

Dendritic cell-based nanovaccines for cancer immunotherapy

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Cancer immunotherapy critically relies on the efficient presentation of tumor antigens to T-cells to elicit a potent anti-tumor immune response aimed at life-long protection against cancer recurrence. Recent advances in the nanovaccine field have now resulted in formulations that trigger strong anti-tumor responses. Nanovaccines are assemblies that are able to present tumor antigens and appropriate immune-stimulatory signals either directly to T-cells or indirectly via antigen-presenting dendritic cells. This review focuses on important aspects of nanovaccine design for dendritic cells, including the synergistic and cytosolic delivery of immunogenic compounds, as well as their passive and active targeting to dendritic cells. In addition, nanoparticles for direct T-cell activation are discussed, addressing features necessary to effectively mimic dendritic cell/T-cell interactions.

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Introduction

Cancer immunotherapy is a promising treatment strategy based on the stimulation of the immune system to attack tumor cells. To generate life-long immunity against tumor cells, priming of tumor-specific cytotoxic effector as well as memory T-cells is essential. Naïve T-cells can be activated by antigen-presenting cells (APCs), in particular dendritic cells (DCs), which can present tumor antigens both on major histocompatibility complex (MHC) class I and class II proteins for interaction with cytotoxic CD8⁺ and helper CD4⁺ T-cells, respectively [1]. Nowadays, several DC subsets have been identified each with distinct antigen processing capabilities: CD8α⁺/DEC205⁺ DCs, which can efficiently cross-present antigens on MHC class I as opposed to CD8α[−]/DCIR2⁺ DCs that mainly process antigen onto MHC class II [2,3].

To date, most DC-based tumor immunotherapeutic strategies involve *ex vivo* loading of DCs with tumor-associated antigens and immune-stimulatory agents (adjuvants) and subsequently re-injecting them into the patient for *in vivo* T-cell activation [4]. Alternative approaches are based on the *ex vivo* expansion of tumor antigen-specific T-cell clones that are then adoptively transferred into the patient [5]. However, both techniques require the use of autologous cells, and are therefore labor extensive and costly.

To overcome these drawbacks, a more pharmaceutical approach explored combined nanotechnological and biochemical advances to develop nanovaccines. Nanovaccines are nanoscale complexes that accommodate both antigens and immune stimuli that can activate T-cells or DCs upon their *in vivo* administration (Figure 1) [6].

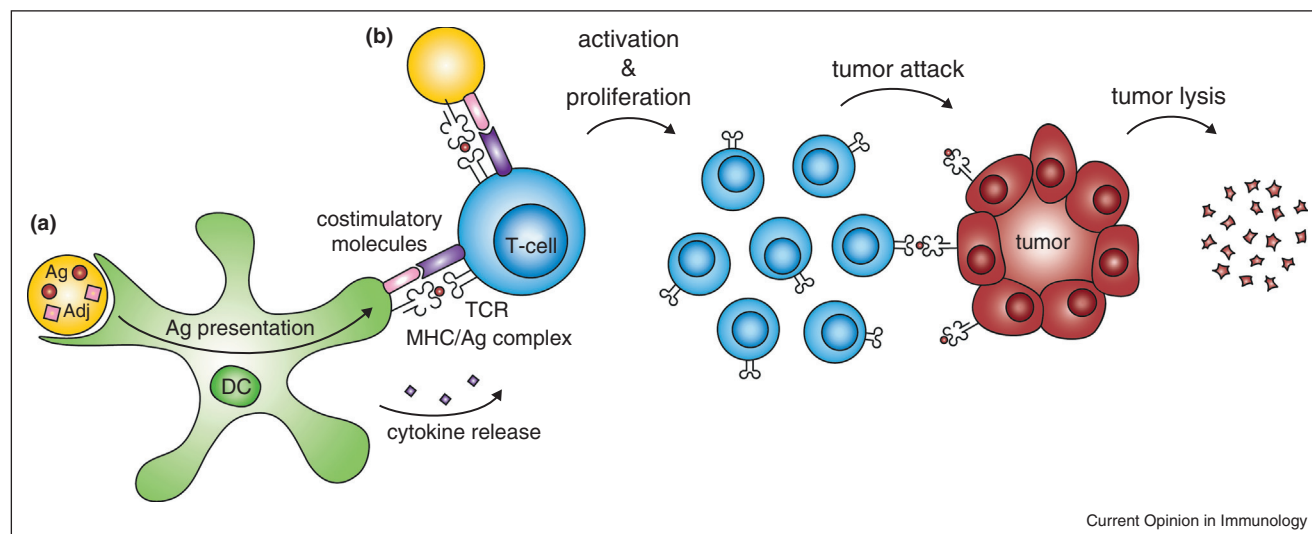
This review focuses on the use of nanovaccines to generate T-cell mediated active anti-tumor immune responses. First, important features of nanovaccines for DC activation will be discussed, including the effect of co-delivery of antigens and adjuvants and their intracellular routing. Next, strategies for passive and active targeting of nanovaccines to DCs will be addressed. Finally, the possibility of exploiting nanovaccines for direct T-cell activation will be discussed: DC-mimics that operate as artificial APCs.

Nanovaccines for co-delivery of antigens and stimulatory molecules

Most tumor-associated antigens explored thus far are endogenous self-antigens with limited immunogenicity. Therefore, to induce tumor immunity rather than tolerance, these antigens should be accompanied by strong adjuvants that boost DC activation, for example, toll-like receptor (TLR) ligands (Figure 1) [7,8]. Importantly, Blander and Medzhitov have shown that it is crucial to deliver antigens and adjuvants into the same intracellular compartment [9].

Nanovaccines are an excellent platform to achieve such synchronized delivery to DCs (Figure 2a). For example, chemical linkage of CpG to ovalbumin (OVA) specifically enhanced the production of cytotoxic T-lymphocytes (CTL) when compared to free OVA and CpG and could inhibit the growth of OVA-expressing tumors in mice [10,11]. Another interesting strategy was reported by Li *et al.*, who exploited the adjuvant nature of aluminum-oxide nanocrystals and decorated these with tumor antigens to generate CTLs capable of eliminating established tumors in mice [12^{*}]. Furthermore, various types of

Figure 1



Nanovaccines for cancer immunotherapy. Nanovaccines (yellow) can be designed to (a) deliver tumor antigens (Ag; red) and adjuvants (Adj; pink) to dendritic cells (DC; green) for antigen processing and subsequent presentation on major histocompatibility complex (MHC) molecules on DCs to the T-cell receptor (TCR) on T-cells (blue) or (b) present tumor antigens directly to T-cells. Antigen presentation in combination with immune-stimulatory molecules (pink) results in tumor-specific T-cell activation and expansion. These T-cells migrate to the tumor (red) and upon tumor antigen recognition, tumor lysis is induced.

adjuvants (MPLA, polyIC, CpG or PAM₂CAG) have been loaded onto antigen-containing polymer-based and lipid-based nanoparticles, resulting in powerful anti-tumor immune responses [13–16]. Interestingly, recent studies showed that the immune response induced by antigen-loaded liposomes could be drastically improved by further processing of liposomes into cross-linked multilamellar vesicles [17]. Although physically linking antigen and adjuvant is thought favorable in order to induce powerful cellular immunity, by contrast Kasturi *et al.* showed that antigen and adjuvant in separate nanocarriers resulted in stronger humoral responses [18].

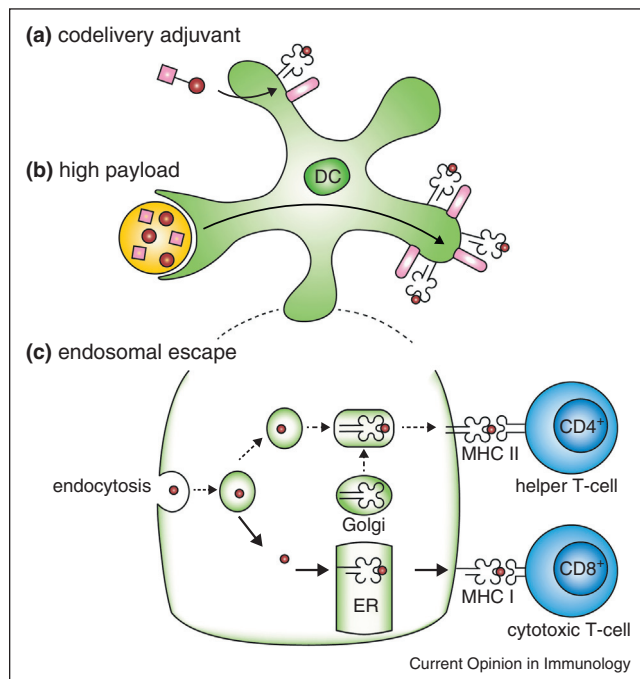
Other attractive characteristics of nanocarrier systems are, first, protection against unwanted antigen degradation or systemic immune activation by soluble adjuvants and, second, the high dose of immunogenic cargo that can be incorporated into a single nanovaccine (Figure 2b). Rettig *et al.* recently demonstrated that, *in vitro*, monocyte activation could be improved by increasing nanocarrier size and thus antigenic payload, indicating that uptake of a few large high-payload particles was sufficient to trigger cells [19]. Furthermore, immunization of mice with either liposomal or poly(lactic-co-glycolic-acid) (PLGA) constructs that can accommodate many TRP2 tumor-antigens per particle provided better protection against tumor growth than free TRP2 at similar doses [13,15]. An additional advantage of using biodegradable polymeric particles, such as PLGA, is the sustained slow release of antigens from the nanocarrier after uptake by DCs [20].

Nanovaccines for improved cytosolic antigen delivery

Following nanovaccine uptake and processing by DCs, tumor-antigens can be presented as MHC class I/II peptide complexes. For effective immunotherapy, antigens should preferably be loaded onto MHC class I in order to prime CD8⁺ T-cells. So far, most nanovaccines developed are internalized via the endocytic pathway, thereby directing antigens to the MHC class II pathway, instead of class I [2,21]. As antigen-MHC class I complex formation takes place in the endoplasmic reticulum (ER), particular emphasis was given to the design of nanovaccines that promote antigen escape from endosomes into the cytosol to improve MHC class I (cross-)presentation (Figure 2c).

Antigens can be encapsulated in virus-like particles, composed of viral envelope proteins [22]. These have preserved their natural ability to fuse with lipid membranes, thereby shuttling antigens from endosomes into the cytosol. However, considering the immunogenic risk of viral constituents, attempts have been made to develop synthetic peptide-based fusogenic particles [23]. Similarly, polymeric nanoparticles based on amphiphilic poly(γ -glutamic acid) were able to promote endosome–ER fusion for enhanced antigen-loading on MHC class I [24].

Furthermore, conventional liposomal vaccines have been modified to facilitate the transport of antigens to the

Figure 2

Strategies to enhance cytotoxic CD8⁺ T-cell priming by dendritic cell (DC)-targeted nanovaccines. **(a)** Nanovaccines that facilitate co-delivery of adjuvants (pink) with tumor antigens (red) to the same cellular compartment improve DC maturation and activation. **(b)** Increasing the immunogenic payload of antigens and adjuvants delivered by a single nanovaccine enhances the DC's immune-stimulating potency. **(c)** Upon uptake of a nanovaccine via endocytosis, nanovaccines that actively promote release of antigens into the cytosol enhance presentation of antigens on major histocompatibility complex (MHC) class I molecules, and therefore priming of CD8⁺ T-cells. Antigens that remain inside endosomes are loaded onto MHC class II molecules resulting in CD4⁺ T-cell priming.

cytosol. The incorporation of cell-penetrating peptides, such as R8, resulted in highly improved MHC class I-mediated T-cell responses [25]. In addition, liposomes have been equipped with pH-responsive moieties, such as cholesteryl hemisuccinate or polyglycidol, which trigger liposome destabilization and promote lipid membrane fusion under mildly acidic conditions [26]. Such liposomes release their contents into the cytosol upon encounter of low pH within endosomes, thus improving MHC class I antigen loading and recognition by CD8⁺ T-cells [26,27].

Passive targeting of nanovaccines to DCs

Another key factor for successful clinical application of nanovaccines is to achieve efficient uptake by DCs. Major populations of DCs are found in lymphoid organs, that is, lymph nodes and spleen, but DCs also occupy peripheral tissues, including skin [28]. As DCs have the natural ability to phagocytose foreign material, passive targeting of DCs can be achieved by directing nanovaccines to sites

rich in DCs. For this, the interplay between nanovaccine size and their route of administration has been explored.

Nanovaccine size has a major impact on its local distribution especially when administered into the skin (Figure 3a). Small nanoparticles (<100 nm) are quickly transported into the lymphatic system by interstitial flow to interact with lymph node resident DCs [29–32]. Importantly, upon arrival in the lymph nodes, small nanocarriers are cleared slower than free antigens, thereby enhancing their interaction time with DCs [30,33]. Larger constructs (>500 nm), instead, are physically trapped in the skin and are predominantly internalized by skin-DCs or monocytes, which subsequently migrate to the lymph nodes [31,32,34]. Nanovaccines of intermediate size (100–500 nm) showed both free and cell-based drainage to the lymph nodes [29,33]. Therefore, the induced immune response critically depends on nanovaccine size, which creates a trade-off between the extent of passive DC targeting and the immunogenic payload delivered per nanoparticle [29]. Yet, especially for larger vaccine carriers, an important fraction remains at the injection site [31,34].

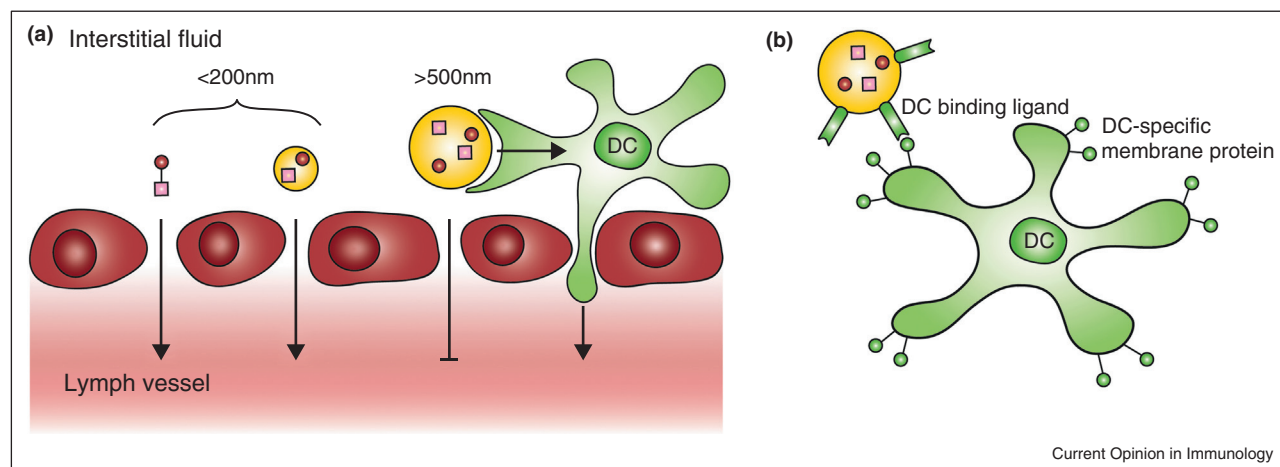
To overcome retention in the skin, direct administration into the lymph node might offer an attractive route to enhance passive DC targeting. Indeed, larger vaccine particles (>300 nm) demonstrated prolonged retention in the lymph node [35]. However, the majority of the nanovaccines was phagocytosed and degraded by macrophages, rendering them unavailable for activation of DCs [33,36]. As an alternative route, injection into the blood is less size dependent and an easy way to reach both blood and splenic DCs. Indeed, although they might suffer from macrophage uptake, strong immune responses were observed by a variety of particles [37,38,39].

Active targeting of nanovaccines to DCs

To improve the targeting of DCs, nanovaccines can be decorated with ligands that specifically bind DC surface receptors (Figure 3b). For example, targeting of tumor-antigen DNA-containing complexes to CD40 or MHC class II on DCs significantly prolonged the survival of mice upon tumor-challenge [40,41]. Similarly, several C-type lectin receptors (CLRs), extensively expressed by DCs, are amongst the most popular targets. Antibodies and ligands have been developed that bind to, for example, the mannose receptor (MR), DEC205, CLEC9A, Langerin and DCIR2 [42].

Mannose-functionalized liposomes that target MRs showed higher uptake by DCs than conventional liposomes thereby enhancing the anti-tumor response [39]. As MRs are also expressed on macrophages and other cell types, more DC-restricted receptors have also been explored. Importantly, specific DC subsets can be targeted by selection of specific CLRs. Idoyaga *et al.* showed

Figure 3



Passive and active targeting of nanovaccines to dendritic cells (DC). **(a)** Passive targeting of nanovaccines to DCs upon intradermal or subcutaneous injection is dependent on nanovaccine size. Nanoparticles up to 200 nm can diffuse from the interstitial fluid across the lymphatic endothelium (red) into lymph vessels. Subsequently nanovaccines are transported to lymph nodes, thereby targeting lymph node resident DCs. Nanoparticles larger than 500 nm cannot traverse the endothelium and are trapped at the injection site. Here, skin-resident DCs (green) can take up the nanoparticles and transport them to the lymph node for antigen presentation to T-cells via dermal DCs. **(b)** Active targeting of nanovaccines to DCs involves functionalization of nanoparticles with ligands or antibodies that bind specifically to DC surface receptors, thereby directing nanovaccine uptake toward DCs.

that, in mice, antigens could be directed to the splenic CD8 α^+ DC subset by antigen conjugation to antibodies against DEC205, Langerin or CLEC9A. By contrast, DCIR2 antibodies appeared to specifically target CD8 α^- DC [43^{••}]. As discussed above, murine CD8 α^+ DCs are specialized in cross-presentation of antigens on MHC class I, which is required for activation of CD8 $^+$ tumor-specific CTLs [2]. Therefore, targeting nanovaccines to CLRs found on CD8 α^+ DCs might be favorable for anti-tumor immunotherapy. Indeed, in mice, DEC205-targeted delivery of tumor-antigen loaded PLGA-particles or liposomes to DCs resulted in T-cell activation at a much lower antigen dose than non-targeted nanoparticles [37[•]], and importantly provided better protection against engraftment of tumor metastasis than control liposomes [44].

In humans, BDCA3 $^+$ DCs are regarded the cross-presenting counterpart of the murine CD8 α^+ DCs [45]. However, several studies also attribute cross-presenting functionality to other human DC subsets, which makes the choice for a particular target cell in the human system difficult [42].

Nanovaccines for direct T-cell activation

An alternative immunotherapeutic approach is the development of nanovaccines aiming at direct T-cell activation instead of via DCs (Figure 1). Mainly, two strategies have been explored to induce direct proliferation and activation of T-cells. One of these strategies is to expand tumor antigen-specific T-cells *ex vivo* after

isolation of lymphocytes from cancer patients. Re-injecting the expanded T-cells back into the patient is thought to boost their anti-cancer activity [46–49]. Alternatively, instead of adoptive transfer of T-cells, nanovaccines have been designed for *in vivo* induction and activation of tumor-specific CTLs [50^{••},51]. In both cases, the complexes are designed to mimic the antigen-presenting and T-cell activating capacity of natural APCs, and are referred to as artificial APCs (aAPCs). Artificial APCs encompass both cell-based and acellular technologies. Some of these are tumor antigen specific, whereas others are nonspecific T-cell ‘amplifiers’ [52–55]. Here, recent advances in aAPC design are summarized.

Cellular aAPC

Cellular aAPCs are generally derived from primary or transformed human cells or xenogeneic cells such as murine fibroblasts or insect cells [52,56[•],57,58]. They are engineered through retroviral or lentiviral transduction to introduce MHC molecules that interact with T-cell receptors (TCR) as well as co-stimulatory molecules [59–61]. Because of their capacity to induce tumor rejection in mice, some human cell-based aAPCs are now explored in clinical trials to treat cancer patients [56[•]].

In spite of extensive progress in the cellular aAPC field, there are major drawbacks that are preventing them from wide spread application. Cellular aAPCs are mainly derived from tumor cell lines or xenogeneic cells. However, the use of cell lines with tumorigenic potential may result in tumor growth originating from the aAPCs. Also,

the use of allogeneic or xenogeneic aAPCs may elicit an immune response against the aAPCs, which would drastically limit their efficacy [52].

Acellular aAPC

Alternative strategies have been explored to overcome these limitations of cellular aAPCs. Polymers, solid beads, liposomes and exosomes have all been used as synthetic scaffolds for the development of acellular or synthetic aAPC [54,62–64]. The advantages of such acellular aAPCs over cellular aAPCs for clinical applications are first, their control over ligand uploading, second, easy and high quality production without any further manipulation, and third, long-term storage without the loss of activity.

The introduction of mono-disperse spherical polymer or solid bead-based aAPCs loaded with T-cell stimulatory ligands has revealed a totally new platform for designing aAPCs [54,64,65]. Bead-based aAPCs have shown to be more efficient than natural APCs in both adoptive and active immunotherapy against cancer [54]. However, the rigid surface of bead aAPCs restricts dynamic movement of the TCR and costimulatory molecules on the T-cell surface occurring during immunological synapse formation at the aAPC/T-cell engagement site [52]. Hence, a major disadvantage of bead aAPCs is surface rigidity, which may reduce their overall efficiency.

Efforts to improve the dynamics of acellular systems resulted in development of lipid-based synthetic aAPCs where T-cell stimulatory ligands were conjugated to liposomes, supported planar membrane structures and exosomes [62,63]. Exploiting lipid bilayer surfaces provides mobility to ligands allowing these aAPCs to more closely mimic natural APC, especially when interacting with T-cells. Interestingly, lipid-based aAPCs have proven more efficient for *in vivo* rather than *in vitro* expansion of T-cells [66,67]. This might be explained by their small size (50–90 nm) and lipid bilayer composition, which facilitate their migration to their sites of action, that is, lymph nodes and tumor zones.

Future perspectives: harnessing synergisms of nanovaccines targeting DCs and T-cells

Recent advances in nanovaccine development have demonstrated their enormous potential for cancer immunotherapy. Different approaches where T-cells were activated either directly or via DCs, were able to elicit strong anti-tumor responses, demonstrating proof of principle and paving the way for clinical translation. Clinically, virus-like particles have been used for the co-delivery of tumor-derived antigens and adjuvants to DCs. Similarly, antigen–antibody constructs targeted to DCs have already been successfully tested in patients [68–70]. In the field of aAPC-design for direct T-cell activation, a panel of human cell-based aAPC has entered clinical trials [56,71].

Furthermore, combining aAPCs for T-cell activation with nanovaccines that target DCs might act synergistically. Administration of aAPCs in cancer patients might induce instantaneous but temporary bulk expansion of T-cells against the specific cancer antigen, causing rapid reduction of the cancer load. Subsequent treatment with a nanovaccine targeting DCs may induce a multifaceted and more long-lasting immune response to further reduce cancer load and to induce memory responses to prevent cancer recurrence. We believe that combining both strategies holds great promise for future immunotherapy to improve the life of cancer patients.

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