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*Reviewing the rationale for the design of particulate vaccines and how particle characteristics influence internalization, processing and antigen presentation by APCs.*



# Particulate vaccines: on the quest for optimal delivery and immune response

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**Subunit vaccines offer a safer alternative to traditional organism-based vaccines, but their immunogenicity is impaired. This hurdle might be overcome by the use of micro- and nanodelivery systems carrying the antigen(s). This review discusses the rationale for the use of particulate vaccines and provides an overview of antigen-delivery vehicles currently under investigation. It further highlights the cellular uptake, antigen processing and the presentation by antigen-presenting cells because these processes are partially governed by particle characteristics and eventually determine the immunological outcome. Finally, we address the attractive concept of concomitant delivery of antigens and immunopotentiators. The condensed knowledge could be an asset for rationally designing antigen-delivery vehicles to obtain safe and efficacious vaccines.**

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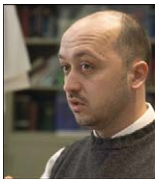

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## Introduction

The implementation of vaccines to prevent infectious diseases can be considered as one of the most successful *tours de force* in medicine. Since the seminal works of Jenner, Pasteur and other scientists, the incidence of numerous infectious diseases, such as mumps, measles, polio, tetanus, diphtheria, hepatitis, etc, has been significantly reduced and, in some cases, elimination has been achieved – the classic example being small pox [1,2]. In the early days of vaccination live attenuated or inactivated pathogens were used to generate a long-lasting immunity. Traditional live attenuated vaccines do not need the 'help' of adjuvants because they comprise not only antigen(s) but also bacterial or viral

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1359-6446/06/$ - see front matter  doi:10.1016/j.drudis.2011.04.006

compounds that efficiently activate several components of the innate immune system and thereby enhance immunity. To improve general safety and reduce reactogenicity of vaccines, well-defined antigenic subunits of the causative infectious agents are increasingly considered in new vaccine developments.

Well-defined and purified subunit vaccines are mostly based on specific peptides or proteins. Recent technological advances have enabled the consistent production of bulk quantities of subunit vaccines [3]. Although these vaccines possess a better safety profile, they are inherently less immunogenic than traditional whole-cell vaccines. Accordingly, modern vaccines require effective delivery systems and adjuvants (immunostimulatory molecules), reviewed in Refs [4,5]. Currently available vaccine adjuvants are effective at inducing certain types of antibody responses but fail to elicit efficaciously cell-mediated immunity. Cellular immune responses are, however, needed to prevent or treat infections caused by viruses, intracellular bacteria or protozoa, cancer and possibly autoimmune diseases as well.

In the search for better vaccines, 'particulate vaccines' represent a promising concept to surmount the aforementioned obstacles [6]. Formulation of antigens in particles in the viral or bacterial size range offers some attractive features, including protection of the antigen against degradation, facilitated uptake by antigen-presenting cells (APCs) through passive or active targeting, depot formation and co-delivery of antigens and adjuvants to the same APC, which could assist in directing the type of immune response desired, as will be discussed later.

In this review we provide an overview of the particulate antigen delivery systems under investigation and elaborate on particulate antigen delivery systems as a strategy satisfying many criteria of vaccine development. We will focus on the influence of particle characteristics on the underlying mechanisms involved in internalization, processing and antigen presentation by APCs, which all impact the outcome of the immune response. As a starting point we will summarize the biological processes involved in immune response development upon vaccination. This paper could set the scene for bridging the biological and pharmaceutical requirements of particulate vaccines and might serve as tool for their further rational development.

## Generation of immunity: the biological scene

In the early phase of the host immune response, macrophages (Mϕ) and dendritic cells (DCs), which are APCs, capture pathogens and particulate material by phagocytosis, as illustrated in Fig. 1. DCs are widely regarded as the most potent and versatile APCs in the immune system, having a superior capacity to capture and process antigens for presentation [7], although Mϕ can also fulfil these functions [8,9].

DCs are present as sentinels in the periphery where they sample the environment for foreign antigens. Classically, DCs exist in immature and mature states, with maturation leading to a wide spectrum of phenotypes. Immature DCs are capable of antigen uptake and express, at the surface, only low levels of class I and class II major histocompatibility complex (MHC) and co-stimulatory molecules, with the latter representing nonspecific signals, the so-called 'second signal', for T-cell activation. DC maturation can be triggered by numerous factors including pathogen-associated molecules, for example lipopolysaccharide (LPS), dsRNA, bacterial DNA and cytokines such as granulocyte-macrophage colony-stimulating factor and tumor necrosis factor

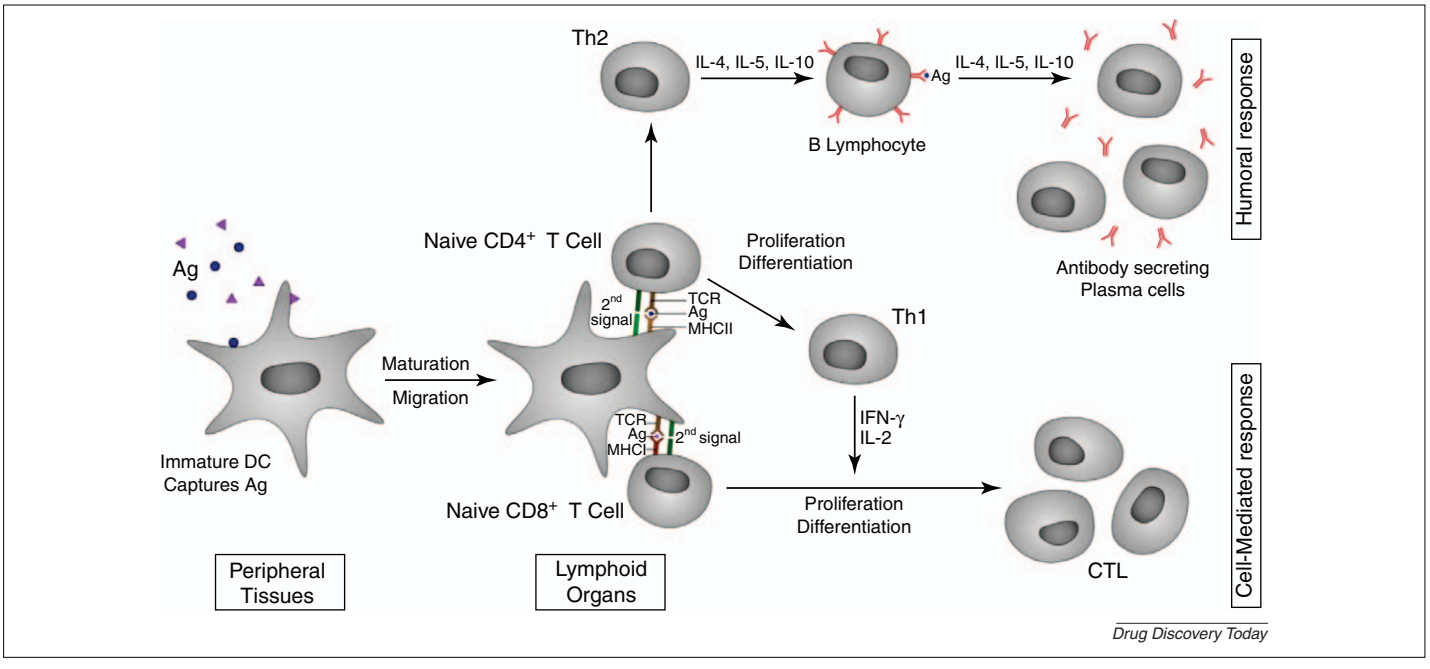


**FIGURE 1**

Schematic overview of antigen presentation and Th priming. Immature DCs screen the peripheral tissues; upon encounter of DCs with antigen and PAMPs, migration to the lymph nodes and maturation is induced. In the lymphoid organs, Ag presentation via MHC class II and class I molecules accompanied by a second signal leads to activation, proliferation and differentiation of respectively Ag-specific $CD4^+$ and $CD8^+$ T-cells. Activation of $CD4^+$ helper T-cells results in secretion of cytokines and subsequent activation of $CD8^+$ T-cells or B lymphocytes. The humoral branch of the immune response comprises activation of B lymphocytes followed by differentiation into antibody secreting plasma cells. Whereas the cellular response involves activation of $CD8^+$ T-cells followed by proliferation and differentiation into cytotoxic T-cells.

(TNF). Transformation into mature DCs is accompanied by a reduced capacity for antigen capture, presentation of more MHC molecules at the surface, change in expression pattern of chemokine receptors, upregulation of co-stimulatory molecules (CD80, CD86) and T-cell adhesion molecules (CD58, CD48) [7], and production of crucial cytokines such as interleukin(IL)-12. Besides DC maturation, 'homing' of DCs from distal tissues to the draining lymph nodes is crucial for fulfilling DC functions because it is in the lymph nodes where DCs encounter and activate T cells [10].

After ingestion of free or particle-associated antigen by APCs the antigen is degraded in endosomes or phagosomes, yielding antigenic peptides for presentation in the cleft of MHC molecules to naïve T cells, as depicted in Fig. 1. Peptides derived from proteins acquired from the extracellular environment (e.g. vaccine proteins) are generally loaded onto MHC class II molecules, whereas peptides generated from endogenously synthesized proteins are typically presented on MHC class I molecules. However, in 1976 Bevan described the loading of exogenous peptides onto MHC class I molecules with subsequent induction of a peptide-restricted $CD8^+$ T-cell response [i.e. a cytotoxic T-cell (CTL) response] [11]. This process is a unique feature of DCs and Mϕ and is referred to as cross-presentation. The loading of exogenous antigens onto MHC class I molecules therefore offers an attractive pathway to elicit cytotoxic T-cell responses.

Following the uptake and degradation of exogenous antigen by APCs, and the loading of antigen fragments onto MHC class I or II molecules, peptide–MHC complexes are transported to the cell surface. Upon cellular contact between a DC and a T cell the antigen–MHC complex is recognized by T-cell receptors (TCRs) (Fig. 1). Additionally to TCR stimulation, a second or co-stimulatory signal must be delivered to the same T cell to induce clonal expansion of the T cell and differentiation into effector and memory cells. Absence of co-stimulation results in anergy.

In adaptive immunity two branches of reactions can be distinguished, namely humoral (antibody) and cell-mediated (cytotoxic T-cell) responses. Upon activation naïve $CD4^+$ T cells can differentiate into either Th1 or Th2 cells, which differ in the type of cytokines they produce. Th1 cells produce, for example, interferon (IFN)-γ and IL-2, whereas Th2 cells typically secrete IL-4, IL-5 and IL-10. Naïve $CD8^+$ T cells become, upon appropriate stimulation, CTLs, which can kill altered host-cells (e.g. by perforines, granzymes) and produce cytokines such as IFN-γ and TNF-α. B cells that recognize antigenic peptide–MHC class II complexes and are co-stimulated by $CD4^+$ T cells or cytokines undergo clonal expansion and differentiation and ultimately become antibody-secreting plasma cells.

In addition to the Th1 and Th2 cells, other effector $CD4^+$ T cells exist. For example, Th17 cells are involved in immune responses against fungi and extracellular bacteria and typically produce IL-17, IL-21 and IL-22. However, the Th17 cells are not only involved in host defense, especially on mucosal surfaces, but also play a crucial part in autoimmune pathogenesis [12].

Finally, regulatory T cells ($T_{reg}$) fulfil an indispensable function in the maintenance of immunological self-tolerance and immune homeostasis. $T_{reg}$ exert a suppressive effect on immune responses, which is mainly mediated through cell–cell contact and the secretion of transforming growth factor-β or IL-10 [13].

## Particulate antigen delivery systems

### *The promise of delivering particulate antigens over soluble antigens*

One advantage of particulate vaccines over soluble antigens arises from their facilitated uptake by APCs. Antigens associated with particles mimic the particulate nature of pathogens. Indeed, particulate vaccines are typically a few hundred nanometers to a few microns in size; these dimensions are comparable to those of common pathogens, against which the immune system has evolved to react, and they are readily taken up by APCs. Usually, particles larger than 0.5 μm are internalized by APCs via phagocytosis (with an upper size limitation of 5–10 μm) (Fig. 2), whereas uptake of soluble antigen or smaller particles is primarily mediated by endocytosis [14]. Internalization of particulate vaccines through phagocytosis into phagosomes has important consequences because phagosomes are known to be competent organelles for antigen cross-presentation [15,16]. This makes particulate vaccines attractive for inducing cellular immune responses, in contrast to soluble antigens which are preferentially presented by the MHC class II pathway and are only poorly cross-presented. Soluble proteins can be internalized into different populations of early endosomes having different maturation kinetics [17]. The endocytic process itself

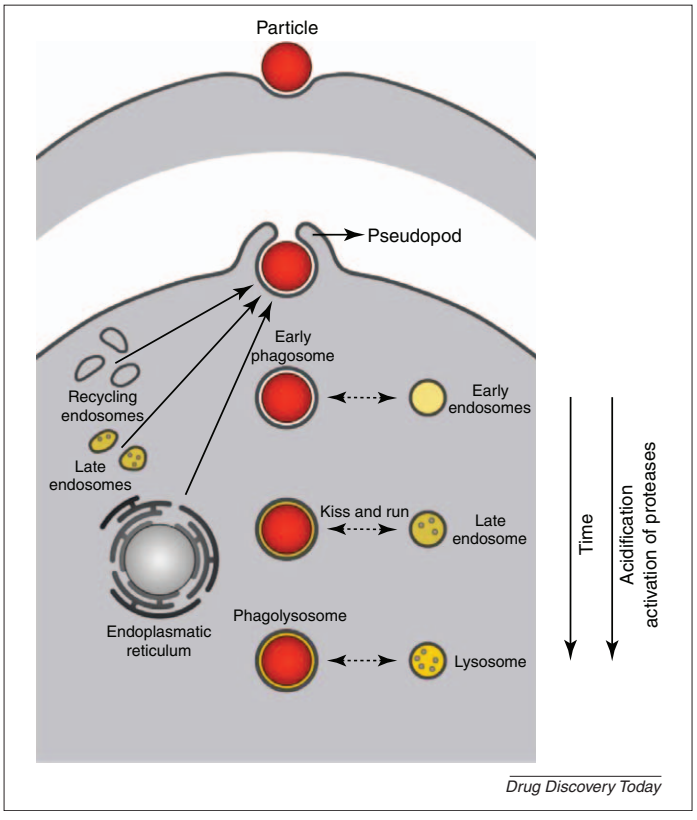


**FIGURE 2**

Phagocytosis of particulate vaccines by APCs involves extension of pseudopods around the particle, resulting in engulfment of the particle and the formation of a phagosome. Beside the plasma membrane, this process also requires contribution of intracellular membranes of multiple origin. Through phagolysosome biogenesis, the phagosome matures by fission and partial fusion with endocytic compartments in 'kiss and run' events and acquires increasing degradative properties. Degradation of the phagocytosed particulate vaccines generates peptide antigens for loading on MHC molecules and subsequent antigen presentation at the cell surface.

directs the antigen into distinct populations of early endosomes and thereby dictates the intracellular processing and presentation of the antigen. Yet, it remains unclear how the endocytic process responsible for the uptake of soluble antigen determines the pre-early endosome sorting and the subsequent loading on MHC class I or MHC class II molecules [17,18]. By contrast, particulate antigens contained in phagosomes can enter the MHC class I and MHC class II pathways [14].

During transport to the lymph nodes soluble antigen is susceptible to premature degradation by proteolytic enzymes. Association of antigens with carrier particles can protect against such degradation, although the degradation products of the polymeric particles can also alter or destroy the associated antigen [19]. Other attractive features of particulate antigens include: (i) the possibility to deliver relatively large quantities of particle-associated antigen inside the APCs; (ii) a prolonged intracellular [20,21] or extracellular [22] release leading to prolonged antigen presentation compared with soluble antigen; (iii) concomitant delivery of antigen and immunostimulatory components to the same APC (antigen and adjuvant being associated with the same particle) [23–27], aiming at directing the immune response toward the cellular or humoral arm.

Depending on the nature of the antigens and the particle characteristics, the antigens can be localized on the outside, be encapsulated and/or be dispersed in the particle. The precise location of the antigen is likely to influence the immune response. On the one hand, surface-bound antigen will be exposed to proteolytic enzymes, which can result in premature degradation and, on the other hand, it can interact directly with B cells, which can present an advantage in booster administrations.

## Vaccine emulsions

Water-in-oil (w/o) emulsions have a long history in vaccine formulation. As early as 1916, Le Moignic and Pinoy described a vaccine w/o emulsion of inactivated *Salmonella typhimurium* broth in mineral oil, which increased the immune response [28]. In 1937 Jules T. Freund described a mineral oil mixed with a broth of inactive *Mycobacteria*, which is now known as Freund's complete adjuvant (FCA) – the w/o emulsion minus *Mycobacteria* is referred to as Freund's incomplete adjuvant (FIA). FIA is still applied in veterinary medicine but has been prohibited for use in human vaccines because of severe adverse events. In the 1990s a squalene oil-in-water emulsion, MF59, was developed and generated good antibody titers [29,30], demonstrating good tolerability and general safety [31]. MF59 is currently approved in Europe for influenza vaccines. The exact mechanism of adjuvanticity is not completely understood to date. It has been proposed that the uptake of emulsion droplets with associated antigen by APCs could stimulate cytokine production and a subsequent enhanced immune response [32].

## Particulate vaccines based on lipids and lipid–saponin mixtures

### *Liposomes*

Liposomes are spherical vesicles composed of phospholipid bilayers surrounding an aqueous compartment; their size ranges from 50 nm to several micrometers. Antigens and, optionally, immunomodulating compounds can be encapsulated within the aqueous lumen, linked to the liposomal surface [33] or embedded in the lipid bilayer [34], all of which can protect the antigen from the surroundings. Variations in size, composition and physicochemical characteristics can render liposomes a versatile platform for antigen delivery, as discussed in depth elsewhere [35]. Integration of viral envelope proteins into the lipid membrane of the liposomes has proved to be effective for directing antigen into the MHC class I pathway. The viral envelope proteins are generally glycoproteins from, for example, influenza or hepatitis A virus; their integration into the liposomal lipid bilayers yield the so-called virosomes or immunopotentiating reconstituted influenza virosomes (IRIVs). Virosomes are fusogenic (i.e. the viral proteins such as hemagglutinin induce fusion of virosomal and endosomal membranes, which facilitates the escape of antigens from the endosomes into the cytosol of APCs) [36]. In liquid form, liposomes have limited stability and tend to aggregate and fuse together, an issue that can be solved to some extent through lyophilization. Unsaturated liposomal lipids can also degrade, which results in leakage of encapsulated antigens [37]. The few virosomal human vaccines presently on the market (against influenza and hepatitis A [38,39]) show good immunogenicity, good safety and tolerability.

Among the innumerable types of liposomal vaccine formulations studied over the past 30 years, cationic liposomes appear to be particularly immunogenic. For example, liposomes made of dimethyldioctadecylammonium (DDA) and the immune-modulating glycolipid trehalose dibehenate (TDB) efficiently promoted the cell-mediated and the humoral immune responses [40]. This type of liposome combined with a tuberculosis antigen is currently being evaluated in a Phase I study [41].

### *Immunostimulating complexes (ISCOMs)*

ISCOM matrices are negatively charged, spherical, cage-like nanoparticles composed of phospholipids, cholesterol and Quil A saponine, with the latter being derived from the bark of *Quillaja saponaria* [42]. Loading of antigens into such colloidal structures yields the ISCOMs. Hydrophobic antigens can be embedded or anchored directly into the lipidic colloidal domains, whereas hydrophilic antigens require modification for efficient entrapment [43,44]. ISCOMs were initially administered parenterally; later, their mucosal administrations {i.e. oral [45] and intranasal (i.n.) [46]} have been quite successful. In particular, the i.n. administration of ISCOM-based vaccines elicited antigen-specific mucosal IgA responses efficiently, as well as serum IgG antibody [47] and CTL responses [48]. The capacity of ISCOMs to induce CTL responses is related to facilitated antigen entry into the cytosol of APCs, which probably results from an interaction between ISCOMs and the lipid membranes of endosomes or phagosomes. The question whether the saponin component or the lipids are responsible for this phenomenon remains to be elucidated [49].

An interesting feature of ISCOMs is their good stability under varying conditions [50]. ISCOMs have been evaluated in clinical trials in humans where they induced strong humoral and CTL responses even at very low antigen doses [51–54]. Formulations were well tolerated without any serious adverse events being reported [51,53]. Despite their potential and good performance in clinical trials ISCOM-based vaccines are thus far approved for veterinary use only [55].

## Particulate vaccines based on polymers

Many different polymer types have been used to prepare micro- and nanoparticles for antigen delivery. Here, we will focus on some of the most widely studied and more recent and novel types of polymeric particulate antigen-delivery vehicles.

### *Vaccine particles based on polymers of lactic acid and glycolic acid*

Owing to a long history in medical applications (suture material, drug delivery implants and microspheres) [56,57], the biodegradable poly(D,L-lactide) (PLA) and poly(D,L-lactic-*co*-glycolic acid) (PLGA) are probably the most studied materials for parenteral and mucosal antigen delivery. The initial motivation of using PLGA particles for antigen delivery resided on the achievable prolonged and pulsatile antigen release in view of developing single-shot vaccines, which would obviate the need for multiple booster immunizations [58]. Despite substantial research efforts and interesting scientific results the feasibility of single-shot vaccination has not been demonstrated clinically, and further exploration of this concept was stopped for scientific, technological and economical reasons [59]. Recently, PLGA particles have been studied mainly for the delivery of antigens to DCs and Mϕ aiming at inducing cytotoxic T-cell responses. PLGA microparticles are efficiently phagocytosed by APCs *in vivo* [60]. In addition, the particle surface has been modified to influence maturation and activation of APCs upon particle uptake. As an example, PLGA microparticles were decorated with antibodies against CD40 to stimulate DCs, which induced upregulation of the CD83 maturation marker [61,62]. PLGA nano- and microparticles have been shown to mediate prolonged antigen presentation by DCs [63].

Besides the encouraging immunological performances of PLGA-based microparticles and nanoparticles, the stability of encapsulated protein antigens has been found to be an important issue. Indeed, some protein antigens tend to aggregate or degrade upon entrapment into PLGA or during release from the matrix. In the encapsulation process antigens are exposed to organic solvents (e.g. dichloromethane) that are required to dissolve the polymer, and also to shear stress [64]. The (bio)degradation of the PLGA particles, which occurs through hydrolysis of the ester bonds, not only controls antigen release but also creates an acidic and potentially harmful microenvironment [65]. These problems have been partially solved by optimized manufacturing methods [66,67] or addition of stabilizing agents such as $Mg(OH)_2$, other proteins, surfactants or sugars [68–71]. Another approach to prevent protein instability is by adsorption of antigens to the surface of preformed particles to avoid exposure of the proteins to organic solvents and shear forces during the emulsification step [72].

### *Layer-by-layer (LbL) capsules*

In recent years there has been interest in exploring so-called polyelectrolyte microcapsules as vaccine carriers [73]. These microcapsules are fabricated by LbL-coating [74] of template nanoparticles or microparticles (containing antigens and/or other bioactive compounds) with polymers, usually oppositely charged polyelectrolytes. After coating the template core is removed yielding 'hollow' capsules with antigens captured in the aqueous lumen. LbL capsules are promising antigen carriers because they are built from biodegradable and biocompatible polyelectrolytes [75,76], produced by a fully aqueous procedure involving minimal stress for protein antigens and exhibit relatively high loading efficiencies. However, there are currently few reports on the most important formulation and process parameters that influence protein encapsulation. Own recent data [77] show that both the microcapsule composition and protein characteristics (e.g. IEP, MW) determine the achievable encapsulation efficiency.

The potential of LbL capsules for antigen delivery has recently been demonstrated with capsules made of poly(methacrylic acid) (PMA) modified with thiol groups ($PMA_{SH}$) that were converted into disulfide bonds between the polymer layers [78,79]. The $PMA_{SH}$ capsules were stable in the oxidative extracellular environment but degraded under the reducing conditions inside the cells, and thereby released the encapsulated antigens. Loaded with oligopeptide antigens, the capsules were shown to activate $CD8^+$ T lymphocytes *ex vivo* resulting in the production of IFN-γ and TNF-α [78]. With the encapsulated model antigen ovalbumin (OVA) significantly higher $CD4^+$ and $CD8^+$ T-cell proliferation was induced in mice as compared with an equivalent dose of soluble OVA [79].

Because LbL capsules have only recently gained attention for antigen delivery purposes, and despite promising preliminary results with LbL capsules, their real potential for eliciting immune responses needs to be demonstrated in further studies. It is still unclear as to which way and to what extent the type of polymers, the size and charge of the capsules, the furnishing with immunostimulatory molecules or the route of administration influence the immunogenicity of the antigens delivered by LbL capsules.

### *Chitosan particles*

Chitosan and derivatives are, after PLGA, probably the next most studied nano- and microparticles for antigen delivery [80,81]. Chitosans are cationic polysaccharides that are considered to be biocompatible, biodegradable and nontoxic [82]. Chitosan itself is insoluble in water at physiological pH, but chemical substitution at the amine or hydroxyl groups can yield water-soluble derivatives such as *N*-trimethyl-chitosan (TMC) [83,84]. Chitosan nanoparticles and microparticles have attracted most attention for mucosal immunization through the oral or i.n. routes. The potential for mucosal delivery is related to the mucoadhesive property of chitosans, which prolongs the residence time, and enhanced absorption of chitosan particles [85–87]. After oral delivery chitosan particles are internalized by M-cells from Peyer's patches located in the intestinal tract, being part of the gut-associated lymphoid tissue [88]. Chitosan particles mediated an improved mucosal and systemic antibody response after oral [87] and i.n. [86] administration. It has been shown recently that TMC possesses, in addition to the mucoadhesiveness, intrinsic adjuvant activity, which was not found for nonsubstituted chitosan [89].

### *Microgels and nanogels*

Microgels are another class of systems with potentially favorable properties for antigen delivery. Acid-sensitive microgels made from biocompatible acetalated dextran have been proposed as a novel material that degrades inside the phagosomes of APCs [90,91]. It is assumed that the breakdown of such microgels increases the phagosomal osmotic pressure, which will cause disruption of the phagosomal membrane and release of protein

antigen into the cytoplasm [90,91]. Interestingly, it was found that the degradation properties of the acid-sensitive microgel particles influence the *in vitro* processing and related MHC class I and MHC class II presentations by DCs [92]. Microgel particles with varying degradation kinetics exerted a significant impact on the MHC class I presentation efficiency (i.e. the faster degrading particles promoted MHC class I presentation) a phenomenon that could not be explained fully.

Microgels and nanogels have also been produced from poly(-acrylamide) (PAA) that was cross-linked with acid-labile moieties [91]. Upon ingestion by APCs the lower pH caused particle degradation and fast release of antigen from the particles. OVA-loaded pH-sensitive microgel particles were co-administered with free CpG oligonucleotides to assess the impact of particle size on T-cell activation *in vivo* [91]. T-cell proliferation and antigen-specific cytotoxicity were found to be size-independent, in contrast to results with hydrophobic latex particles [93,94]. The co-delivery of OVA and CpG oligonucleotides from the PAA microgel particles elicited a strong cytotoxic T-cell response *in vivo* [95].

Further, poly(ethylene glycol)-based nanogels were developed, which were loaded with OVA and surface-functionalized with CpG oligonucleotides [96]. The nanogel particles were prepared through an aqueous two-phase system without organic solvents; the process yielded a high protein load (75% of dry mass). DCs pulsed with the OVA-loaded nanogel particles activated naïve $CD4^+$ and $CD8^+$ T cells *in vitro* more efficiently than did soluble protein [96].

Altogether, hydrogel particle-based formulations appear promising for antigen delivery. Their preparation does not require the use of organic solvents or shear conditions that could harm sensitive antigens or immunomodulators. However, further studies are needed to assess in more detail the potency of the formulations to induce T-cell and B-cell immunity against infectious agents or tumor cells.

## Trafficking of particulate antigen formulations to the lymph nodes

Antigens have to reach the lymph nodes for recognition by immune cells and induction of an adaptive immune response [97]. Immature DCs and Mϕ catch soluble or particulate antigenic material in the peripheral tissues and carry them to lymph nodes via lymphatic vessels for presentation to naïve and memory T cells. Soluble antigens can drain spontaneously into lymph capillaries and travel to lymph nodes where DCs and T cells are abundant. Spontaneous draining of particulate vaccines is less likely because it is size-restricted [i.e. only nanoparticles (<100 nm) can leave the interstitial matrix via the interstitial flow and be transported through the lymphatic vessels into the lymph nodes] [98,99]. With this challenge in mind, 25 nm and 100 nm polypropylene sulfide nanoparticles loaded with OVA have been explored as antigen delivery systems. After intradermal injection the 25 nm particles reached the lymph nodes with a 10-fold higher efficiency than the 100 nm particles. Intradermal injection of the 25 nm particles in mice elicited humoral and cell-mediated immunity, with the anti-OVA IgG levels being similar to the titers achieved with an intradermally administered OVA-alum formulation, whereas the antibody titers elicited with the 100 nm particles were significantly lower [100]. Another research group observed that ISCOMs reached the draining lymph nodes from the site of injection more rapidly and persisted longer than soluble antigen [101].

Although passive targeting of lymph-node-resident DCs might appear attractive, most nano- and microsized particulate antigen formulations to date are too big for spontaneous transport to the lymph nodes. Instead, most particles are internalized by endocytosis or phagocytosis into APCs circulating in the peripheral tissues and then transported to the lymph nodes [102]. This transport is crucial for eliciting a proper activation of the immune system; when migration of DCs through the lymphatic vessels is inhibited only poor T-cell responses are observed [103].

Although it is clear that most particulate vaccines need to be internalized in the periphery and transported by DCs to generate an immune response, the question on how the route of vaccine delivery influences the type and magnitude of the elicited immune response remains largely unanswered. Recently, different particulate antigen delivery systems have been tested via various routes of administration in mice; the results revealed that the route of administration particularly affected the kinetics and strength of the $IgG_{2a}$ response associated with Th1 immunity [104].

## Targeting particulate antigens to DCs

Immature DCs in the periphery screen their environment continuously for foreign antigens. They dispose of diverse endocytotic internalization mechanisms for capturing foreign material that operate in a size-dependent manner. In general, virus-sized particles (20–200 nm) are taken up by receptor-mediated endocytosis [105], whereas larger particles (>0.5 μm) are ingested via phagocytosis or macropinocytosis. Macropinocytosis is a receptor-independent uptake mechanism restricted to few cell types. It is constitutively carried out by DCs [106], whereas Mϕ acquire this capacity upon stimulation [107]. Phagocytosis is initiated by interaction of ligands with receptors on the surface of the APC [108]. DCs and Mϕ express a variety of surface receptors for recognition and uptake of manifold antigens and particles. For synthetic particles their opsonization by albumin, immunoglobulins or other serum proteins is probably an important step for receptor-mediated phagocytosis [109,110].

One strategy to facilitate uptake by DCs involves coupling of a DC receptor ligand to the antigen itself or to the particulate delivery vehicle. Targeting of soluble antigens is also promising because soluble antigens are internalized by endocytosis and, thus, get presented preferentially through the MHC class II pathway [111]. It has been shown that soluble antigens can also elicit strong CTL responses *in vivo* after antibody-mediated targeting to the DEC-205 DC surface receptor [112].

*In vivo* studies revealed that delivery of targeted particulate vaccines to DCs enhances immune responses. For example, subcutaneously injected targeted liposomes induced stronger humoral and CTL responses than nontargeted liposomes [111,113,114]. Different ligands have been reported for the targeting of antigen-loaded liposomes. For example, phosphatidylserine was incorporated into liposomes to facilitate interaction with surface receptors on monocytes [33,115]. Ligands with terminal mannose, fucose or *N*-acetylglucosamine can promote phagocytosis by binding to lectin-like receptors at the surface of DCs, such as the mannose receptors DEC-205 and DC-SIGN [116–118]. Another strategy involves the coupling of monoclonal antibodies

to liposomes, forming so-called immunoliposomes [119]. Further, targeted PLGA-based nanoparticles grafted with antibodies (targeting DC-SIGN) on their surface were reported for enhanced antigen delivery; compared with nontargeted PLGA nanoparticles, the internalization by DCs seemed to occur more efficiently, at least *in vitro* [120]. However, most particulate vaccines are probably too large to diffuse freely through the peripheral tissue matrix, which results in inefficient targeting. Despite the many interesting reports on targeted antigen delivery, the potential of targeted particulate vaccine delivery has to be further explored and validated. Targeted antigen delivery might enhance not just efficacy but also the specificity of interaction between antigen-loaded particles and the surface receptors of DCs.

## Intracellular fate of particulate antigens

### *Phagosomes and phagolysosomes*

A simplistic view of phagocytosis is the internalization of 'foreign particles' into a plasma-membrane-derived organelle (i.e. the phagosome). This view has, however, further developed in recent years (Fig. 2). Pseudopod extensions around the foreign particles are originating not only from the outer cell membrane but also from intracellular membranes that are recruited to the surface to contribute to the formation of the phagosome membrane [121–123]. As for the endoplasmic reticulum (ER) membrane, its recruitment for phagosome formation remains controversial [123–125].

In a next step, nascent phagosomes proceed through a series of changes to acquire the capacity to degrade their content. During this maturation phagosomes sequentially interact with early endosomes [126,127], late endosomes [128,129] and lysosomes [130], and ultimately become phagolysosomes. The interaction events are orchestrated by signals expressed by the phagosomes. There is evidence that newly formed phagosomes preferentially interact with early endosomes; as maturation proceeds they interact with late endosomes and attain characteristics of late endosomes, which are refractory to early endosomes [131–134]. Material transfer through interaction of phagosomes and endocytic organelles is described by the 'kiss and run' hypothesis [135].

Phagosomes are highly dynamic organelles. During phagolysosome formation not only does extensive remodeling of the phagosomal membrane occur but the lumen of the phagolysosomes also acidifies [136], with the pH dropping from ~7 to <5; such acidification is optimal for the activity of the hydrolytic enzymes, which are acquired during the interaction events. Within the pathway of phagosomal maturation, proteolytic activity is accurately tuned because hydrolases are not acquired simultaneously, but sequentially at different time points of the dynamic process [137]. This balance in proteolytic activity is achieved in favor of the generation of peptide antigens for MHC presentation and avoiding the complete degradation of the epitopes.

### *Phagocytosis of antigen particles*

Phagocytosis of particulate antigens is triggered by the interaction between the particles and cell surface receptors. Phagocytosis of particles devoid of specific ligands requires their coating by opsonins (opsonization), a process that occurs spontaneously upon injection of most particles into the dermis, subcutis or muscle. Opsonin molecules include components of the complement system (e.g. C3b), antibodies, albumin and mannose-binding lectin. Opsonins then interact with different receptors located on the surface of APCs, such as the complement [138] and $F_c$ receptors [139].

The type of ligand–receptor interaction mediating phagocytosis by APCs influences the further processing of the internalized particles and subsequently the immune response. Observations in mice [140,141] led to a 'phagosome autonomous' model, according to which signaling proteins taken up in APC phagosomes dictate the subsequent phagosomal maturation. Beside phagocytic receptors, co-receptors such as Toll-like receptors (TLRs) (e.g. TLR2 and TLR4) also seem to influence the phagosomal maturation in favor of antigen presentation and induction of an immune response [140]. Thus, formation and subsequent maturation of phagosomes in DCs are related to the characteristics of the ingested particulate antigens. In this context, several groups have investigated the effect of surface charge, hydrophobicity and size of particulate antigens on the mechanism of phagosome formation and/or the induced immune response, as outlined below.

The importance of surface charge of particles on phagocytosis by Mϕ and DCs was demonstrated *in vitro*, with cationic particles being more efficiently phagocytosed than negatively charged particles [142–144]. A similar effect was seen with cationic and anionic liposomes [115]. In mice, positively charged liposomes induced a more efficient CTL response than did negatively charged or neutral liposomes [145]. Unsurprisingly, positively charged particles bind efficiently to the negatively charged APC surface, which results in enhanced phagocytosis. However, surface charge does not seem to be the only factor determining phagocytosis. This has been shown in an early study with microspheres of similar size and varying surface charges, which were phagocytosed by Mϕ to comparable extents [144]. This important study demonstrated that phagocytosis depends not only on particle size and surface charge but also on the chemistry of the bulk or surface material of the particles.

In an attempt to show how particles influence the phagosome formation in APCs some groups have examined the engulfment of particles with varying surface characteristics by transmission electron microscopy. Hydrophilic microparticles seemed to be ingested by APCs without pseudopod formation by a so-called 'sink-into-the-cell-like manner', ending up in loose contact with the phagosomal membrane [132,146]. By contrast, phagocytosis of hydrophobic and positively charged particles involved active pseudopod formation resulting in a tightly apposed membrane, probably as a result of the high affinity of the particle surface for the cell membrane [147]. This second mechanism to engulf particles was also noticed with latex beads [132] and lipid vesicles [105].

The nature of the ingested particles influences not only phagosome formation but also the kinetics of APC maturation, which might be related to the interaction between the particle surface and the phagosomal membrane. After particle uptake the phagosome vacuole matures according to the 'kiss and run' hypothesis (Fig. 2). The controlled acidification and increasing proteolytic activity in the phagosome have important consequences for the subsequent generation of epitopes. With hydrophilic particles this process seems to be fast (i.e. characterized by a rapid interaction with lysosomes and a drop of phagosomal pH [132,146]). By contrast, hydrophobic or positively charged particles are engulfed

by a closely fitting membrane, which hinders maturation and is accompanied by only minor acidification [132,146]. It is expected that the kinetics of APC maturation affects the type of immune response.

The kinetics of APC maturation is probably also influenced by the size of particulate antigens. In a recent study, it was shown that 50 nm polystyrene beads, loaded on their surface with antigen, co-localized rapidly with lysosomes, in contrast to 500 nm and 3 μm beads that remained in a neutral or only slightly acidic phagosomal milieu [148]. This is in agreement with an earlier observation where 500 nm antigen-loaded lipid vesicles did not traffic into lysosomes. Thus, the differences in APC maturation kinetics according to the size (or other properties) of ingested particles might suggest the simplistic view that microparticles could favor Th1-type and antibody responses, whereas nanoparticles generate Th2-type responses [105,148,149].

## Humoral and cellular immune responses: antigen presentation by MHC molecules

After internalization, antigen degradation and loading of antigen-derived peptides onto MHC molecules the peptide–MHC complexes are presented at the cell surface of APCs. Antigenic peptides can be loaded onto MHC class II molecules in different compartments of the endocytic pathway [150]. It has been shown that antigens associated to latex beads were presented in MHC class II complexes after phagocytosis, which indicates that not only endosomes but also phagosomes can mediate formation of peptide–MHC class II complexes [151]. Antigen–MHC class II complexes are further transported to the cell surface for presentation to $CD4^+$ T cells. Recent studies on DCs suggest that MHC-class-II-containing compartments form tubule-like structures directed toward the interface between DCs and T cells [152,153], followed by translocation and clustering of these molecules in microdomains at the plasma membrane by a still unknown mechanism [150].

Although the unique capacity of DCs and Mϕ for antigen cross-presentation has been known for some time, the exact mechanisms of the involved intracellular pathway remain unclear. Nevertheless, three main models have been proposed to clarify how exogenous proteins can gain access to MHC class I molecules (Fig. 3). In the first model, called vacuolar pathway or TAP(transporter-associated with antigen presentation)-independent cross-presentation, antigens are degraded by proteases and loaded onto MHC class I molecules inside the endosomes [154,155]. According to the second model antigens gain access to MHC class I molecules

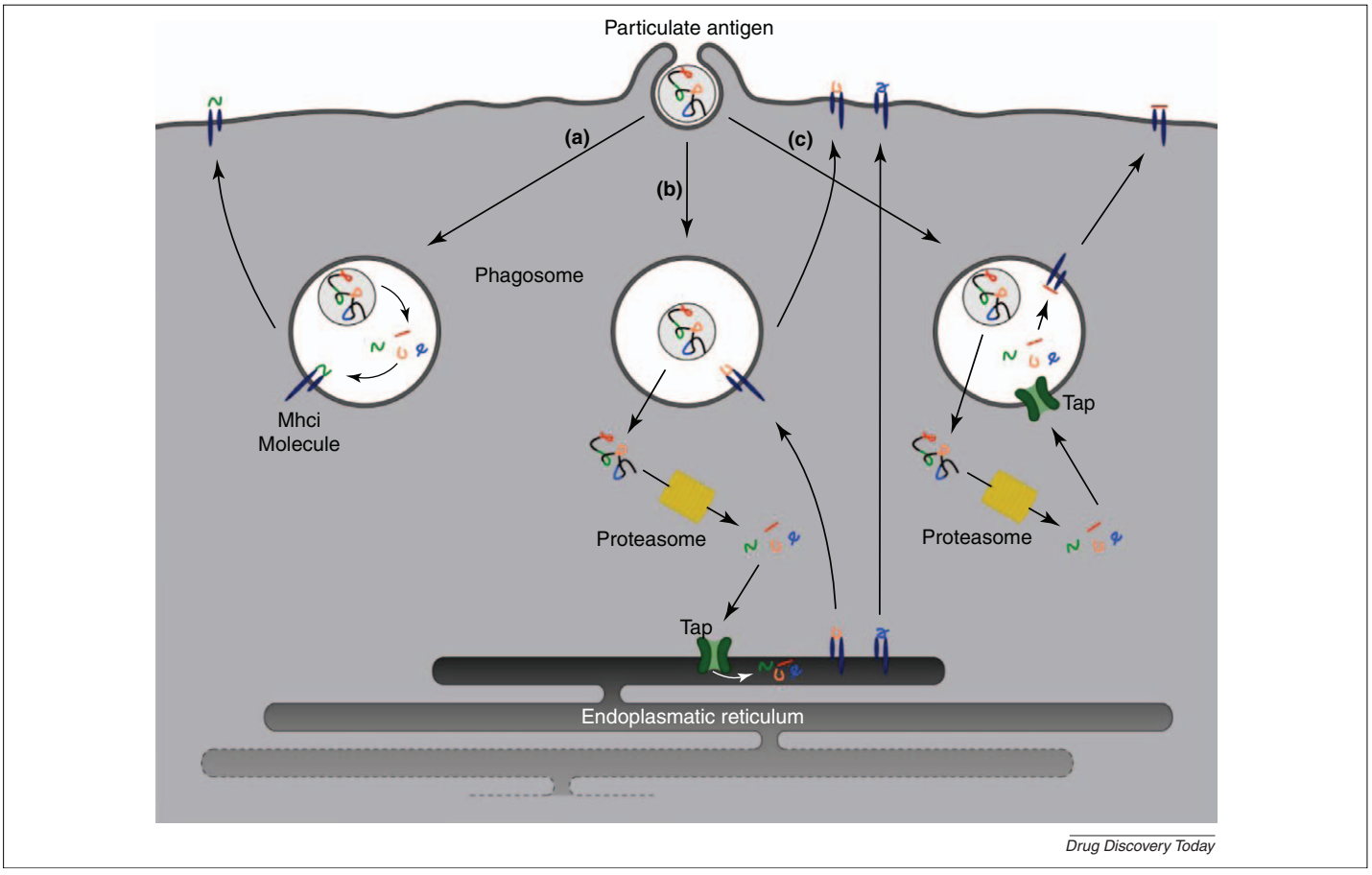


**FIGURE 3**

Potential cross-presentation pathways for antigen loading on MHC class I molecules in DCs. (a) In the vacuolar pathway, antigen is degraded by proteases in the phagosome and loaded within this organelle on MHCI molecules. (b) After internalization in the phagosome, the antigen is transferred to the cytosol and degraded by the proteasome. Antigenic peptides are transported by TAP and loaded on MHC class I molecules in the endoplasmatic reticulum. The complexes are either shuttled directly to the cell membrane or via the endosome. (c) Antigen is degraded by proteasome, transported by TAP into the endosome and loaded there on MHC class I molecules.

by 'escaping' from the phagosomal lumen into the cytosol, where they are processed by the proteasome, followed by TAP-dependent import of the antigenic peptides into the ER lumen, which is equipped for MHC class I loading [156]. Subsequently, the antigenic peptide–MHC class I complexes are either transported directly toward the cell surface or to the endosomes first and from there to the cell membrane. With the third model, antigenic peptides that have gained access to the cytosol and been degraded by the cytoplasmatic proteasome are re-imported into the phagosome in a TAP-dependent manner and loaded at this site onto MHC class I molecules [157,158].

Particulate vaccines appear to be well qualified for entering the cross-presentation pathway in DCs, thereby opening prospects toward induction of cell-mediated immunity. ISCOMs, for example, are efficiently internalized into APCs by endocytosis [159] followed by activation of the APCs [160] and upregulation of MHC class I and II expression [161]. Upon their processing, ISCOMs mediate the induction of cytokine production, especially of IL-2 and IFN-γ which promote Th1-type responses [162]. In various animal models ISCOMs induced humoral and $CD8^+$ CTL responses to different antigens and enhanced the magnitude and rate of the responses [51,163]. Evidence of cross-presentation was also found for virosomes. After receptor-mediated endocytosis virosomes fuse with acidified endosomes, which results in the release of antigens into the cytosol, where the antigenic proteins/peptides can enter the MHC class I pathway and prime CTL responses [164]. The virosomes that fail to escape from the endosome continue on the endolysosomal route to the loading and presentation on MHC class II molecules [38,165]. Finally, there is also evidence that polymeric particulate antigens can enter the cross-presentation pathway in DCs. Numerous studies reported that antigens encapsulated in PLGA microparticles result in antibody [166–168] and CTL [169,170] responses, after systemic [171] and mucosal [172] administration. Furthermore, after uptake by APCs, PLGA microparticles function as an antigen reservoir for stimulation of CTL responses [170]. Our recent studies with antigen-loaded LbL-capsules showed an enhanced stimulation of $CD4^+$ T cells *in vitro* by encapsulated OVA as compared with soluble antigen [73]. In this work, we also observed $CD8^+$ T-cell proliferation at low OVA concentrations suggesting enhanced cross-presentation of antigens delivered by the LbL-capsules [73].

## Co-delivery of antigens and immunomodulators by particles

With the progression toward using safer subunit vaccines also emerged the need to augment or modulate the immunogenicity of the antigens by addition of immunological adjuvants. By definition, vaccine adjuvants are compounds added to vaccines to enhance and/or modulate the immune response [173]. By virtue of their recognition by APCs particulate delivery systems possess important adjuvanticity *per se*. This inherent basic adjuvanticity can be further enhanced or modulated by combination with immune response modulators (e.g. ligands that induce a specific type of immune cell activation by binding to defined receptors).

Probably one of the greatest benefits of particulate antigen delivery systems resides in their capacity to deliver antigens and immunomodulators concomitantly to one and the same APC, which is crucial for efficient immune response stimulation. The selection of the immunomodulator (adjuvant) for co-delivery will determine the preferential induction of Th1 or Th2 responses. The association of immune response modulator molecules with particles minimizes the toxic effects of the former, because of restricted systemic distribution and lower maximal available concentrations. This is particularly attractive because a major bottleneck in immunomodulator development is safety. Indeed, many immunomodulators have demonstrated important immune response potentiating effects but their use in human vaccines is compromised because of safety concerns.

Despite huge efforts to find and develop new vaccine adjuvants, the most widely used and accepted adjuvant in human vaccines is alum [173,174]. Alum describes a group of aluminum-based water-insoluble salts, mostly aluminum hydroxide or aluminum phosphate; the salts are precipitated from an aqueous solution to form colloidal particles appropriate for adsorption of microorganisms or protein/peptide antigens. Since its early use in the 1920s, alum has shown good safety, although it has occasionally been associated with severe tissue reactions, induction of IgE and related allergic and hypersensitivity reactions [174]. Alum predominantly generates Th2 responses, but fails to induce significant cell-mediated immune responses. Because alum-adjuvanted vaccines have to be kept in liquid form (freezing or lyophilization destroys the colloidal structure) they suffer from limited storage stability. Finally, alum-adjuvanted vaccines are effective only upon parenteral administration, but fail in mucosal use. There is general agreement that the adjuvanticity of alum resides on the provision of an antigen depot, the nanosized particulate form appropriate for phagocytosis by APCs, and the induction of a local inflammation with associated further recruitment and activation of APCs at the site of injection [175]. Recent observations indicate that alum induces chemokine secretion and results in priming of Th2 cells producing IL-4, IL-5 and IL-10 [176]. Finally, alum adjuvanticity is also associated with an increase of uric acid concentrations [177] and activation of the NLRP3 inflammasome, which leads to maturation of proinflammatory cytokines including IL-1β [178]. Despite being the most studied and longest used vaccine adjuvant, the complete mode of action of alum is still unknown.

Thanks to the impressive gain in immunological knowledge over the past 30 years some of the 'the immunologist's dirty little secrets' (Charles Janeway) have been unraveled. For example, the discovery of the pattern recognition receptors (PRRs), which are involved in APC activation upon recognition of pathogen-associated molecular patterns (PAMPs), has exerted a great impact on present and future vaccine developments. Ligands that target PRRs are potentially interesting immunomodulators. PRRs include TLRs, nucleotide-binding oligomerization domain receptors (NOD), NOD-like receptors (NLR) and RIG-like helicases (RLH) [179]. Activation of these receptors plays a key part in DC maturation and cytokine production. For example, the TLR9 agonist CpG (a synthetic, unmethylated oligodeoxynucleotide, containing cytosine–guanine motifs, and is present in bacterial but not in eukaryotic DNA) activates DC maturation, facilitates cross-presentation and induces a Th1-biased response [180].

Several TLRs have been studied for enhancing or modulating the immune response upon vaccination, with the most prominent being TLR3, TLR7, TRL9 and TLR4. Although TLR4 is located on the cell surface and, therefore, readily accessible, TLR3, TLR7 and

**TABLE 1**

**Particulate antigen delivery systems incorporating co-adjuvants**

| *Receptor* | Adjuvant | Formulation | Specific outcome | Refs |
|---|---|---|---|---|
| **TLR3** | Poly(I:C) | Liposomes | Liposomes complexed to poly(I:C) were able toefficiently generate strong CD4 and CD8 T cell responses. | [186] |
| **TLR3** | Poly(I:C) | Polyketal microparticles | Increased crosspriming of T lymphocytes by DCs in vitro. | [27] |
| **TLR4** | MPL | Liposomes | Combination of MPL with QS21 saponine induced very strong and persistent humoral and cellular immune response immune responses with acceptable reactogenicity profile. | [182] |
| **TLR4** | MPL | o/w emulsion | Combination of MPL with QS21 saponine induced very strong and persistent humoral and cellular immune response immune responses with acceptable reactogenicity profile. | [182] |
| **TLR7** | 3M-019 | Liposomes | Significantly increased antibody responses and strong CTL response could be induced after s.c. administration in mice | [183] |
| **TLR9** | CpG | o/w emulsion | Increased antibody levels but low cellular response in humans. | [182] |
| **TLR9** | CpG | Liposomes | Co-encapsulation of CpG and tetanus toxoid resulted in higher serum IgG and antitoxin titers but suppressed IgA titers after i.n. immunization of rabbits. | [24] |
| **TLR9** | CpG | Liposomes | Liposomes formulated with CpG elicited strong T cell responses in mice. | [186] |
| **TLR9** | CpG | Liposomes | Increased antibody levels but low cellular response in humans. | [182] |
| **TLR9** | CpG | PLG | CpG adsorbed on the surface of the PLG microparticles was effective for CTL induction against HIV antigens following i.m. immunization of mice. | [23] |
| **TLR9** | CpG | PLGA | PLGA microparticles with surface adsorbed CpG generated a significant CTL response after s.c. immunization of mice. | [26] |

TLR9 are located on the endosome of DCs. Access to the intracellular TLRs requires efficient internalization of the ligands, a criterion that is perfectly met with particulate delivery systems (Table 1). In agreement with these considerations, simple mixtures of antigens and intracellular TLR ligands have generally not promoted the immune response significantly as compared with simple antigen formulations. By contrast, the proper adsorption of one of the most studied TLR4 ligands, namely monophosphoryl lipid A (MPL), to alum-based vaccines was immunologically highly beneficial and led to the formulation of several vaccine products against hepatits B and human papillomavirus infections (Fendrix®, Cervarix®), and also for the treatment of hayfever (Pollinex Quattro®). MPL derived from the LPS of *Salmonella minnesota* has an acceptable safety profile and the capacity to promote Th1 responses [181]. Finally, MPL has also been tested in liposomal formulations that resulted in improved immunogenicity [182].

The TLR9 ligand CpG has been tested in association with different types of particulate vaccine formulations, including PLGA particles and liposomes, where it mediated enhanced immunogenicity [23,24,26]. CpG adsorbed to PLGA microparticles effectively mediated antibody production, whereas CpG alone remained an ineffective adjuvant [23]. In the same study, soluble antigen mixed with free CpG did not induce a potent antigen-specific cellular immune response in contrast to CpG adsorbed on the surface of antigen-loaded PLGA particles; the latter were probably taken up more efficiently by APCs via phagocytosis. Another group reported that co-delivery of an antigen and CpG from the same PLGA microparticle induces a more rigorous CTL response after immunization of mice in comparison with the separate delivery of the antigen and CpG from different PLGA particles [26]. Likewise, CpG co-encapsulated with antigen in liposomes induced protective antibody titers, whereas liposomes loaded with antigen alone failed to do so [24].

Ligands for the intracellular TLR3 and TLR7 were also tested in association with particulate vaccine formulation. For example, Johnston and co-workers showed that ssRNA (TLR7 ligands) encapsulated in liposomes mediated strong cellular responses in mice [183]. The TLR3 ligand poly(inosinic acid)-poly(cytidyl acid), or poly(I:C), a synthetic dsRNA analog, enhances cross-priming of $CD8^+$ cytotoxic T lymphocytes [184,185]. When associated with microparticles [25,27] or liposomes [186] it mediated strong antigen-specific $CD8^+$ T-cell responses.

## Concluding remarks

Traditional vaccines consisting of live attenuated or inactivated microorganisms raise considerable safety concerns shifting vaccine development toward the use of clearly defined subunit proteins that, however, need an appropriate delivery vehicle. Formulation of antigens in nano- and microparticles can be advantageous in various ways: (i) protection of antigens against degradation by enzymes; (ii) facilitating uptake by and/or activation of APCs; (iii) depot formation for prolonged presentation of antigens; (iv) concomitant delivery of antigens and adjuvants to the same APC; and (v) induction of cell-mediated immune responses. Numerous studies have indeed confirmed that particulate vaccines elicit more-effective immune responses than soluble antigens. It has been shown that the physicochemical properties (size and charge) as well as the chemical nature of materials of the particles influence the route of entry into APCs and the efficiency of presentation of antigenic peptides by MHC class I and II molecules. Knowledge of the impact of particle characteristics on phagosome biogenesis is highly relevant for a more rational design of effective antigen delivery systems and vaccine formulations. Therefore, we stress the importance to consider all factors that modulate uptake, processing and presentation of (particulate) antigens by professional APCs in the design of particulate vaccines.

Naturally, however, we cannot provide a recipe for the perfect antigen delivery system because there are only a few studies where different formulations were compared side by side under identical conditions. Therefore, we believe that well designed comparative studies with different types of antigen carriers should resolve the question: which type of carrier performs best for a specific vaccination?

Another interesting strategy in particulate vaccine development involves the concomitant delivery of antigen and immunopotentiators, which can affect the Th1:Th2 balance and extent of the elicited immune response. Although the mechanism of action of most adjuvants is still not fully elucidated, numerous reports support the benefits of concomitant delivery of antigen and adjuvant from the same particle to enhance the immune response. Therefore, to obtain a potent vaccine, it could be necessary to combine a good delivery system with an immunostimulatory adjuvant. Despite extensive research in recent years safety concerns have, however, restricted the application of adjuvants.

With the existing knowledge from the fields of immunology and vaccinology, antigens, adjuvants and delivery systems can now be selected more rationally to achieve optimal protective or therapeutic immune responses against infections and other diseases. Nevertheless, depending on the target disease, antigen type and administration route, formulations must be optimized case by case. Thus, important further efforts are required to fill existing gaps in the puzzle of generating protective and therapeutic immune responses by vaccine tailored formulations. We believe that particulate delivery systems will play an important part in such future endeavors.

## Acknowledgement

The authors thank T. Coppieters for help with illustrations.

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