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Review Article

Nanotechnology-based manipulation of dendritic cells for enhanced immunotherapy strategies

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Abstract

Dendritic cells (DCs) are potent antigen-presenting cells capable of initiating a primary immune response and possess the ability to activate T cells and stimulate the growth and differentiation of B cells. DCs provide a direct connection between innate and adaptive immune response, and arise from bone marrow precursors that are present in immature forms in peripheral tissues, where they are prepared to capture antigens. DCs migrate from the peripheral tissues to the closest lymph nodes through afferent lymphatic vessels to present the foreign antigens, stimulating T-cell activation and initiating a cellular immune response. Moreover, it is known that DCs have an important role in various diseases and conditions involving the immune system, particularly in cancer and autoimmune disorders. For these reasons, targeting nanoparticles (NPs) to DCs provides a promising strategy for developing an efficient balanced and protective immune response. NPs can modulate the immune response and might be potentially useful as effective vaccine adjuvants for infectious disease and cancer therapy. The objective of this review is to present the latest advances in NP delivery methods targeting DCs, the mechanisms of action, potential effects, and therapeutic results of these systems and their future applications, such as improved vaccination strategies, cancer immunotherapy, and immunomodulatory treatments.

From the Clinical Editor: Dendritic cells (DCs) are potent antigen-presenting cells capable of initiating a primary immune response and activating T and B cells. The role of DC-s can be considered as a bridge between innate and adaptive immunity. Targeting nanoparticles (NPs) to DCs can modulate the immune response and might be useful as vaccine adjuvants in infectious disease and cancer therapy.
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Key words: Antigen-presenting cells; Dendritic cells; Nanoparticles; Immunotherapy

Studies on the biological consequences of new nanomaterials suited for biomedical applications are important, particularly those related to the immune system, one of the essential organizing signaling networks involved in health and disease.[1] Therefore, the interactions of nanoparticles (NPs) with the immune system and its potential effects and implications are key questions that must be answered to take full advantage of such approaches. For this reason, before explaining the variety of interplays, mechanisms of action, effects and implications of NP-based interactions with dendritic cells (DCs), we will start with a brief introduction about the functions of the immune system and its cellular components, paying special attention to the important role of DCs.

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The immune system is a dynamic network of cells that must recognize and deal with antigens—foreign substances that stimulate antibody generation—and/or highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated microbial patterns. These processes involve adaptive (also called acquired or humoral) events that take place on the surface of the antigen-presenting cells (APCs), which display foreign antigens coupled with the major histocompatibility complex (MHC). This is recognized by T-cells using their T-cell receptor (TCR) complexes. Also, the recognition by APCs of pathogen-associated microbial patterns elicits an early innate immune response. The immune system contains three types of APCs: macrophages, DCs, and B cells. APCs collaborate with the innate response by cytokine communication, which steers the differentiation of T-helper (Th) cells into Th1 or Th2 subsets.[2] These T cells activate other cells of the immune system such as cytotoxic T cells and macrophages.[3]

DCs connect innate and adaptive immune responses and have particular functions at different stages of maturation. Nonactivated DCs are present in peripheral tissues and are considered

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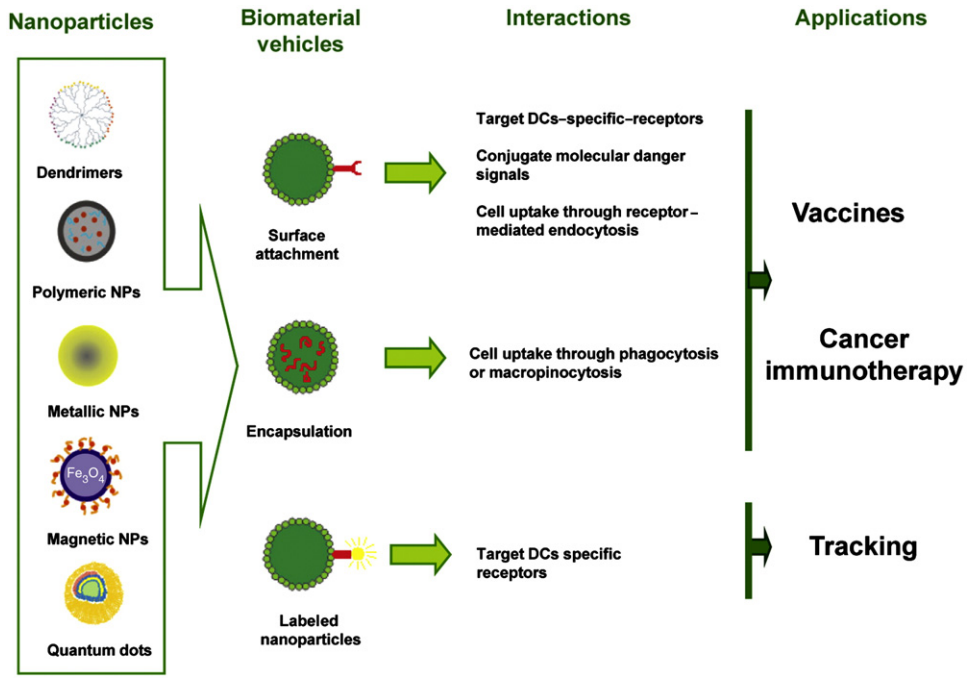

Figure 1. Dendritic cells (DCs) and nanoparticle (NPs) targeting. The use of multifunctional nanosystems based on NPs (dendrimers, polymeric NPs, metallic NPs, magnetic NPs, and quantum dots) affords convergence of technologies (surface attachment, encapsulation, or labeled NPs) for simultaneous or sequential target-specific delivery of multiple drugs, antigens, or tracking molecules to DCs. The interactions involve the targeting of DC-specific receptors, and uptake of NPs through receptor-mediated endocytosis, phagocytosis, or macropinocytosis, so as to achieve a specific desired response in applications in the fields of vaccine research or cancer immunotherapy, where DCs are known to be key immune-based regulators.

immature cells that capture self-antigens.[4] When the antigen is processed and finally the antigen-loaded DC matures and moves to the secondary lymphoid organs, it induces antigen-specific immunity. Here, they present the antigen to T cells, initiating T cell–mediated immunity by intracellular signaling mediated by the MHC and co-stimulatory molecules.[5]

In this review we will summarize the capabilities of engineered NPs in the modulation of DCs and the latest advances in targeting DCs. Particular attention will be paid to the potential clinical usefulness of NPs in this context, as well as their efficacy in infections and cancer. NPs can modulate cellular and humoral immune responses and would be potentially useful as effective vaccine adjuvants for the therapy of infectious diseases and cancer.[6] Through a critical analysis of the current state of knowledge, we will elucidate the latest data concerning the biological effects of NPs on DCs and the therapeutic results aimed at the development of future DC-based applications, such as vaccines, cell tracking, and immunotherapies.

DCs as a target for nanotechnology applications

A wide variety of NPs can be designed with particular properties (certain size, shape, and composition) to be used as vehicles for specific DC targeting. There are different strategies to use NPs as effective vehicles, such as NPs conjugated to antigens, which are recognized by specific receptors; antigens encapsulated within NPs, which offer the ability to protect the antigen from degradation; and labeled NPs, which are also recognized by specific receptors and permit an effective tracking of their migration (Figure 1). These different strategies can be exploited for a variety of applications such as cancer immunotherapy and vaccines that manipulate the immune system for therapeutic benefits and minimize adverse effects. Tracking is also an important application that can be used to evaluate the correct migration of the NP to its target. Figure 1 summarizes the applications of DCs in immunotherapy that can be further exploited by the interactions with different engineered NPs, particularly dendrimers, polymeric NPs, metallic NPs, magnetic NPs, and quantum dots. Although there is no accepted international definition for NPs, it is generally assumed that NPs are particles having one or more structural features on a scale of less than 100 nm that cause their properties to be different from that of the bulk material. All NPs shown in Figure 1 developed for DCs manipulation are included in such a category. The electronic and optical properties and the chemical reactivity of small clusters are completely different from the better known property of each component in bulk or on extended surfaces. This is because a NP has a large surface area in relation to its size and is consequently highly reactive. For this reason the appropriate NP must be designed to be used as an effective vehicle that interacts correctly with DCs, so as to finally develop a desired response.

Mechanisms of action mediated by the interaction between DCs and NPs

NPs conjugated with groups that permit specific recognition of DCs allow a more precise localization of these cells. This

active targeting is based on the use of a wide variety of ligands such as antibodies, polysaccharides, peptides, and drugs that bind to a protein, for example a cell surface receptor. Another way to target DCs is by cellular uptake, capturing the NPs via endocytocis or pinocytosis.[7,8]

An efficient delivery avoiding cytotoxicity during passage through the DC membrane must be arranged to reach the intracellular compartment. For this reason, various biomaterials such as liposomes, polymeric NPs, and quantum dots, among others, are being used to target specifically DCs and finally accelerate their maturation. One type of NPs used as antigen delivery platforms are dendrimers, which are polymers arranged with repeated branched molecules. Dendrimers are a fine example of nanoscale engineering and hierarchical self-assembly. Unlike linear polymers, dendrimers are monodisperse macromolecules. Also, in opposition to the classical polymerization process, which is usually random in nature and produces molecules of different sizes, the size and molecular mass of dendrimers can be specifically controlled during synthesis. Dendrimers display improved physical (including optical and electrical) and chemical properties when compared to traditional linear polymers; in particular, dendrimer solutions have lower viscosity, high miscibility, specific rheological properties, and form a tightly packed ball.

In the field of DCs, a novel mannosylated dendrimer has been demonstrated to have a strong binding avidity by mannose-binding receptors. After binding to DCs, this mannosylated dendrimer ovalbumin was localized in lysosomal compartments subsequently taken up by DCs.[9] Therefore, other bioconjugation strategies can be applied, so dendrimers can act as nanoscale carrier molecules targeting DCs.

Precisely defined and engineered molecules with specifically designed functions can be attached to NPs and activate DCs Toll-like receptors (TLRs). Toll-like receptors are a type of pattern recognition receptors that recognize molecules present in pathogens and tumors.[10] An example of this strategy can be highlighted in the case of polyethylenimine-based small interfering RNA nanocomplexes (siRNA-PEI NPs), which activate specific TLRs expressed by tumor-associated DCs.[11] Other NPs are designed to avoid trafficking to degradative lysosomes, which are one of the most important cell barriers for effective gene transfer. Therefore, a high transfection efficiency can be achieved with NPs such as polymer nucleic acid complexes,[12] used to prevent degradation. Moreover, most NPs are trapped in endosomes by receptor-mediated endocytosis. For example, p24-coated NPs (HIV p24 protein, on the surface of surfactant-free anionic D,L-lactic acid NPs) induced a potent antibody response and accumulated in endosomal compartments, where some of their membranes were degraded but did not disrupt the nanoparticle structure.[13] Another example of an antigen carrier trapped in endosomes is the methoxypolyethylene glycol and poly(lactide-coglycolic acid) (PEG-PLGA) NP, which encapsulates a recombinant hepatitis B antigen able to react with an anti-HBs antibody.[14] Magnetic NPs have also been demonstrated by transmission electron microscopy images and confocal microscopy to internalize DCs and localize mainly in the lysosomal compartment.[15] Other properties of the NPs have to be kept in mind; for example, the size can have a significant effect on the endocytic process. This has been investigated with magnetic NPs, and it has been concluded that endocytosis is not size-selective within a size range of 10-200 nm.[15] Other data about size-selective targeting of DCs with polystyrene NPs have been reported to show that large particles (500-2000 nm) traffic to the lymph nodes in a DC-dependent manner, whereas small NPs (20-200 nm) and virus-like particles drain freely to the lymph nodes, where they are taken up by lymph node–resident DCs and macrophages.[16]

An alternative strategy to specifically activate DCs can be carried out indirectly, by interacting with other cell types. This is the case of natural killer T (NKT) cells, which display potent antiviral and antimetastasis function after activation of DCs.[17,18] For this reason, α-galactosylceramide (a glycolipid antigen presented by CD1d receptors on NKT cells)-functionalized NPs are an attractive DC-mediated immunomodulatory strategy. The NKT cells, thus stimulated by TCR signaling, may finally activate DCs by upregulating co-stimulatory molecules and intracellular trafficking via the MHC class I pathway.[19]

The effect of NPs on DCs

An extensive study of the diverse effects produced by the activation of DCs is needed for the development of new nanotechnology applications. The effects produced by NPs on the maturation of DCs have been examined by studying the expression of co-stimulatory molecules and receptors such as MHC class I and II molecules, the production of cytokines and chemokines, and downstream signaling pathways. Although the amount of information is scarce, we explain here the variety of effects produced by various NPs and various conditions, describing in vitro and in vivo studies.

Most of the comprehensive studies have been done with polymeric NPs. The cargo substance is dissolved, entrapped, encapsulated, or attached to NPs and, depending upon the method of preparation, NPs, nanospheres, or nanocapsules can be obtained. Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a polymer membrane, whereas nanospheres are matrix systems in which the drug is physically and uniformly dispersed (Figure 1). Recently, polymers have been designed primarily for medical applications, in particular in the field of controlled release of bioactive agents. Many of these materials are designed to degrade within the body, such as as poly(γ-glutamic acid) NPs (γ-PGA-NPs) or PLGA NPs. As biodegradable polymers, they avoid the safety problem of elimination from the human body. The effects of these NPs on the maturation of mice spleen DCs were examined.[12] The spleen is a secondary lymph node that contains lymphoid-resident DCs that develop in situ and stay there as immature cells, unless they encounter a pathogen. After treatment of these DCs with γ-PGA-NPs, a dose-dependent increased expression of co-stimulatory molecules such as CD40, CD80, CD86, and MHC class I was observed. The results also showed detectable levels of inflammatory cytokines and chemokines such as tumor necrosis factor-α, interleukin-6 (IL-6), RANTES, and macrophage inflammatory protein-1α (MIP-1α) after γ-PGA-NPs treatment and indicate that γ-PGA biodegradable NPs can induce the

Table 1
Modulation of DCs co-stimulatory molecules, soluble mediators, and receptors after treatment with different NPs

NPs	Size (nm)	Cell type	Co-stimulatory molecules	Soluble mediators	Receptors involved
Poly(γ-glutamic acid)	~210	Spleen DCs	CD40 CD80 CD86	TNF-α IL-6 RANTES MIP-1α	MHC I
Carbon black	14–56	Bone marrow DCs	DEC205 CD80 CD86		MHC II
Mannosylated dendrimers ovalbumin		Bone marrow DCs	CD40 CD80 CD86		
Poly(d,l-lactide-co-glycolide)	500–1000	Bone marrow DCs		IL-2	
siRNA nanocomplexes	40–60	Tumor-associated DCs	CD80		MHC I MHC II TLRs

DCs, dendritic cells; IL-1, IL-6, interleukin-1 and interleukin-6; MHC I, II, major histocompatibility complex classes I and II; MIP-1α, macrophage inflammatory protein-1α; NPs, nanoparticles; TLRs, Toll-like receptors; TNF-α, tumor necrosis factor-α.
NPs can modulate DCs co-stimulatory molecules, soluble mediators, and receptors depending on their composition, size, and shape. As shown in the table, most NPs increase the expression of co-stimulatory molecules such as CD40, CD80, and CD86. It has also been detected that different NPs modulate soluble mediators such as TNF-α, IL-6, RANTES, MIP-1α and IL-2, and upregulate receptors MHC I, MHC II, and TLRs.

maturation of spleen DCs. The signaling pathways involved in the maturation of spleen DCs by γ-PGA-NPs were also studied, and it was suggested that the activation could be induced through turning on nuclear factor–κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways.[20] NF-κB activation begins with the phosphorylation, ubiquitination, and degradation of the inhibitory IκB complex in the proteasome. For this reason the activation of NF-κB was determined by IκB degradation, demonstrating a significant IκB degradation. The activation of the MAPK signaling pathway was studied by examining phosphorylated forms of p38, SAPK/JNK, and ERK of the MAPK pathway, and the results demonstrated by western blot that these molecules appeared in a dose-dependent manner.

The activation of bone marrow DCs (BMDCs) with other NPs has also been investigated. The expression of cell surface molecules on BMDCs exposed to carbon black NPs[21] and mannosylated dendrimers ovalbumin[9] showed a marked increase in DEC205 and CD86, and also tended to increase the expression of MHC class II and CD80 surface molecules expression. In comparison with carbon black NPs, mannosylated dendrimers ovalbumin NPs moderately stimulated the upregulation of CD40, CD80, and CD86. The effect of carbon black NPs on allogeneic T-cell stimulating capacity of BMDCs was also studied, and the results show a considerable proliferation of T cells, increased by the addition of BMDCs.[21]

These examples show how different NPs can activate immature DCs (derived from spleen and bone marrow) by increasing the expression of co-stimulatory molecules and other receptors involved, but other strategies can be developed to modulate DCs. For example, NPs can also be useful by enhancing the efficiency of presentation by DCs. NPs are loaded with specific antigens, which are recognized by DCs that subsequently mature. For this reason a biodegradable polymer, PLGA, was generated to deliver antigen using the MHC class I antigen presentation pathway. Mouse BMDCs were incubated in the presence of PLGA-encapsulated ovalbumin, and one of the effects observed was the stimulation of T-cell IL-2 secretion and an increase of ovalbumin that was released from endosomes into the cytoplasm, increasing the access of exogenous antigen to the MHC class I loading pathway and finally enhancing the antigen presentation by MHC class I.[22] Similar effects have been reported in vivo with recombinant hepatitis B surface antigen encapsulated with PEG-PLGA co-polymers NPs. In this study BALB/cNCr mice were used for the delivery of encapsulated antigen, which resulted in a much more efficient uptake of soluble antigen. Therefore, the concentrations of serum antibodies were higher when compared with mice that received a nonencapsulated antigen treatment, most probably because of a slow release of the encapsulated antigen, thus facilitating a better access and a prolonged delivery to the immune system.[14]

Other in vivo studies have been made with siRNA-PEI NPs. siRNAs are potent sequence-selective inhibitors of transcription that are introduced into cells and mediate post-transcriptional gene silencing of a specific target protein.[23] The expression of CD 80 and of MHC classes I and II on tumor-associated DCs from mice bearing ID8-Defb29/Vegf-A tumors (a model for ovarian cancer research) was analyzed by flow cytometry, and the results indicated that this siRNA nanocomplex was enough to activate tumor-reactive lymphocytes and exert direct tumoricidal activity driven by siRNA-PEI NPs that had been DC-modified.[11] This study also demonstrated that the NPs uptake stimulated multiple TLRs that were part of the mechanism of action to transform tumor-resident DCs into potent APCs with tumoricidal activity.[11]

As described before, NPs can activate DCs by binding to them specifically and stimulating the expression of co-stimulatory molecules, soluble mediators, and receptors (see Table 1). But NPs can also be loaded with specific antigens and work as APCs, enhancing the efficiency of presentation by DCs. Moreover, for targeting tumors, DC can be designed, with siRNA to downregulate a specific gene and at the same time enhance their APC capacity and tumoricidal activity. Finally, in

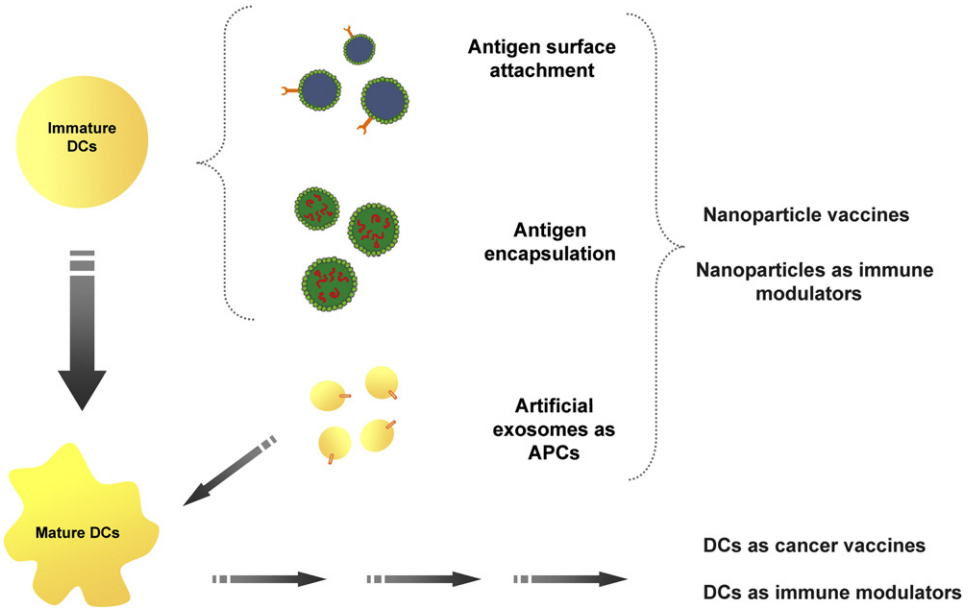

Figure 2. Modulation of dendritic cells (DCs) by different nanoparticles (NPs)-based strategies for immunotherapy applications. When a DC is fully mature, it provides to the rest of the immune system the information about the tumor contained in the NPs functionalized with antigen surface attachment or encapsulation, in particular to cytotoxic T lymphocytes. Exosomes are nanovesicles released by different cell types including DCs. Nanoengineered artificial exosomes acting as artificial antigen-presenting cells (APCs) are a very promising approach to established mature DCs for tumor-associated immunosuppression and tumor immunosurveillance.

the near future the synthesis and characterization of polymeric, metal, and magnetic NPs including hydrophobic quantum dots will allow the modulation and study of DCs by conjugated polymer–quantum dot composites. The coupling of these two active components produces composite materials with unique optical properties that hold potential to display, detect, and deliver multiple inputs to DCs.

Nano-engineered exosomes to exploit DC-mediated immune intervention

Exosomes are vesicles released by different cell types, particularly APCs such as DCs, B cells, and macrophages. They are enriched in MHC class I and II molecules and co-stimulatory molecules, and are considered an alternative pathway of antigen delivery and presentation. The use of exosomes engineered to modulate the immune system against tumor antigens is a potent application for cancer immunotherapy.[24] DC-derived exosomes are able to mediate and modulate immune responses in vivo by semi-direct T-cell activation, and it has been reported that exosomes are also secreted by living tumor cells that could contain and transfer tumor antigens to DCs.[25,26] Using nanotechnology, artificial exosomes (30-100 nm in diameter) have been designed by coating liposomes with an optimized amount of MHC class I and peptide complexes and a smart range of ligands for adhesion, early activation, late activation, and survival of TCRs simulating a DC-derived exosome. These artificial exosomes can be traced in vitro and in vivo by fluorescent and magnetic resonance imaging, and function semi-directly with natural APCs, producing the semi-direct T-cell expansion ex vivo.[25] The expansion of functional antigen-specific T cells by artificial exosomes is a novel system with potential clinical applications for the treatment of autoimmune diseases. Tumor-derived exosomes are also very attractive because of their possibilities to promote an efficient polyepitopic antigen presentation and therefore are very convenient as cancer vaccines. Biochemical and immunological analyses have been done with tumor-derived exosomes. The exosomes released by tumor cell lines in supernatants revealed that most mouse tumor cell lines constitutively release exosomes containing as major components MHC class I molecules, tetraspanins, hsp 70-80 and LAMP 1.[27] Thus, tumor-derived exosomes work as an efficient tumor antigen delivery system that can be used in the future as a platform to expose target molecules containing tumor rejection antigens for T-cell cross priming, relevant for cancer immunotherapy.[26,28]

Immunotherapies: vaccines and oncological research

A variety of NPs have been designed with different properties to target or use DCs for diverse applications by different schemes (Figure 2). Immunotherapy has two main (passive or active) strategies to mobilize the immune system. Passive strategies include targeting tumors by NPs, taking advantage of tumor-hyperpermeable cells, which have enhanced permeability and retention effects. Moreover, active strategies have attempted to stimulate specific antitumor or immune responses with specific antigens to ultimately develop safe applications that permit effective treatment of cancer and immune disorders or diseases.[29] Targeting antigens to APCs such as DCs is an efficient vaccine strategy when NPs are

designed as an antigen carrier and also as a potent adjuvant inducing a specific immune response. The use of vaccines to induce antibodies and neutralize disease-related proteins offers the capability to treat several diseases. Particulate vaccines, because of their particulate nature and dimensions, are taken up by DCs, inducing a T-cell and antibody response. The structural similarity of tailor-made viruslike particles to the original counterparts induces T-cell responses and makes them attractive as vaccine candidates for cancer and chronic antigen-mediated diseases.[30,31] Specific antigens encapsulated by NPs have been used as delivery systems to DCs. This is the case, for example, in recombinant hepatitis B surface antigen vaccines[14] or the p24 human immunodeficiency virus type 1 (HIV-1) protein.[32] Other strategies have been designed to induce a cellular immune response against antigens by using pulsed DCs with antigen-loaded NPs for cell therapy vaccination.[33] These strategies use DCs as a vaccine, generating DCs ex vivo, loading them with selected antigens, and, subsequently administering them to patients. Some studies have already been conducted with this approach. DCs have been loaded with HIV-1 p24 proteins adsorbed on the surface of surfactant-free anionic PLA NPs, and humoral and cellular immune responses were analyzed,[13] observing in CBA/J mice vaccinated with p24-NPs DCs a generation of specific levels of serum IgG and intestinal IgA as well as a specific $CD4^+$ T-cell proliferation in the spleen and mesenteric lymph nodes. This novel delivery tool can also be effective in other immunotherapy approaches, including in cancer therapy. In vitro generation of DCs loaded with tumor-associated antigens has been investigated against human glioblastoma multiforme, an aggressive primary brain tumor. The objective was to achieve a cancer stem cell targeting to enhance the antitumor immunity.[34] In this study, NPs were not used only to load DCs with the antigen but instead to regulate the antigen release into the DCs and to develop a controlled response.

It has been reported that the injection of exosomes derived from DCs presensitized with tumor peptides induced a potent antitumor immune response with a final eradication of established tumors.[35]

At present clinical trials are continuing using DCs for immunotherapy of recurrent cervical cancers. Here, DCs are pulsed with synthetic peptides that represent cytotoxic T-lymphocyte epitopes of human papillomavirus type 16 E7. This study is currently recruiting participants. Other clinical studies in phase I–II are being carried out for a DC vaccine pulsed with multiple peptides for recurrent malignant gliomas. The objective is to determine the safety and induction of the immune response of these vaccinations. (Data were obtained from http://clinicaltrials.gov/, a service of the U.S. National Institutes of Health). Therefore, NPs can contribute to a better design of medical applications by a controlled release of a specific agent with more efficient and specific targeting, affording at the same time the opportunity to track them to obtain information about their biodistribution.

At the moment several research groups are actively trying to combine a variety of functions into NPs as platforms for targeting different DC compartments and to develop diverse strategies to modulate specific immune responses. A major effort toward successful NP-based therapeutics will be needed to avoid extensive and nonspecific immunostimulatory or immunosuppressive reactions to the nanomaterials once administered into the body, and an extensive study of the diverse effects produced by the activation of DCs is needed for the development of new nanotechnology applications. Also, these developments include some degree of risk, and bringing a product to the clinic entails identifying all possible hazards, characterizing and quantifying the associated risks, including probabilities and severities, reducing risks to an acceptable level, balancing any remaining risk against benefit to the patient, and communicating effectively and appropriately the nature of such remaining or "residual" risks. This implies the future development of new or adapted methods appropriate to assess new medicinal products involving NPs. Although many questions still require extensive investigation, the available data suggest that a variety of NPs can be engineered to become part of the next generation of vaccines and immunomodulatory platforms.

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