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# Nanoparticle formulated vaccines: opportunities and challenges

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Vaccines harness the inherent properties of the immune system to prevent diseases or treat existing ones. Continuous efforts have been devoted to both gaining a mechanistic understanding of how the immune system operates and designing vaccines with high efficacies and effectiveness. Advancements in nanotechnology in recent years have generated unique opportunities to meet the daunting challenges associated with immunology and vaccine development. Firstly, nanoparticle formulated systems provide ideal model systems for studying the operation of the immune system, making it possible to systematically identify key factors and understand their roles in specific immune responses. Also, the versatile compositions/architectures of nanoparticle systems enable new strategies/novel platforms for developing vaccines with high efficacies and effectiveness. In this review, we discuss the advantages of nanoparticles and the challenges faced during vaccine development, through the framework of the immunological mechanisms of vaccination, with the aim of bridging the gap between immunology and materials science, which are both involved in vaccine design. The knowledge obtained provides general guidelines for future vaccine development.



## Introduction

In recent decades, advances in immunology, molecular genetics and nanotechnology have revolutionized vaccine research. From the experimental demonstration of a cross-neutralizing influenza vaccine[1] to the clinical translation of cancer vaccine therapy,[2] nanoparticle (NP) vaccines have been at the forefront of these innovations. Nanoparticle vaccines are those with particle-like morphologies and size ranges of a few to a few hundred nanometers. They have demonstrated great potential for vaccination as they can be conveniently tailored to resemble natural targets in the immune system using parameters such as size, shape, surface modification,[3] *etc.* for optimized bio-distribution and interactions with immune cells.[4]

The material aspects of nanoparticle vaccines have been thoroughly reviewed in recent articles.[5–7] However, as published works on nanoparticle vaccines accumulate, it has become clear that common mechanisms underpin the advantages shown by nanoparticle formulations. These mechanisms are conserved across material systems and are deeply connected to the fundamental workings of the immune system. Understanding these specific features of the interactions between nanoparticles and the immune system could lead to unique opportunities for improving vaccine designs.

The aim of this review is to consolidate the existing information about the immune mechanisms relating to vaccination, which is frequently scattered throughout the vast and established field of immunology.

We will begin with basic discussion about the biological aspects of immunity and vaccination, highlighting knowledge that nanoparticle-based research has contributed to the field of immunology research, so that the connections between nanoparticles and the immune system can be appreciated. Using a framework based on the immunological mechanisms, or key steps, involved in vaccination, we focus on major pathways involving dendritic cells (others, such as macrophage mediated, *etc.*, are beyond the scope of the present review), and further elaborate how notable NP vaccine demonstrations achieved superior efficacy through the exploitation of a known number of beneficial interactions. Finally, we highlight the challenges relating to current nanoparticle formulated vaccines and suggest future directions in the field of nanoparticle formulated vaccine development.

## A brief introduction to immune response

The immune system consists of a collection of cellular and soluble components (Table 1). It is responsible for maintaining homeostasis[8] and implementing defense against any

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Table 1 The soluble and cellular components of the immune system

| |
|---|
| **Innate immune system** |
| *Soluble components* |
| Complements, antimicrobial peptides and other components inactivate microbes *via* mechanisms such as pore formation on microbial membranes or the depletion of essential nutrients. Furthermore, some of these components enhance the phagocytosis of pathogens, activate phagocytes through cell surface receptors, or generate signaling molecules for immune system activation.[9,31] |
| *Cellular components* |
| These consist of various types of cells specializing in different immune functions. Natural killer (NK) cells eliminate infected or cancerous cells.[32] Neutrophils destroy bacteria and microbes.[10] Eosinophils, basophils and mast cells repel multicellular parasites.[8] Macrophages maintain the local immunological environment through the secretion of cytokines and chemokines that induce inflammation for local defense or to facilitate tissue repair.[33] Dendritic cells act as sentinels, collecting pathogens at tissues for delivery to adaptive immune cells in the secondary lymphoid organs.[34] Monocytes act as reserves that replenish macrophages and dendritic cells during inflammation.[34] Finally, innate lymphoid cells integrate and amplify cytokine responses.[22] |
| **Adaptive immune system** |
| *Antibodies* |
| Antibodies (immunoglobulins, Igs) are soluble components of the adaptive system that bind and neutralize microbes. There are five broad subtypes of Igs: IgA, IgD, IgE, IgG and IgM. IgD and IgM are antibodies in the first line of defense, which are secreted by B cells immediately after the recognition of an antigen.[35,36] IgD and IgE can bind to mast cells and basophils to potentiate an antigen-specific anti-parasitic/allergic response. IgG and IgA are major subtypes that are secreted for the neutralization of viruses and microbes.[9,31,37,38] IgG is circulated in the blood while IgA is localized at mucosal linings.[9,34] |
| *Cellular components* |
| B cells express antigen-specific B cell receptors (BCRs), which recognize antigens based on their three-dimensional structure. Naïve B cells residing in lymph nodes are activated to produce antibodies, also known as immunoglobulins (Igs), upon the recognition of their cognate antigens. Follicular T helper cells assist with Ig class switching, affinity maturation and the differentiation of activated B cells into plasma cells and memory cells.[39] $CD4^+$ T cells express T cell receptors (TCRs), which recognize antigen peptides presented on the major histocompatibility complex class II (MHC II) molecules of antigen presenting cells (APCs). The recognition of cognate antigens by naïve $CD4^+$ T cells leads to activation and differentiation into an effector cell type called T helper (TH) cells, which are the central coordinators of the immune response.[9,10,40,41] $CD8^+$ T cells recognize through TCRs and CD8 antigen peptides present on MHC I complex molecules, which mainly present peptides derived from intracellular proteins. Activated effector $CD8^+$ T cells (also known as cytotoxic T cells) induce the death of infected or cancerous cells.[42] |



detected abnormalities. These abnormalities can be infection by pathogens,[9,10] the emergence of cancer cells,[11] or misconstrued normal signals that can lead to autoimmunity.[12]

Pathogens entering the body are detected by macrophages, which recognize molecular features of pathogens and the damage they do to body tissues. Macrophages recruit other innate immune cells to eliminate the invading pathogens through an innate immune response. Simultaneously, pathogens and the associated damage that they cause are detected by dendritic cells (DCs), which sample the pathogens and migrate to a lymph node. There, dendritic cells present antigens to T cells, resulting in the activation of T cells, which function as central coordinators of the immune response to pathogens or of the cytotoxic cells that eliminate pathogen-infected body cells. Cognate pathogen antigens that diffuse or are carried by dendritic cells to the lymph nodes are recognized by B cells, which stimulate the generation of antibodies. These antibodies neutralize pathogen toxicity through binding and enhance innate cell phagocytosis. Together, the innate and adaptive immune functions can lead to the clearance of pathogens and the resolution of infections.[8–10]

Cancer cells emerge from random mutations that lead to changes in genetic expression and the acquisition of stem cell-like phenotypes that allow uncontrolled cellular replication.[13] Natural killer (NK) cells and cytotoxic T cells detect cancer-associated changes and execute the cell-mediated killing of the cancer cells. As the result of a certain genetically dependent probability, some cancer cells survive immunosurveillance and further acquire an immunosuppressive phenotype.[11] These cancer cells not only escape into the immune system but also recruit innate suppressor cells and regulatory T cells (Treg) to protect and nurture their growth,[14] developing into malignant tumors.

Autoimmune diseases arise from complex multifactorial mechanisms that direct a defensive immune response towards normal tissue. The pathogenesis of autoimmunity involves the generation of self-reactive B and T cells that escape the central tolerance process.[15] Cumulative exposure to autoantigens or analogues from the environment can trigger the activation of these self-reactive B cells or T cells, mounting an immune response and directing adaptive and innate cells against the target normal tissue. Using multiple sclerosis as an example, self-reactive T cells escape the central tolerance process due to alternative expression[16] or the selective destruction[17] of autoantigens in the thymus. Cross-reactivity between these autoantigens and foreign antigens from pathogens presumably leads to self-reactive T cell priming.[18] The subsequent priming and boosting of these self-reactive T cells leads to TH1-dependent neurodegenerative pathologies.[19] The issue is multifactorial, as polymorphism in many immune process-related genes increases the risk of multiple sclerosis.[20] Treg cell dysfunction also contributes to the development of multiple sclerosis.[21]

## Learning from nanoparticle-based model systems: an advanced understanding of the immune system

Conventional immunology knowledge is built upon *in vitro* and *in vivo* experiments based on small molecular tools. Good examples include studies of immune cell activation with isolated cytokines[22] or pathogen components.[23]

The study of the immune system using nanoparticle-based model systems has advanced our understanding in more than one way. Unlike small molecules, the volume and surface area of nanoparticles enable them to be equipped with multiple functionalities, so that their role is no longer limited to the bio-chemical field. The same features also allow them to simulate natural pathogens in terms of morphology and a number of physical/chemical properties; these parameters can then be adjusted in a controlled manner so that the respective effect(s) on the functioning of the immune system can be independently studied.

For understanding the pathways of various immune responses, nanoparticles are commonly used as carrier systems for key components of pathogens. It was soon realized that both physical and chemical interactions between the nanoparticle carrier and the immune cells determine the resulting immune response. Repetitive arrays of antigens, a natural feature of pathogen surfaces, stimulate stronger B cell responses than random aggregates.[24] Using self-assembled protein nanoparticles that arranged influenza HA antigens with octahedral symmetry, Kanekiyo *et al.* demonstrated that defined symmetry is also an important factor that stimulates B cells for antibody generation[1] (Fig. 1a), raising interesting questions about how the geometrical features of viruses may play a role in immune cell stimulation. The loading of pathogen-relevant features on nanoparticles with defined symmetry and density has suggested that there is a geometrical requirement for the optimal stimulation of immune cells. Another example showed that a monomeric CpG molecular adjuvant required presentation on nanoparticle surfaces to perform optimally.[25] Gungor *et al.* demonstrated that assembling CpG ligands into nanorings further enhances the adjuvant effect[26] (Fig. 1b). These results suggest a possible spatial/geometrical relationship between the ligand and the receptor microdomain when immune cells communicate with pathogens in the initial stage.

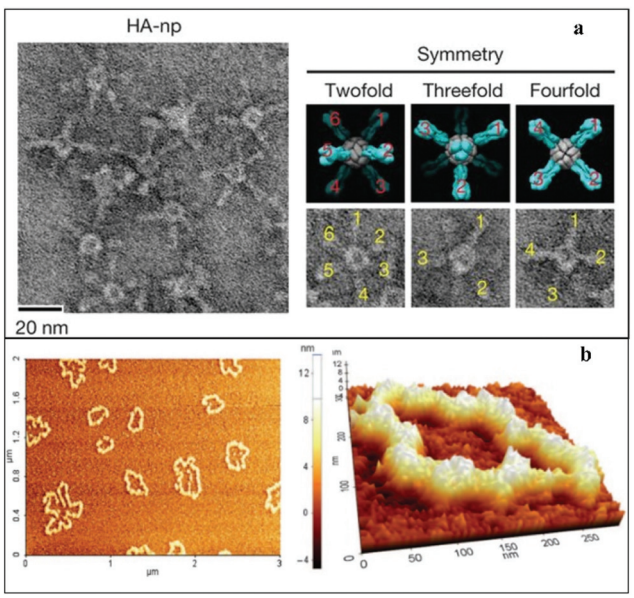


**Fig. 1** (a) The left panel shows a transmission electron microscope (TEM) image of a self-assembled influenza haemagglutinin nanoparticle (HA-np). The right panel shows a schematic diagram illustrating the symmetry of a HA-np along various symmetry axis in the top row with corresponding TEM images of the HA-np in the corresponding orientations in the bottom row. The pale blue colour in the schematic diagrams represents HA molecules and the grey colour represents the ferritin core. (b) Atomic force microscope (AFM) images of a CpG oligodeoxynucleotide and peptide self-assembling into a nanoring structure; the left panel shows multiple nanorings at low magnification and the right panel shows a single nanoring at high magnification. Fig. 1a is reprinted from ref. 1 with permission from Springer Nature, copyright: 2013. Fig. 1b is reprinted from ref. 26 with permission from The American Association for the Advancement of Science, copyright: 2014.

The study of lymphatic transport using nanoparticles of predefined sizes has enriched our understanding of how different biological entities may access and interact with adaptive immune compartments.[27–30] Reddy *et al.* first demonstrated size-preferential lymphatic transport using polypropylene sulfide (PPS)-based nanoparticles with sizes of 25, 40 and 100 nm.[27] By applying this finding to vaccine applications, they further demonstrated that the effective draining of the 25 nm, but not 100 nm, PPS nanoparticles to lymph node would translate into stronger B cell response in mouse model.[28]

Artificial antigen presenting cells (aAPCs) based on engineered nanoparticles have recently emerged as an important model system for understanding the communication between DCs and T cells. The construction of particle-based aAPCs generally includes the decoration of the nanoparticle surface with DC membrane proteins, such as epitope-bound major histocompatibility complex (MHC) molecules and costimulatory ligands, which are involved in signalling between DCs and T cells. Various bioconjugation techniques have been developed to chemically link the proteins to nanoparticle surfaces.[43] The presentation of these signalling molecules on nanoparticle surfaces allows interactions with T cells under conditions with closer resemblance to the native ones, compared to freely dissolved ligands, as the signalling receptors on the T cells are clustered together in submicron-sized domains.[44–46]

The origin of aAPCs can be traced to genetically modified cell lines expressing MHC molecules and co-stimulatory molecules for cancer therapy.[47,48] These cell lines allowed the tailored and reproducible activation of peripheral blood cytotoxic T cells (CTL), but they were limited to *ex vivo* applications due to biological safety concerns, such as the possibility of a severe immune response against the foreign cell lines. Synthetic particle-based aAPCs avoid these biological hazards, while maintaining the flexibility to present a predefined set of signalling molecules for T cell interactions. Thus, they have been employed to study T cell activation *in vitro*[49] and *in vivo*.[50,51] Particle-based aAPCs have been applied to *in vitro* studies of the biophysical requirements of T cell interactions.[52] Using PLGA microparticles, Sunshine and co-workers studied the presentation of MHC and costimulatory molecules on spherical and ellipsoidal particles. They observed that the interaction cross-sections between T cells and the two particles were different, leading to differential T cell activation.[53] This indi-

 

cated a dependence on the area or geometry of the interaction cross-section between T cells and APCs for optimal T cell activation. In another novel application, Santamaria and co-workers engineered iron oxide nanoparticles with surface decoration consisting of MHC molecules complexed with autoantigen peptides to mimic immunosuppressive dendritic cells.[50] The density of the MHC-peptide complex on the particle surface was found to be crucial for controlling the stimulation of Treg.[51]

## Definition of vaccines and their categories

Vaccines make use of the inherent properties of the immune system to bring about protective and therapeutic effects. The essential components of a vaccine formulation are antigens and adjuvants. They respectively provide the adaptive immune system with the identities of disease agents, to allow a specific response to be mounted, and information about the nature of a disease, to allow an effective immune response to be directed. Antigens are mainly proteins for T cell responses (but they can take different forms, such as polysaccharides, nucleic acids or some synthetic materials, for B cell responses). Adjuvants can be small immunogenic molecules, molecular genetic tools, such as mRNAs or siRNAs, or specially designed synthetic materials, such as nanoparticles. The aim of a vaccine is to supply dendritic cells, which act as adaptive response initiators, with the appropriate combination of disease-specific antigens and adjuvants that is required to mount a properly polarized T helper cell response, and to induce the required sets of complementary effector cells, such as cytotoxic T cells and B cells.

Fig. 2 illustrates the requirements of protective correlates for different diseases.[54] Some viruses and bacteria only require vaccines to induce a protective level of antibodies (generated by B cells) against surface proteins or secreted toxins to confer protection. Examples include human papillomavirus (HPV), hepatitis A virus (HAV), hepatitis B virus (HBV), diphtheria, and pneumococci. Others require the induction of a $CD4^+$ or cytotoxic T cell ($CD8^+$) response to prevent infections. While $CD4^+$ T helper cells coordinate the action of the innate and adaptive systems against disease agents, $CD8^+$ cytotoxic T cells eliminate infected/mutated cells. For cancer, a combination of $CD4^+$ and cytotoxic T cell responses against tumor cells is required for efficacy.

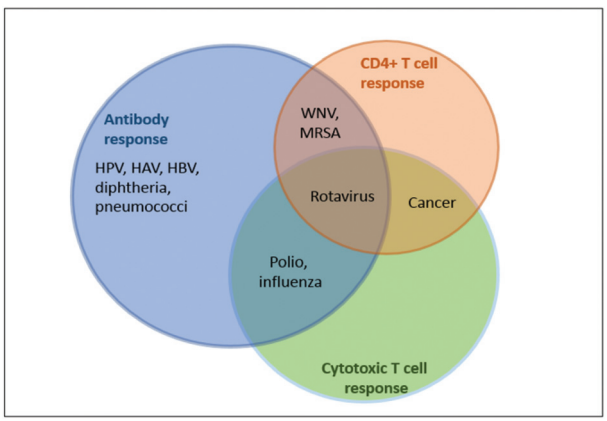


Fig. 2 The requirements of protective correlates for different diseases. HPV: human papillomavirus; HAV: hepatitis A virus; HBV: hepatitis B virus; WNV: West Nile virus; MRSA: multiple-drug resistant *Staphylococcus aureus*.

There are two categories of vaccines: prophylactic and therapeutic. Prophylactic vaccination, also known as protective vaccination, refers to vaccines used against common pathogens. It is based upon the induction of immunological memory against a disease agent (mediated by specific antigens) and the ability of memory cells to mount a rapid response upon subsequent exposure to the relevant pathogens.[55] Unsurprisingly, whole attenuated live pathogens contain all the antigens and natural adjuvants that are needed to induce robust responses,[56] so they have become one effective platform for vaccine development. The most notable example is whole attenuated yellow fever vaccine YF-17D, which is known to induce life-long protection from yellow fever based on a single vaccination.[57]

However, not all pathogens can be used as live vaccines. Some are too virulent and others are inherently immune-evasive.[23,58] These situations require strategies to deliver antigens outside of the original pathogens. Antigens can be purified from pathogen cultures or produced *via* genetic recombination. Alternatively, antigens can be supplied in the form of peptides that can be presented by MHC molecules[59] or they can take the form of genetic material (such as DNA and RNA) that can be translated back to the native protein forms using the intrinsic protein expression machinery within the cells of the body.[60,61] These antigens, when formulated with extrinsic adjuvants, are called subunit vaccines.[62] Nanoparticles are found to serve as excellent carrier systems for subunit vaccines and, in many cases, they can simultaneously provide the adjuvant effect. These features make nanoparticle formulated vaccines very attractive when developing new generations of protective vaccines.

In comparison, therapeutic vaccines are aimed at restoring immune system homeostasis by using the basic formula of vaccines, that is, antigen and adjuvant combinations, to elicit therapeutic effects against various chronic diseases, such as cancer and autoimmune diseases, in which the failure of the immune response serves as one of the root causes.

Cancer immunotherapy involves the activation of the immune system to prevent cancer development and/or treat developed cancer. In the ideal case, it enables the targeted attack of specific tumor cells by immune cells without causing damage to normal tissue. In this way, primary and secondary tumors resulting from metastasis are addressed.[1,2]

Cancer vaccines belong to the big cancer immunotherapy family, which also includes therapeutic monoclonal anti-

bodies,[63] recombinant cytokines,[64] immune checkpoint inhibitors,[65] and adoptive cell therapy.[66] The relatively easy implementation of the cancer vaccine approach means that it stands out as an attractive method to harness the immune system for cancer treatment. Other benefits of cancer vaccines are their low toxicity and high specificity when compared to conventional treatments, such as chemotherapy and radiotherapy. Current developments in cancer vaccines have come from a focus on strategies to provide personalized antigens and to improve the delivery vehicles and adjuvant formulations; developments relating to the latter two are very similar to those seen in the protective vaccine field.

In many autoimmune diseases,[12,15] Treg impairment is a common theme. Reconstitution of Treg functionality was observed to improve prognoses in many relevant animal models.[21] Currently, treatment options for autoimmune diseases are limited to non-specific immunosuppressors, increasing the risk of infections or cancers.[20] The development of therapeutic vaccines that induce disease-specific Treg for the suppression of autoimmunity may revolutionize the treatment of autoimmune diseases.[67]

Tolerance induction through immunization has long been known to be within the realm of possibility, as the immunization of animals with an overdosed amount of antigens has led to antigen-specific immune tolerance instead of resistance.[68] Under the overdosing condition, thymic tissue may acquire and subsequently present the injected antigen for Treg induction. This is generally regarded as the mechanism underlying the phenomenon. However, immunization under overdose conditions is not suitable for clinical applications for obvious safety and practical reasons.

An alternative approach utilizes the tolerogenic properties of skin Langerhans cells to induce immune tolerance.[67,69] This involves the injection of MHC-restricted peptides of self-antigens into the dermal layer, where Langerhans cells are located, but the random ignition of autoimmunity in clinical trials suggests that these dermal APCs may adopt non-tolerogenic phenotypes, making the strategy less reliable.

Several approaches for obtaining therapeutic vaccines against autoimmune diseases, including nanoparticle formulated ones, are under active development.[70] Inherently, the ability of nanoparticles to simultaneously carry multiple types of cargo allows for the co-delivery of immunosuppressive drugs to reliably ensure a tolerogenic APC profile. To that end, the co-delivery of self-antigens with clinically available immunosuppressive drugs[71] and preclinical tolerogenic signalling pathway ligands[72] has resulted in the antigen-specific suppression of autoimmunity.

During homeostasis, interactions between T cells and dendritic cells, which present antigens with subthreshold co-stimulation, lead to T cell anergy against self-antigens.[41] More recently, it has been demonstrated that nanoparticles decorated with MHC I-peptide[51] and MHC II-peptide complexes[50] can mimic the dendritic cells that present antigens with subthreshold co-stimulation. This provides a novel option for the induction of immune tolerance.

## Key steps in the vaccination pathway: the advantages and challenges relating to nanoparticles

There are a few key steps in the vaccination pathway that leads to the induction of $CD4^+$ and $CD8^+$ T cell responses. It begins with the capture of antigens by immature dendritic cells. Dendritic cells are generated in the bone marrow from haematopoietic stem cells. They then enter the circulation and arrive at tissue,[73] searching for antigens.[34] The processing of pathogen- and damage-associated molecular signals captured alongside the antigens leads to their maturation and the migration of the dendritic cell to the lymph nodes. There, dendritic cells interact with T cells to present antigens together with co-stimulatory factors and release cytokines for T cell activation (Fig. 3).

### The capture of antigens by DCs

Common mechanisms of antigen uptake by DCs involve phagocytosis[74] and macropinocytosis.[75] These are approaches taken by many immune cells, including DCs, macrophages and neutrophils.[73] Both mechanisms play important roles in the clearing of microbes by macrophages and neutrophils, and in the collection of antigens by dendritic cells. Macropinocytosis involves the encircling of the surrounding liquid by protrusions on the cell surface, forming micron-sized macropinosomes.[74] The major uptake pathway for freely dissolved proteins or peptide antigens is macropinocytosis.[75] It has been found that small nanoparticles with a size range of a few tens of nanometers can also take this endocytic pathway to enter immune cells.[76] For larger particles, such as microbes and cell debris, internalization mainly involves the deposition

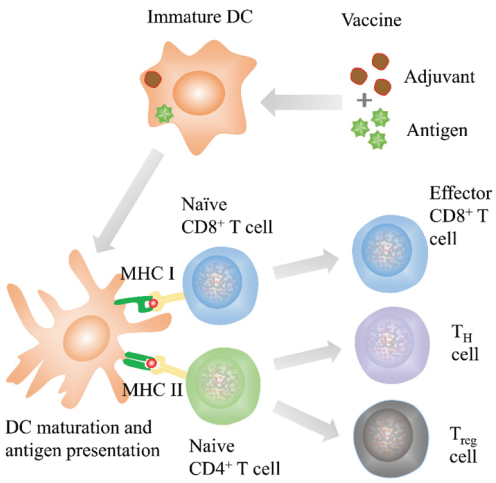


Fig. 3 A schematic illustration of the key steps in the vaccination pathway.

of the cell membrane along the boundary of the target, eventually forming a phagosome that closely encircles and internalizes the target.[74]

More efficient cellular uptake is reported to occur in the case of nanoparticle-carried antigens[77] (compared to small-molecule based ones). One explanation for this phenomenon is higher affinity between the nanoparticle surface (with specific surface functionalization or charge modulation) and the cell membrane. For example, the uptake rate of naked mRNA with negative charge is less than 1 in 10 000 initial input molecules.[78] The uptake of these antigens can be significantly improved (up to 80–95%) by adopting nanocarriers with positive surface charge.[79] The surface functionalization of nanocarriers with ligands for DC-specific surface receptors, such as CD11c, CD40, and DEC205, was also found to be effective for facilitating their uptake by DCs.[80,81] For instance, nanocarriers modified with anti-DEC-205 antibodies could deliver antigens to DEC-205 positive DCs efficiently.[27] The surface modification of nanoparticles with antibodies, peptides or receptor ligands could simultaneously lead to the targeting and activation of DCs.[6]

A number of receptors have been found to mediate phagocytosis.[74,82] A list of representative phagocytosis receptors is given in Table 2. Receptor targeting has also been found to modulate the processing of antigens in the next key step, leading to favourable outcomes, such as the increased cross-presentation or sustained presentation of antigens.[83] However, the *in vivo* responses to receptor targeting are species-specific, and direct translation from animal studies to humans is frequently not straightforward.[83]

The immense potential and complexity of receptor targeting have been exemplified in two recent studies. In the first study, Conniot and co-workers explored the adoption of mannosylated NPs for cancer vaccine applications.[84] Consistent with previous studies, mannose functionality on nanoparticles leads to DC-targeting *via* mannose receptors, enhancing the delivery of antigens and adjuvants carried by the nanoparticles and resulting in a stronger anti-tumor response compared to a non-mannosylated control. However, detailed analysis of the tumor microenvironment revealed that immunization with mannosylated nanoparticles also led to an unforeseen influx of myeloid-derived suppressor cells (MDSCs), which can negatively impact the vaccine efficacy. Consequently, the research team included an MDSC inhibitor, ibrutinib, in the immunization approach and demonstrated that this novel treatment method could allow the optimal application of a mannosylated cancer vaccine. In the second study, the induction of a humoral response by glycosylated HIV antigens in monomeric form or oligomerized into nanoparticles was studied.[85] These antigens target APCs through mannose receptors. Both the monomeric and oligomeric forms induced similar levels of follicular T helper cell response, suggesting similar efficiency for targeting dendritic cells. However, they induced different levels of humoral response. The cause of these differing humoral responses was traced to follicular dendritic cells from draining lymph nodes. This intriguing result suggests that different cell types may have geometrical or size requirements during receptor recognition, allowing for the potential fine tuning of targeting using physical properties.

Table 2 Representative receptors for phagocytosis

| Receptor | Cognate ligand |
|---|---|
| Antibody receptors (*e.g.*, FcRγI and FcRγIIA) | Antibodies aggregated with pathogens and toxins |
| Complement receptors (*e.g.*, CR1, CR3, and CR4) | Activated complements bound to pathogens |
| Lectin receptors (*e.g.*, dectin 1 and CD205) | Polysaccharides on the pathogen surface |
| Toll-like receptors (TLR) | Pathogen-associated TLR ligands |
| Integrins | Miscellaneous; some integrins can bind denatured proteins or plastic surfaces |

The optimum size of nanocarrier for cellular uptake usually varies with material composition and is cell-type dependent. Nanoparticles of ~100 nm are considered the most favorable for cellular uptake by dendritic cells.[86] This size matches those of the natural targets of dendritic cells: viruses and bacteria. Generally speaking, when the size of nanoparticles exceeds 500 nm, they tend to be taken up less efficiently by DCs and ingested mainly by macrophages[7,81]

The morphological features and mechanical properties of NP carriers are also found to affect their cellular uptake efficiencies. Geometrical shape is a highly studied morphological parameter with respect to cellular uptake. As many polymer-based and inorganic-material-based nanoparticles can be synthesized with different sizes and aspect ratios, the effects of geometry on uptake have been evaluated using various cell types.[87–90] While rod-shaped nanoparticles and prolate ellipsoids are phagocytosed similarly to or less favourably than spherical nanoparticles, oblate ellipsoids are more efficiently phagocytosed than spheres.[88–90] Niikura *et al.* compared the uptake efficiencies of spherical, rod-like and cube-like Au NPs by DCs and found that rod-shaped Au NPs had a slightly higher uptake efficiency by DCs. In contrast, cube-like Au NPs were the least efficiently internalized.[91] Generally speaking, a larger contact area between the nanoparticle and plasma membrane likely results in a high NP anchoring probability on the cell membrane; however, the internationalization of NPs also relies on the likelihood of membrane ruffling, which is an energy dependent process.[92,93]

Governed by similar principles, soft deformable nanoparticles result in enhanced uptake efficiencies.[81] In a recent report, Xia *et al.* demonstrated that a soft pliable microparticle made of PLGA-stabilized squalene emulsion delivered 3 times more antigen than that of a solid PLGA microparticle.[94]

Other than phagocytosis, nanoparticle systems are known to enter cells by other endocytosis pathways.[95] It is generally accepted that nanoparticles no larger than 200 nm in size can undergo clathrin-mediated endocytosis.[96] Caveolae-mediated uptake possibly imposes stricter limits on particle size, as caveolae-dependent invagination usually involves particles no

larger than 80 nm.[97] However, discrepancies exist in the literature reports, and these respective sizes could be cell line dependent. Further complications can arise when *in vivo* uptake is considered, as different cell types compete for a limited supply of nanoparticles. It is worth pointing out that certain endocytic pathways, such as the caveolae-mediated pathway, can direct the engulfed cargo to specific intracellular vesicles,[97] possibly modifying the intracellular processing pathway undertaken by the nanoparticles. Consequently, the surface engineering of NPs aimed at specific endocytic pathways may provide new opportunities for vaccine design.

Additional advantages of nanoparticle-based antigen delivery come from differences between the pharmacokinetics of nanoparticles and freely dissolved antigens. Freely dissolved antigens are easily consumed in multiple ways, mainly *via* diffusion into body fluids and circulation and subsequent dilution. In comparison, the bio-distribution of nanoparticles is largely decided by their size. Nanoparticles of <5 nm are known to easily exit circulation *via* the renal system, but those of larger size (20–200 nm) are found to have long circulation times.[98] In circulation, nanoparticles of larger sizes generally have an increased probability of being captured by the mononuclear phagocyte system (MPS), such as by macrophages in the spleen of Kupffer cells in the liver.

Of particular importance for some vaccine applications is the likelihood of nanoparticles becoming enriched in lymph nodes. This is important, as enhanced lymphatic transport allows direct access to lymphoid node-resident dendritic cells, enhancing antigen uptake and presentation, to B cells, enhancing the humoral response,[28] or to T cells, allowing immunomodulation.[50,51] Nanoparticles of 10–100 nm injected into tissue (*i.e.*, *via* intramuscular, intradermal, subcutaneous or intraperitoneal injections) are found to be readily carried across the lymphatic endothelium by interstitial fluid, while being too big to diffuse into the blood circulatory system. These nanoparticles are found to have higher lymph node transport efficiencies[99] (Fig. 4). In one implementation of this effect, 10 nm, tumor antigen-loaded, disc-shaped nanoparticles made of synthetic high density lipoprotein (sHDL) with effective LN-targeted delivery were found to significantly enhance the anti-tumor immune response compared to freely dissolved antigens,[100] which possibly diffused into body fluids and were diluted due to their small size (<2 nm in size).

The retention of antigens by appropriate nanoparticle formulations at an injection site can also significantly enhance antigen uptake and presentation by dendritic cells, forming the basis of an immunogenicity boost in the case of many nanoparticle formulations.[77] In tissue, nanoparticles larger than 200 nm are effectively retained by the extracellular matrix, so their transport to lymph nodes[99] must be aided by dendritic cells; that is to say, they need to be endocytosed by DCs before traveling to the lymph nodes. Retention at the injection site is also one reason why aggregated antigens show higher efficacy than freely dissolved ones.[101]

The successful loading of antigens on nanoparticle carriers is a prerequisite if one wishes to take full advantage of nanoparticle carriers for efficient antigen delivery to DCs. Antigens come in different forms, including proteins/peptides, DNA, and mRNA, with diverse chemical and physical properties. These biological polymers may vary wildly in terms of their physical and chemical properties, as their sequences and compositions can change, making their solo application challenging. Loading onto nanoparticles can provide a versatile platform for the universal handling of different antigens. A summary of the different types of materials used for antigen loading and the associated vaccination effects can be found in Table 3. Protein and peptide antigens can be encapsulated into nanocarriers, conjugated with nanocarriers *via* charge-based or chemical interactions, or absorbed on the surfaces of nanocarriers.[16] DNA and mRNA inherently induce a strong MHC I-mediated $CD8^+$ T cell response,[78,102] which is critical for cancer vaccine efficacy. For these antigens, the protection provided by the nanoparticle carrier is important for antigen delivery *in vivo*; in fact, the usage of nanoparticle vehicles has particularly enabled the application of mRNA antigens for vaccine applications,[61] as mRNA otherwise degrades as a result of RNases in the extracellular space. Loading these polynucleotide-based antigens onto nanocarriers requires extra considerations compared to protein-/peptide-type antigens, as DNA and especially mRNA are chemically labile and cannot survive many harsh chemical/physical manipulation techniques when being loaded onto nanocarriers.[79,103]

Lipid-based nanoparticles, which have already been approved by the FDA for various nanomedical applications, provide a viable solution for the loading of nucleotide antigens.[103,104] These nanoparticle carriers are typically prepared using amphiphilic lipid materials containing polar head groups and nonpolar tails.[105] Positively charged lipids, like DOTAP (1,2-dioleoyl-3-

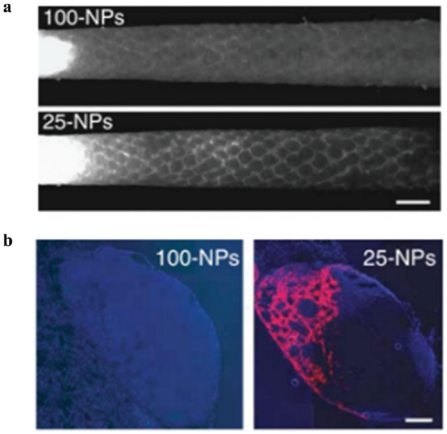


**Fig. 4** Small nanoparticles effectively diffuse across the lymphatic epithelium to reach draining lymph nodes after injection. (a) Fluorescence microlymphangiography images of a mouse tail after the co-injection of fluorophore-labelled 25 nm and 100 nm nanoparticles. The 25 nm NPs enter the lymphatic network more efficiently than the 100 nm NPs. Scale bar: 1 mm. (b) Fluorescence microscopy images of draining lymph nodes. The 25 nm NPs reach the lymph node more efficiently than the 100 nm NPs 24 hours after injection. Scale bar: 200 μm. Reprinted from ref. 28 with permission from Springer Nature, copyright: 2007.

 

Table 3 The classification of nanoparticles used for vaccination

| Type | Representative example | Features | Ref. |
|---|---|---|---|
| Organic based nanoparticles (soft) | Liposomes | Th easy cytosol delivery of DNA/mRNA-based antigens | 2, 104 and 110 |
| | Polymers | The easy loading of multiple cargo types with different forms and functions; self-adjuvanticity in special cases | 111–113 |
| Inorganic based nanoparticles (hard) | Mesoporous silica | Multisite cargo loading; the controllable release of cargo; self-adjuvanticity | 114 |
| | Magnetic nanoparticles | Vaccine delivery together with imaging capabilities (MRI) | 115 and 116 |
| | Gold nanoparticles | Vaccine delivery together with imaging capabilities (CT) | 117 and 118 |

trimethylammonium-propane) and DOTMA (1,2-di-*O*-octadecenyl-3-trimethylammonium propane), are commonly used for the encapsulation of polynucleotide antigens *via* electrostatic interactions. Such encapsulation can be done without the involvement of harsh processes.[2,106] Other carrier options for loading polynucleotide antigens include the use of cationic polymers, which also encapsulate *via* electrostatic attraction.[107,108] For example, polyetherimide (PEI) was used for HIV antigen-encoding mRNA delivery, and a strong systemic and mucosal HIV-specific immune response was reported.[109]

The aforementioned antigen loading strategies are *ex situ* processes; that is, the loading of antigen takes place during the nanoparticle vaccine preparation process (Fig. 5a). Recently, a very different approach has emerged, in which nanocarriers tailored for antigen capturing *in vivo* can be injected without preloaded antigens.[119] These novel types of particle capture tumor antigens released during the immunogenic death of tumor cells (Fig. 5b). A number of therapeutic methods, such as photodynamic/photothermal therapies, radiotherapies, and certain chemotherapies, are now known to cause tumors to undergo immunogenic cell death, releasing tumor-associated antigens.[120] A detailed discussion of the biological processes involved in programmed cell death can be found in a recent review article.[121] Such an *in situ* antigen loading strategy has the merits of being cost-effective and robust compared to conventional cancer vaccines employing *ex situ* loading schemes, as the latter require tumor neoantigen personalization to maximize vaccine efficacy.[122] As such, nanoparticles that can promote immunogenic cell death and effectively catch antigens are of immense research interest.[123–126] For example, Min and co-workers developed PLGA-based antigen-capturing nanoparticles (AC-NPs) that enable the binding of tumor-associated antigens *via* chemical or physical interactions. They showed that these AC-NPs could promote the uptake and presentation of tumor antigens by DCs, resulting in the robust activation of $CD8^+$ T cells.[125] In another example, Wang and co-workers used phospholipid-stabilized micelle nanoparticles to deliver indocyanine green, and induced tumor cell death and antigen release *via* photodynamic therapy. Tumor antigens were captured *in situ* by the maleimide groups on the nanoparticle surfaces, and this approach demonstrated a therapeutic effect against metastatic cancer in a mouse model.[126]

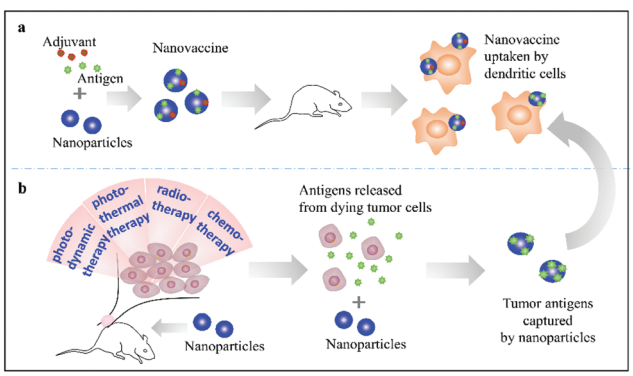


Fig. 5 Loading schemes of nanoparticle formulated vaccines. (a) *Ex situ*: loading pre-prepared antigens (adjuvant) on nanoparticles, followed by their administration to mice, in which the nanovaccine will be captured by dendritic cells. (b) *In situ*: antigens are generated during tumor cell death induced by various therapeutic methods. They are captured by nanoparticles administrated to the mice, forming a nanovaccine that can undergo DC uptake.

## Presentation of antigen by DCs

Antigens acquired by DCs are processed for antigen presentation *via* two different pathways: the MHC II antigen presentation pathway for $CD4^+$ T cell activation; and the MHC I presentation pathway for $CD8^+$ T cell activation. A schematic diagram can be found in Fig. 6 illustrating the major MHC I and II pathways.

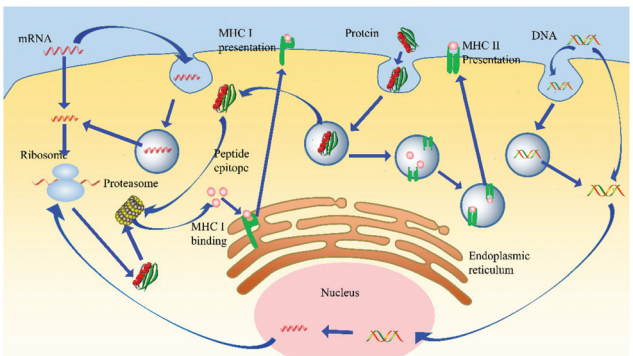


Fig. 6 Different antigen presentation mechanisms; note: the phagosomal cross-presentation pathway, a minor mechanism involving antigens binding to an MHC I complex recycled to phagosomes, is not included in this illustration.

MHC II molecules are exclusively expressed by dendritic cells, macrophages, monocytes, B cells and mucosal epithelial cells; of these, DCs are responsible for migration to lymph nodes for interactions with T cells, leading to the initiation and propagation of an adaptive response.[37] MHC II molecules are synthesized as membrane-bound complexes in the endoplasmic reticulum (ER) with an invariant chain (Ii) that blocks the MHC II peptide-binding site. The MHC II-Ii complex is shipped from the ER, through the Golgi apparatus to the plasma membrane, followed by clathrin-mediated endocytosis back into an antigen-containing endolysosomal compartment for post-translational processing by proteases in the antigen-processing compartment. While the antigens are being digested into peptides fit for presentation, the Ii chain is digested into a short blocking peptide, and its subsequent removal opens up the binding site of MHC II for antigen peptide binding. MHC II acquiring antigen peptides are then shipped to the membrane surface, where presentation to $CD4^+$ T cells occurs.[127]

MHC I molecules are ubiquitously expressed by all nucleated cells in mammalian bodies. The MHC I pathway mainly presents peptides derived from intracellular antigens (cytosolic and membrane proteins) that can self-originate or be introduced extrinsically by vectors such as viruses or nanoparticle DNA/mRNA carriers. Peptide segments of antigens are obtained through the digestion of the respective protein antigen by cytosolic proteasome. These peptide segments are then translocated to the endoplasmic reticulum (ER) lumen by transporters associated with antigen processing (TAP) to bind with MHC I,[128] which is on the luminal side of the ER. Alternatively, a cross-presentation pathway allows for the presentation of endocytosed antigens on MHC I molecules. This involves the transport of protein antigens from the *endo*-lysosomal compartment to the cytosolic space.[129] Another minor cross-presentation pathway involves antigen processing in membrane-bound vesicles, such as phagosomes, using the same antigen processing enzymes of the MHC II pathway, and binding with MHC I complexes recycled to these vesicles. Resulting MHC I-peptide complexes are then transported to the plasma membrane for antigen presentation, in a fashion that resembles the MHC II pathway.[17] Cross-presentation is mainly performed by the $CD8^+$ DC subset.[130]

Common nanoparticle carrier systems enter cells *via* endocytic pathways; a direct consequence of this is that they reside in membrane-bound vesicles (*i.e.*, endolysosomes) destined for antigen processing and MHC II presentation. Combined with the impact of tissue retention and enhanced cellular uptake discussed above, nanoparticle formulations have the potential to enhance the MHC II presentation of protein or peptide antigens for $CD4^+$ T cell activation.

On the contrary, for antigens delivered in the form of mRNA, requiring translocation into cytosol (transfection) for translation into proteins, the MHC I pathway is the predominant mode of antigen presentation (the details will be elaborated on below). MHC II presentation enhancement has become an important issue related to mRNA antigens. It has recently been achieved using sequence level modifications that induce ER translocation and the subsequent localization of the translated antigens in the antigen processing compartment for MHC II presentation. One prominent example of an ER translocation signal is the MITD signal sequence developed by the Sahin group.[2,131]

When mRNA or DNA antigens are used, their translation to encoded proteins in the cytosol of dendritic cells is a first requisite for subsequent antigen presentation (Fig. 6). This requires the transfection of a polynucleotide from the extracellular space into the cytosol. In the case of DNA, it must also enter the nucleus to be transcribed into mRNA.[132] This nuclear transport of DNA occurs efficiently only during mitosis, when the disintegration of the nuclear envelope and the reforming of the nucleus allows for the passing of the antigen into the nucleus.[133] Otherwise, complex strategies, such as chemically conjugating a signaling peptide containing the nuclear localization signal to the DNA, have to be used to enhance the nuclear transport efficiency.[134] The use of viral transfection vectors may enhance nuclear transport, but this may also induce life-threatening immune pathologies or increase the risk of cancer.[135] As DCs are non-dividing *in vivo*, the transport of DNA from the cytoplasm to the nucleus remains a daunting challenge for DNA-based vaccine development.

Compared to DNA, mRNA antigens work directly in the cytoplasm without the need for nuclear entry.[78] A number of nanoparticle-based strategies have been developed to enable the cytosolic delivery of mRNA.[79,103] One strategy employs the proton sponge effect, which enables the vesicle escape of endocytosed nanoparticles, for cytosolic delivery.[108] This involves a positively charged carrier that can absorb protons during the acidification of endosomes, resulting in osmotic swelling and the subsequent disruption of endosomes. Polyetherimide (PEI)[109] and poly-β-amino ester (PBAE)[136] are common examples of material systems that enable proton sponge effects.

Another strategy for the cytosolic delivery of polynucleotide antigens involves the use of cationic lipid-based carriers. Electrostatic interactions between the cationic lipid and anionic endosomal membrane can cause the disruption of the vesicle membrane and thus allow for the intracellular delivery of the carried cargo.[103,137] Alternatively, cationic lipid-based carriers can be formulated to fuse directly with the plasma membrane, releasing the polynucleotide cargo into the cytosol across the cell surface.[138,139] Cationic lipids, such as lipofectamine, are commercially available, and their high RNA transfection efficiencies are well documented.[140] However, several studies have shown that cationic lipid-based delivery systems are associated with *in vivo* toxicity. Several methods have been used to reduce the toxicity of cationic lipids. One is to reduce the ratio of cationic lipid in the nanoparticle formulation by adding neutrally charged helper lipids, such as 1,2-dioleoyl-*sn*-*glycero*-3-phosphoethanolamine (DOPE).[110,141] Helper lipids also play an important role in the endosomal escape process.[110] Another method to reduce toxicity is to replace strongly cationic lipids with weakly cationic lipids, like 1,2-dioleoyl-3-dimethyl-ammonium-propane (DODAP).[142] Coating cat-

 

ionic lipid nanoparticles with a layer of hyaluronan has also been found to reduce *in vivo* toxicity.[143]

Decorating cationic lipids on the surfaces of other nanoparticles has been found to enable cytosolic delivery. Kim *et al.* coated fusogenic liposomes onto the surface of porous silicon NPs (pSiNPs).[144] They demonstrated that the fusogenic pSiNPs underwent fusogenic uptake to reach the cytoplasm directly, whereas non-fusogenic pSiNPs underwent endocytosis and then localized in the cytoplasm. As such, the fusogenic pathway for direct cytoplasmic release attained higher transfection efficiency than the endosomal uptake route. This delivery strategy avoids the endocytic pathway and prevents mRNA antigen degradation in endosomes.

As the cytosolic delivery of proteins and peptides is a key step in cross-presentation (Fig. 6), nanoparticle formulations that enable cytosolic delivery through endosomal escape or membrane fusion can be utilized to increase the MHC I presentation of protein and peptide antigens. Similar to the cytosolic delivery of mRNA, cationic lipids[145] and polymers[146] have been applied to improve the MHC I presentation of protein and peptide antigens. Another established strategy is to adjust the degradability of nanoparticle carriers. On the cellular level, adjusting the degradability of nanoparticles can lead to antigens being directed to different endolysosomal compartments, skewing antigen presentation towards the MHC I or MHC II pathway.[147] Animal studies have shown increased efficacy in cancer and vaccinia virus models.[100,111,148,149]

The induction of the $CD8^+$ T cell response required for vaccination against cancer and viral infections is an important topic. To that end, various strategies developed over the years have been devoted to improving the MHC I presentation of nanoparticle-carried antigens. These include the use of MHC I-restricted peptides and mRNA antigens, and cross-presentation enhancement strategies (including receptor targeting and the tuning of nanoparticle properties, as discussed above). Currently, none of the above-mentioned strategies have shown a definitive edge over the others. As each of the strategies is inherently tailored for a specific type of nanoparticle design, these strategies may remain open options in the foreseeable future to allow for different nanovaccine designs to achieve useful $CD8^+$ T cell responses.

### Dendritic cell maturation

Immature DCs perform interstitial patrols while actively sampling the environment for antigens.[34] Their migration to the lymph nodes, where T cells are located, for antigen presentation is enabled by a switch to a mature phenotype. This phenotype change, unique to dendritic cells among the phagocytes, is called maturation.

Immature dendritic cells can switch to a mature phenotype in the presence of proinflammatory cytokines,[10,75] pattern recognition receptor (PRR) ligands,[23] immune-stimulating exosomes,[150] or complement[31]- or immunoglobulin[151]-decorated particles. Signalling cascades induced by these stimulants activate transcription factor NF-κB, which leads to phenotype changes, including a lowering of phagocytotic capacity due to the redistribution of cellular resources for migration,[34] a loss of surface adhesiveness,[152] and the expression of C–C chemokine receptor (CCR7) for lymph node directed haptotaxis.[34] These result in the active migration of mature dendritic cells from tissue into the lymphatic system. Furthermore, NF-κB-mediated maturation leads to the upregulation of MHC II synthesis and the redistribution of MHC II complexes from the endolysosomal compartment to the plasma membrane, enhancing MHC II presentation to $CD4^+$ T cells.[127] Also accompanying these steps in the maturation process are the expression of co-stimulatory molecules on the plasma membrane[130] and an increase in the cytokine production capacity,[73] both of which are essential to the formation of $CD4^+$ and $CD8^+$ T cell responses and memories.

In the steady state, immature dendritic cells undergo maturation through spontaneous Ικκβ transcription factor activation. Ικκβ-mediated maturation leads to the lymph node migration of dendritic cells, promoting peripheral tolerance towards self-antigens.[153] Thus, the induction of dendritic cell maturation through NF-κB, but not Ικκβ, activation is essential for the efficacy of protective and therapeutic cancer vaccines. For therapeutic cancer vaccines, the stimulation of dendritic cell maturation is particularly important, as co-stimulatory molecules on dendritic cells can reactivate T cells exhausted by chronic exposure to tumor antigens.[41]

Using nanoparticles that co-deliver PRR ligands with antigens is a popular strategy for inducing antigen-presenting DC maturation. Most common PRR ligands developed for vaccine applications have been shown to work well with nanoparticle formulations, improving immune responses and vaccine efficacies. These include the TLR3 ligand polyinosine–polycytidylic acid (poly(I:C)),[154] the TLR4 ligand monophosphoryl lipid A (MPLA),[56] the TLR7/8 ligand imiquimod[56] and TLR9 ligand CpG oligonucleotides.[100] Synergism allowing the co-delivery of multiple PRR ligands using a single nanoparticle has been reported.[56] In designing nanoparticle carriers for PRR ligands, one should pay attention to the idea that different members of the PRR family reside in different cellular compartments.[23,155] This means that nanoparticle designs need to cater for specific delivery requirements, such as intracellular delivery for nucleotide-binding oligomerization domain-like receptor (NLR) ligands, cell surface delivery for TLR4 ligands, and endosomal release for TLR3, 7/8 and 9 ligands. Furthermore, geometrical requirements, especially size requirements, for the optimal stimulation of endosomal receptors, such as TLR9,[25,26] should be considered during particle design.

On the other hand, multi-modality nanoparticle formulations can be acquired in different manners. Some nanoparticles have inherent adjuvanticity, due to their intrinsic chemical/physical parameters (Table 3). A number of nanoparticle systems are known to intrinsically stimulate an inflammasome response.[5] These are generally particles with sharp and pointy edges, such as alum[156] and various crystalline particulate materials, which can destabilize the phagosomal membrane.[157] In fact, alum is commonly used as an adjuvant

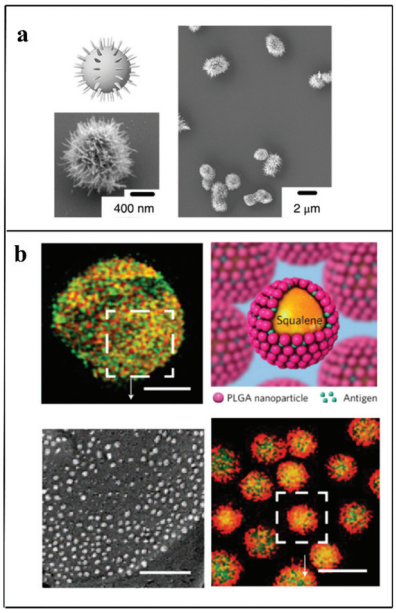


Fig. 7 (a) The morphology of spiky titanium oxide nanoparticles that induce an inflammasome response. Top left panel: a schematic diagram of a spiky titanium oxide nanoparticle; bottom left panel: a scanning electron micrograph of the nanoparticle under high magnification; right panel: a scanning electron micrograph of the nanoparticle under low magnification. (b) The morphology of pliable nanoparticles. Bottom right panel: a confocal microscopy image of the nanoparticles; scale bar, 5 μm; top left panel: a structured illumination microscopy image of a single particle; scale bar, 1 μm; green, squalene core, DiO-labelled; red, PLGA nanoparticles, Cy5-labelled; bottom left panel: a cryo-scanning electron microscopy image of a magnified section of the nanoparticle; scale bar, 500 nm; top right panel: a schematic diagram of the nanoparticle composition, made of a squalene core stabilized by a Pickering emulsion of PLGA nanoparticles. Fig. 7a is reprinted from ref. 158 with permission from Springer Nature, copyright: 2018. Fig. 7b is reprinted from ref. 94 with permission from Springer Nature, copyright: 2018.

for vaccines on the market,[62] although its nanoparticle morphology has not been consciously emphasized. Titanium oxide-based particles with spiky surfaces (Fig. 7a) have also been shown to stimulate an inflammasome response, trigger DC maturation, and enhance vaccine effects with respect to influenza protection and tumor reduction.[158]

Recently, mechanical force is recognized as an important regulator of cell signalling in the immune system.[82] In accordance with this, deformability is considered another contributor to the adjuvant effect. The effects of nanoparticle deformability on the immune response were tested when Xia *et al.* designed pliable microparticles, based on a PLGA nanoparticle-stabilized Pickering emulsion system, for loading antigens[94] (Fig. 7b). The enhanced immune response was partially ascribed to DC maturation stimulated by the pliable particles compared to a non-pliable control.

Adjuvanticity may also come from the specific chemistry of nanoparticle surfaces.[28,113,159] For example, Luo *et al.* found that cationic polymer-based nanoparticles functionalized with the azepanyl group could stimulate stimulator of interferon genes (STING) family intracellular receptors, resulting in simultaneous cytosolic antigen delivery and dendritic cell activation.[113] The same STING activation strategy using heterocyclic amine ligands has also been applied to lipid-based nanoparticles for mRNA delivery to enhance anti-tumor immunity.[159]

It should be emphasized that naïve $CD4^+$ T cells differentiate into effector T helper cells if and only if dendritic cells present their cognate antigens together with signals driven by pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMP) or cytokines. If no such signals are provided, naïve $CD4^+$ T cells consider the antigen self-derived and go into an anergic state.[15] This is one of the major loopholes that pathogens[58] and tumor cells[14] employ to achieve immune system evasion.

### Cytotoxic T cell and T helper cell activation

In the case of antigens presented on DCs *via* MHC I and II pathways, and simultaneous signaling *via* co-stimulatory factor and cytokine secretion, the ultimate goal is communication between DCs and cytotoxic T cells or T helper cells, which can lead to the activation of different T cell types.

Cytotoxic or $CD8^+$ T cells recognize antigenic peptides presented on MHC I complexes for activation. They induce the death of infected or tumorous cells using secreted perforin and granzymes, or cell surface CD95L in the FAS-mediated cell death pathway.[42] The assistance of $CD8^+$ T cells by $CD4^+$ T helper cells is required for the optimal activation of cytotoxic T cells.[160] Nevertheless, it has also been reported that the activation of DCs by appropriate PRR ligands, such as poly(I:C), can sufficiently activate CD8 T cells in the absence of $CD4^+$ T helper cells.[37]

$CD4^+$ T helper (TH) cells are activated by the MHC II presentation of the cognate antigen. They can be further subdivided into 3 well-characterized subtypes, each specializing in defense against a specific set of pathogens. Their main features are listed in Table 4. After MHC II activation, the differentiation (or polarization) of naïve $CD4^+$ T cells into different TH cell subtypes is determined by the cytokine and co-stimulatory molecule profile supplied by DCs. Table 4 lists the infectious agents (stimuli of DCs and the innate immune system) that drive TH cell polarization into each corresponding subtype. Also listed are the PRR subsets involved and the cytokines that stimulate the polarization process. Each TH cell subtype then produces a characteristic set of effector cytokines, which act on a series of accessory cells to bring about an appropriate immune response against the infectious agent.

The central coordinator functions carried out by TH cells are best demonstrated using the antiviral TH1 response as an example.[37] Viral infections drive the polarization of $CD4^+$ T cells into the TH1 subtype. The process starts with the detection of viral RNA and DNA by the PRRs (*e.g.* TLR3, 7/8 or 9) of dendritic cells. Upon recognition of these viral PAMPs, dendritic cells are activated for maturation. The process of maturation is augmented by type I interferons secreted by infected

 

Table 4 A list of features of currently recognized T helper cell subtypes

| TH subtype | Stimulus | Major PRRs | Cytokines driving polarization | Effector cytokines | Main accessory cells | Ref. |
|---|---|---|---|---|---|---|
| TH1 | Viruses, bacteria | TLR3, 7, 8, 9 | IL-12 | IFNγ | NK cells, macrophages, and neutrophils | 37 |
| TH2 | Parasites, allergens | TLR2, TLR4, NLRs | IL-4 | IL-4, IL-5, IL-13 | Basophils, eosinophils, and mast cells | 8 |
| TH17 | Bacteria, fungi | Dectins | IL-6 and TGFβ | IL-17 | Macrophages and neutrophils | 9 and 10 |

tissue cells. This leads to dendritic cell upregulation of co-stimulatory factors and the release of IL-12 during antigen presentation to naïve $CD4^+$ T cells. IL-12 drives the differentiation of activated $CD4^+$ T cells to adopt TH1 polarization. TH1 cells then produce IFNγ, which propagates a series of antiviral responses: on infected cells, IFNγ drives the upregulation of the MHC I presentation pathway, facilitating the recognition and killing of infected cells by cytotoxic T cells. It also induces the expression of antiviral genes to suppress viral replication in infected cells. IFNγ enhances the generation of antibodies by B cells for viral neutralization. Furthermore, IFNγ promotes NK cell activity to assist in the clearance of infected cells and acts on macrophages to facilitate the clearance of infectious cell debris. IFNγ also induces signaling by epithelial cells to recruit neutrophils. This general theme of the TH cell coordination of the immune response through cytokine secretion is well conserved in TH2 polarization against parasitic infections and TH17 polarization against bacterial infections.

Apart from cytokines, co-stimulatory molecules on mature dendritic cells are known to play important roles in the activation of $CD4^+$ T helper cells.[41] The co-stimulatory molecules CD48, CD58, CD70, CD80, CD86 and LIGHT promote the activation of naïve $CD4^+$ T cells into effector T helper cells. CD70 and LIGHT are important for inducing TH1 polarization, while SLAM, TIM and CD30L stimulate TH2 polarization. At the end of their life spans, a portion of effector T cells differentiates into memory cells, which provide recall (or secondary) responses during subsequent antigen encounters.[40] The strength of the recall response and the length of time that the memory survives are generally linked to the strengths of cytokine and co-stimulatory molecule stimulation.[62]

The volume and surface area associated with any nanoparticle system provide a natural platform for the co-delivery of antigens and various forms of molecular adjuvants, such as TLR ligands, which in recent decades have been found to be a critical component for generating a potent immune response and long lasting memory.[62] The advantage of antigen and molecular ligand co-delivery by nanoparticles is the spatial and temporal overlap of arrival at the target dendritic cells, allowing for simultaneous processing and associated T cell activation.

Without nanoparticle carriers, the co-delivery of molecular ligands with antigens is usually achieved *via* chemically conjugating molecular ligands to antigens.[161] While this method has its own merit, nanoparticle formulations of the same molecular ligands and antigens can deliver similar results[162] but with greater flexibility for fine-tuning formulations through adjusting the nanoparticle parameters and ligand compositions.

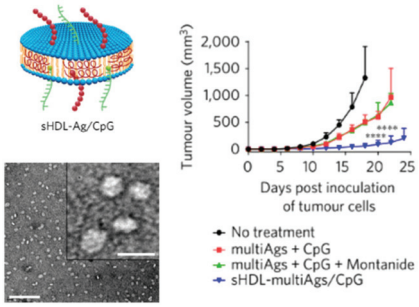


Fig. 8 The co-delivery of antigens (Ags) and CpG adjuvant on synthetic high-density lipoprotein (sHDL) nanodiscs induced strong tumor suppression. Top left panel: a schematic diagram showing the preparation and structure of the nanodisc nanoparticles; sHDL, high density lipoprotein; bottom left panel: the transmission electron microscopy imaging of a nanoparticle showing the nanodisc-like morphology; scale bar, 100 nm; right panel: animal immunization with the sHDL-multiAgs/CpG nanodiscs strongly suppressed tumor growth compared to no treatment and a physical mixture of antigens and adjuvants. This figure is reproduced from ref. 100 with permission from Springer Nature, copyright: 2016.

Indeed, co-delivery by nanoparticle carriers can result in a significantly improved immune response[5] due to the spatial and temporal overlap of multiple functionalities. This has been demonstrated in both humoral and cell-mediated immune responses with many different nanoparticle formulations, regardless of the administration route.[25,100,148,149,163,164] For example, the synergism of co-delivered TLR ligands results in a far stronger response than the co-administration of the same ligands,[56] as the latter approach cannot guarantee the simultaneous availability of the TLR ligands at the same location due to different pharmacokinetics. In a recent study, Kuai *et al.* employed synthetic high-density lipoprotein (sHDL) nanodiscs to co-deliver peptide antigens and adjuvant CpG.[100] The nanodisc nanoparticles induced a higher level of antigen specific T cell response than a physical mixture of the same components (Fig. 8).

## Conclusion and future perspectives

Nanotechnology provides useful tools for accessing the fundamental working principles of the immune system in an unpre-

cedented manner, enabling the precise and independent control of material parameters that may have an effect on the immune process. With current trends in immunological research moving toward tackling immune cell interactions from the perspective of mechanical properties[82] and geometrical arrangements[41] at the sub-micron level, nanotechnology will play an increasingly important role in future discoveries in immunology. While rational vaccine development highly relies on a mechanistic understanding on the immune system, the knowledge gained through the application of nanotechnology to immunological studies will no doubt translate into new strategies for vaccine development.

The working mechanism of vaccines requires the incorporation of more than one functionality, that is, both antigen and adjuvant must be present. Both antigens and adjuvants can come in a variety of forms and may prefer specific delivery sites. In this regard, nanoparticles allow for the safe accommodation of multiple functionalities and offer versatile strategies to satisfy specific delivery requirements. The co-delivery of PRR ligands was one of the earliest breakthroughs related to nanoparticle vaccines,[56] and remains an indispensable part of most nanoparticle vaccine implementation strategies. Vaccines with enhanced efficacy have been repeatedly tested and verified. In many cases, such enhancements originate from the safe loading of the cargo (antigen and adjuvant), avoiding their quick degradation in the blood circulatory system, and improved antigen uptake as a result of the interactions between the nanoparticles and DCs. It is worth pointing out that conventional *ex situ* loading strategies largely rely on the availability of effective antigens, the processing of which can be costly. In comparison, recently developed *in situ* antigen loading strategies for cancer vaccines have demonstrated great potential, but the key issues of how to optimize the specific antigen generation and capturing capabilities of the nanoparticle carriers remain open questions. The triggered and/or programmable delivery of the loaded cargo is a unique feature of nanoparticle formulations and has been widely employed in nanomedicine design for a wide range of therapeutic applications. Nevertheless, this strategy has been less explored in the case of nanoparticle formulated vaccines, despite its obvious importance; for example, the temporal order of antigen and adjuvant delivery is critical, as the adjuvant will boost DC maturation and matured DCs no longer prefer antigen uptake.

One of the most studied aspects of nanoparticle formulated vaccines is the adjuvant effects of the nanoparticles themselves. In addition to chemical examples, many physical features of nanoparticles have been found to have strong adjuvant effects. For example, MHC I presentation enhancement *via* nanoparticle decomposability tuning has been applied and verified in various protective and therapeutic models that require a strong cytotoxic T cell response.[100,148,149,163] However, the biological processes behind these immunomodulatory phenomena are currently not fully understood. Nanoparticles with sharp edges that destabilize the endolysosomal structure for inflammasome response initiation are also known to enhance vaccine efficacy in general.[158,165] The mechanisms that underlie the immunomodulation effects of these nanoparticle systems can potentially improve vaccine designs. On the other hand, it is worth pointing out that among the key immunological components of vaccination, the manipulation of MHC II presentation is a largely neglected direction. The application of novel nanoparticle-based strategies may provide long overdue insights into the natural workings of MHC II presentation modulation and open up new ways to enhance responses to existing vaccines.

Humoral immunity is a topic of intense interest because it is a key protective correlate for many vaccines against pathogens. The development of a humoral response is a process that involves multiple steps and cellular components. In terms of the currently known key steps, it involves: (1) the development of follicular T helper cells (TFH); (2) antigen capture and B cell activation; (3) the formation of a germinal center; (4) B cell class switching; and (5) affinity maturation, with the involvement of B cells, TFH cells and follicular dendritic cells (FDCs) in the process. Further discussion of these biological processes can be found in a number of in-depth reviews.[166–168]

The enhancement of the humoral response by nanoparticle formulated antigens is a well-documented and reproducible phenomenon, and a number of criteria have been established for the enhancement of humoral immunity through nanoparticle formulations. These are mentioned above in this article and include: (1) optimal nanoparticle size (~40 nm in diameter); (2) the surface decoration of antigens in a repetitive and geometrical pattern; and (3) the co-delivery of antigens with molecular adjuvants, such as TLR ligands. The underlying mechanisms explaining these criteria on a cellular level, however, are not completely clear. For instance, efficient lymphatic transport is identified as the reason behind the enhancement of the immune response when using small nanoparticles. From experiences related to experimental cancer and viral nanovaccines, we are certain that small, lymph node-targeting nanoparticles can enhance T cell responses through the DC-T cell axis. But are there contributions from interactions between these nanoparticles and B cells, and from those with FDCs? Currently, many relevant observations are obscured by the sensitivity of B cells to geometrical arrangements of antigens on nanoparticle surfaces. Likewise, many experiments involving antigen geometry did not take differences in size into account. To further complicate matters, some studies suggest that the post-translational glycosylation status of antigens can affect the involvement of cell types.[85] Understanding the underpinning mechanisms of NP-enhanced humoral responses could provide insights for the further improvement of nanovaccines and the true rational design of vaccines for humoral immunity. This will pose challenges to future researchers and will likely require more controlled *in vitro* experiments examining the interactions between nanoparticles and the relevant cell types, and *in vivo* models that allow subsets of the key steps to be tested.

While a majority of research has focused on the interactions between nanoparticles and dendritic cells, aimed at

 

simulating real pathogens using nanoparticles, less effort has been paid to modulating the communication between DCs and T cells. In this regard, the application of artificial antigen presenting cells to immunological studies and nanovaccine development is particularly interesting. Nevertheless, current aAPC models are confined to the interactions between surface receptors, such as MHC–TCR, and costimulatory/coinhibitory interactions. Yet native DC-T cell communication also involves soluble factors (*e.g.*, DC-released cytokines). Combining current aAPC models with nanoparticles that can release signalling molecules in a controlled manner may simulate DC-T cell communication with closer approximation and yield a greater understanding of the biological processes involved.

The future outlook of aAPC research will very much rely on concerted input from molecular biologists and materials scientists. As the construction of aAPCs requires microgram amounts of proteins with high purity and activity, the way forward – moving past commercially available MHC–TCR and various surface factor-binding antibodies – requires the preparation of the myriad surface signaling molecules found on DCs and T cells by molecular biologists.[41] A detailed discussion about preparing membrane proteins can be found elsewhere.[169] At the same time, the devising by materials scientists of nanoparticle models that can act as substrates for specific geometrical arrangements of surface proteins or allow controllable mechanical properties will allow for the testing of observations and speculations related to the geometrical and mechanical requirements of immune interactions. With aAPCs, *in vivo* communications between DCs and T cell could be directly and systematically studied, paving the way for more efficient vaccine development beyond the knowledge of initial antigen-DC communication approaches.

## Conflicts of interest

There are no conflicts to declare.

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