draining lymph node, have been demonstrated

to enhance cytotoxic T-cell responses in pulmonary vaccination [195].

The potential for reaching some regions of the body, such as deep lung, is further complicated by peculiar anatomic properties. In fact, effective pulmonary delivery of particulate carriers demands for engineered devices and inhalable formulations, which will play a crucial role in determining drug deposition along the airways [196].

Skin

Despite the presence of numerous DCs, the skin is difficult to be used as a local vaccination site due to its intrinsic barrier properties. Passive and active permeation enhancement methods have been widely applied to increase the cutaneous penetration, resulting in topical delivery (into skin strata) and transdermal delivery (to subcutaneous tissues and into the systemic circulation) of vaccine particles [197]. In perspectives, the most promising systems for vaccine delivery consist of ultra-small nanoparticles (size smaller than 40 nm) which are able to permeate into skin strata, particles targeted to hair follicle stem cells or combined nanoparticles and microneedle array technologies [197]. Gene gun-mediated transgene delivery system for skin vaccination against melanoma using TAA, cytokines or chemokines, alone or in combination, also provides an attractive approach to increase the cytotoxic T-cell immunity against various cancer diseases [198]. Effective particle-mediated vaccination against melanoma has been achieved both in animals and humans making use of helium-accelerate DNA-coated microscopic gold particles [199,200].

Expert commentary

Recent technological advancement in particle technologies are driving innovation also in the field of cancer vaccines, with few of these approaches already licensed for or evaluated in humans (TABLE 3). The proper combination of base material, adjuvant, targeting moiety and administration route is needed in a multidisciplinary approach to achieve multifunctional platforms which are able to hit the tumor from different sides by combining different pharmacological strategies. A major effort is needed to appropriately target APCs in order to improve the immunogenicity of the delivered tumor antigens and specifically drive the immune response toward a cellular response (Th1 >> Th2). In parallel, nonspecific immunostimulatory or immunosuppressive responses must be avoided. Moreover, attention should be devoted to the understanding of mechanism underlying immune response by a systems vaccinology approach in order to proceed with a rational engineering of the carrier [201]. Indeed, a successful vaccine is required to take advantage of multiple concurrent strategies to counterbalance the immune suppressive tumor micro-environment, as addressed in other contributions to the present issue. Benefit-risk balance is another key factor to be considered when developing a cancer vaccine which in general favors safety over efficacy. Nevertheless, a controlled higher level of adverse events can be accepted when treating advanced cancers. Finally, the

Table 3. Particle strategies licensed or in clinical trial as cancer vaccines.

Type	Cancer targeted	Development stage	Ref.
<i>Virus-like particles</i>			
HBV	Hepatocellular cancer (preventive)	Licensed	[303]
HPV	Cervical cancer (preventive)	Licensed	[304]
<i>Liposomes</i>			
Conventional liposomes	MUC-1 positive cancer	Phase III discontinued (Stimuvax [®])	[305]
<i>Positively charged particles</i>			
Cationic lipids	Non-small-cell lung cancer	Phase I	[306]
Cationic lipids/DNA complexes	Metastatic melanoma	Phase III (Allovectin-7 [®])	[307]

MUC1: Human mucin 1.

ease and cost of production of particulate vaccines as well as formulation stability should be also considered to move from laboratory bench to the bed side.

Five-year view

The exploitation of particles as cancer vaccine approach in humans is likely to significantly expand in the next 5 years. The several pre-clinical and human clinical trials with micro and nano-particles, showing very promising results, ensure a significant pipeline which needs to be fully evaluated in human clinical trials for possibly moving forward to licensing and marketing. Moreover, constant developments of new particles: to improve antigen presentation in a MHC class-I context (e.g., smart particles); to act themselves as artificial APCs and directly stimulate T-cell response and to optimally target lymph-node DCs, will very likely improve the efficacy of particles as cancer vaccines.

Furthermore, the marketing of non-enveloped VLPs as preventive vaccine for the HPV has represented a major step forward in the exploitation of VLPs in the cancer vaccine field. Similarly, the human clinical trials performed using enveloped VLPs for influenza, RSV, rotavirus will provide essential information on the manufacturing optimization,

safety and immunogenicity on VLP-based vaccines. Inevitably, these advancements will also foster the exploitation of VLP technology for therapeutic cancer vaccines. In general, the efficacy in terms of such particle cancer vaccines will strongly depend on the identification of the appropriate target antigens to be presented and delivered to the immune system, which is representing so far an unprecedented scientific challenge.

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Key issues

- Particles are designed to mimic the structure of microbes and optimally target APCs.
- They display on the surface an array of linear and/or conformational epitopes.
- Particles can be specifically designed in accordance to the administration route and the antigen delivery/release necessity.
- If adequately engineered, particles are efficiently internalized by APC and cross-presented in association with both MHC class I and class II molecules.
- Several pre-clinical and human clinical trials have been and are currently conducted to evaluate safety and efficacy as therapeutic cancer vaccines.
- The first two preventive cancer vaccines licensed for humans (HBV and HPV vaccines) are made of virus-like particles.
- Further technical challenges needs to be overcome to meet all the production and regulatory requirements.
- New advancements will foster development of particle vaccine molecules for human clinical trials.
- Particle technology has a relevant room for playing a relevant role in the cancer vaccinology field of the coming years.

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