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Dendritic Cells: The Tools for Cancer Treatment

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Abstract

During cancer immune editing, the immune system shapes tumor fate in three phases through the activation of innate and adaptive immune mechanisms. After the elimination and equilibrium phase, the escape phase represents the final phase in which immunologically sculpted tumors begin to grow progressively. In this chapter, we will discuss which efforts are made to restore the balance in favor of the immune system making use of dendritic cells (DCs). The first approach is adoptive cell transfer, in which autologous DCs are generated and activated ex vivo. Secondly, we will discuss attempts in which pro-inflammatory or pro-migratory factors are delivered to attract and activate DCs in situ. Both strategies have the general goal to activate and mature DCs able to induce a robust tumor-specific T cell response. In addition, this chapter will discuss the clinical impact of DC-based therapies in cancer treatment focusing on the safety, feasibility, immunological responses, and clinical outcome.

Keywords: dendritic cells, immunotherapy, cancer, cell therapy, in situ vaccination

1. Introduction

The concept of a key role for the immune system in the protection against tumor development is longstanding and was formulated for the first time by Paul Ehrlich. He postulated that the immune system has the ability to suppress the majority of carcinomas [1]. However, the immune system can also promote tumor progression through chronic inflammation, selection of poorly immunogenic variants, and suppression of antitumor immunity. Together, the dual host-protective and tumor-promoting actions of immunity are referred to as cancer immune editing. According to this hypothesis, there are three phases in an immune response against tumors, the elimination, the equilibrium, and the escape phase, referred to as the three Es of cancer immune editing. During

the elimination, phase cells belonging to the innate and adaptive immune system can recognize and kill the tumor cells. When the tumor cells cannot be completely eradicated, the tumor and the immune system achieve a balance. During this equilibrium phase, the immune system controls the tumor cells but cannot eliminate the tumor. In the escape phase, the tumor cells can evade the immune control and develop further toward a tumor with clinical manifestations [2, 3].

DCs play an important role in the interface between innate and adaptive immunities. DCs have the unique capacity to take up antigens and present them in the context of major histocompatibility complex (MHC) I and MHC II in order to activate CD8$^+$ T cells and CD4$^+$ T cells, respectively. In order to be fully activated, T cells must be confronted with different activation signals. The initial interaction between the DC and the T cell, through the MHC and the T cell receptor (TCR), provides the first signal. In addition to MHC–peptide complexes, a costimulatory signal, the so-called signal 2, is required for T cell activation. CD8$^+$ T cells also require cytokine signals (signal 3), produced by macrophages and DCs, at different stages of their activation for the optimal generation of effector and memory populations and for their survival [4, 5]. The absence of signal 2 and/or signal 3 or the presence of immunosuppressive cytokines could either activate T helper 2 cells or elicit immune suppression through the induction and activation of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), or dysfunctional DCs [6]. Tumors can disrupt these signals by increasing the production of immunosuppressive cytokines, by reducing levels of MHC I molecules, and by losing tumor antigens, thereby evading immune responses and eventually escaping immune control.

The development of DC vaccines is based on these properties. A successful therapeutic vaccine is mostly dependent on antigen-specific CD8$^+$ T cells educated to generate cytotoxic T lymphocytes (CTLs) that can directly kill cancer cells, but also a T helper response is important. Antigen-specific helper T cells are needed to sustain the CD8 response and induce longtime memory. The desired properties of vaccine-elicited CD8$^+$ T cells associated with elimination of cancer cells comprise (i) high TCR affinity and T cell avidity, (ii) production of high levels of granzymes and perforin, (iii) trafficking of T cells into the tumor and persistence in the tumor site, and (iv) high proliferation and renewal potential [7]. The components of the immune system necessary for the induction of such effector cells include (i) the presentation of antigen by appropriate antigen-presenting cells (APCs) and (ii) the generation of CD4$^+$ T cells producing cytokines which can help CD8$^+$ T cell proliferation and differentiation [6, 7]. Since DCs play a key role in the recognition and processing of antigens present on the tumor cells and in the induction of T cells capable of eliminating tumor cells, their use in the treatment of cancer is heavily investigated.

In this chapter we will describe different strategies that are explored to strengthen the immune system in the fight against cancer, focusing on the key role for DCs in this process. This chapter can only be a summary due to the constraints of time and space. We refer also to excellent reviews that have recently been published elsewhere [8–10].

2. Ex vivo DC vaccination

In order to generate a DC-based vaccine, autologous DCs (the so-called natural, circulating DC) or monocytes, that are induced to differentiate towards the so-called monocyte-derived

DCs, are isolated from patients by a leukapheresis. In order to fulfill the need for signal 1, these DCs are subsequently loaded ex vivo with tumor-associated antigens (TAAs). Afterward, the DCs are activated with different maturation stimuli, required to give these cells the capacity to provide signals 2 and 3 to the T cells. This ex vivo generated DC vaccine is then reinjected into the patient, in order to activate preexisting tumor-specific T cells and induce de novo antitumor immune responses leading to tumor regression.

Each step in the generation of a DC vaccine (isolation of specific DC subset from the peripheral blood or tumor of the patient, choice of TAA and loading of the DCs, maturation stimuli, and injection route) has an impact on the phenotype of the DCs and their capacity to activate T cells, thereby influencing the efficacy of the therapy. These factors will be discussed in detail below.

2.1. DC subset and source of the DCs

Two main subsets of natural DCs in the blood and lymphoid tissue can be distinguished: the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs). These DC populations can be distinguished based on the differential expression of phenotypical markers, their localization, and specific function. mDCs, also called conventional DCs (cDCs), are characterized by a high expression of MHC II and are efficient in inducing T cell proliferation. This subtype can be further divided into a CD141+/BDCA3+ population, called the cDC1 subset, and the CD1c+/BDCA1+ population, also called the cDC2 subset. Some studies have shown the superior ability of the cDC1 subtype to cross-present exogenous antigens on MHC I molecules, resulting in a robust CD8+ T cell response [11, 12]. The cDC2 subpopulation is the predominant subset present in the blood and has been shown to be a potent CD4+ T cell activator. Due to high expression of TLR3 and TLR8, these cells are able to secrete IL-12, resulting in the polarization of naïve CD4+ T cells to IFN-γ-secreting Th1 cells, a critical step for tumor rejection in many models [13]. IL-12p70 has also been shown to promote natural killer (NK) cell activation, thus contributing to antitumor responses [14]. In contrast, pDCs are characterized by a low MHC II expression and lack of CD11c expression. These cells are important effector cells in immune responses due to their ability to produce high levels of type I interferon. pDCs also express high levels of TLR7 and TLR9 in endosomal compartments enabling them to respond to viral infections [15]. These subtype-specific functions may be harnessed in the design of a DC vaccine in order to elicit potent antitumor responses. Although natural DCs only constitute 1% of peripheral blood mononuclear cells (PBMCs), clinical trials have shown the potential of this approach and the promising clinical efficacy. A study in metastatic melanoma patients used the cDC2 subset or pDC subset isolated from the peripheral blood of the patients and loaded these cells ex vivo with tyrosinase and gp100 peptides. The vaccination approach using the cDC2 subset has been shown to be safe, technically feasible and resulted in a prolonged progression-free survival (12–35 months) in 4 out of 14 patients [16] (NCT01690377, NCT number, or ClinicalTrials.gov identifier). The pDC vaccine has also been shown to be safe and feasible and resulted in the induction of antigen-specific CD4+ and CD8+ T cell responses and a systemic type I IFN signature [17]. The use of natural DCs in the clinic is thus a promising, although challenging, avenue. Currently, several trials are ongoing in different cancer types aiming to compare the effect of using cDCs or pDCs as starting material to generate a DC-based vaccine, including melanoma (NCT02574377) and prostate cancer (PCa) (NCT02692976). Moreover, it has been shown that mDCs and pDCs can work together and

act synergistically, resulting in better antitumor efficacy of the vaccine. For example, a murine in vivo assay demonstrated the superior ability of a vaccine composed of both the mDC and pDC subsets to activate CD8+ T cells and subsequently enhance antitumor responses compared to treatment with only one of the subsets [18]. Also, human mDCs and pDCs are able to cross activate each other. Co-application of DC subsets induced plasma cell differentiation from B cells and secretion of high levels of IFN-γ by peripheral blood lymphocytes and NK cells [19]. Further research is needed to confirm that the combination of different subsets of DCs included in a DC-based vaccine could result in better clinical results in different types of cancer patients compared to vaccines composed of only one subset.

Besides the blood and lymphoid DC subsets, there are two main subsets that have been characterized in the human skin: dermal DCs and epidermal Langerhans cells (LCs). A subpopulation inside the dermal DC population, characterized by the expression of CD14, seems to specialize in developing humoral responses, mainly by inducing naïve CD4+ T cell differentiation into follicular helper T cells. LCs are more efficient in cross-presenting antigens and inducing naïve CD8+ T cell differentiation into effector cytotoxic T lymphocytes [20]. LCs derived from CD34+ hematopoietic stem cells are currently being tested in two phase I clinical trials in melanoma patients and patients with multiple myeloma (NCT01456104, NCT01995708).

The main obstacle to use these naturally occurring DCs to generate a DC-based vaccine is the difficulty to obtain large amounts of cells needed for the vaccine generation. Moreover, it has been shown that DCs isolated from cancer patients are often dysfunctional and impaired in their capacity to process and present TAA and subsequently activate T cells, resulting in the development of anergic T cells. In this regard, the discovery that DCs can be generated starting from PBMCs or CD34+ hematopoietic progenitors allowed the production of clinical grade DCs ex vivo and opened new opportunities in the field of DC-based vaccination in cancer patients. PBMCs can be differentiated toward immature DCs by culturing these cells with GM-CSF and IL-4, while CD34+ progenitors differentiate into DCs in the presence of TNF-α, GM-CSF, and Flt3L. The advantages of these approaches are the ability to overcome DC dysfunction in cancer patients and to obtain large numbers of DCs allowing multiple rounds of vaccination. Although time-consuming and costly, this approach is used in the majority of clinical trials.

2.2. Antigen selection and loading

2.2.1. Types of antigen

TAAs can be divided into overexpressed self-antigens, mutated self-antigens, and tumor-specific antigens. Although they are preferentially expressed by tumor cells, TAAs are oftentimes found in normal tissues. However, their expression differs from that of normal tissues by their higher expression levels in the tumor, by alterations in their protein structure, or by their aberrant subcellular localization within tumor cells. Ideally, antigens included in a cancer vaccine are expressed on the surface of all cancer cells and are immunologically relevant and important in maintaining a malignant phenotype. The first TAA identification was made in the context of melanoma with melanoma antigen family A1 (MAGE-A1) in 1991; its expression is restricted to male germ line cells and trophoblastic cells [21]. MAGE-A1 is a member of a large

gene family, comprising of 25 cancer germ line genes. Cancer-testis antigens (CTA) are one of the most prominent TAAs and include antigens such as the MAGE antigens and NY-ESO-1.

In recent years, with the development of deep sequencing technologies, studies have revealed the presence of antigens resulting from somatic mutations and giving rise to proteins with altered sequence. These mutation-derived antigens, also known as neo-antigens, are tumor-specific and patient-specific. Targeting neo-antigens would overcome self-tolerance, and the induction of low-avidity clones is observed when using non-mutated self-antigens in the vaccine [22, 23]. Since several studies have shown that the mutational load is a predictive biomarker in patients treated with checkpoint inhibitors, adding DC-based vaccines that will directly target neo-antigens during treatment will potentially work synergistically [24–27]. This approach already generated promising results. For instance, a study in stage III melanoma patients, which used DCs pulsed with peptides encoding for neo-antigens after prior treatment with ipilimumab (anti-CTLA-4 antibody), demonstrated that the DC vaccines induced a diverse neo-antigen-specific T cell receptor repertoire (see 2.5. for other examples). The ultimate aim of a DC-based vaccine is not only to induce an immune response against the antigens included in the vaccine but also to result in antigen spreading leading to a broad immune response capable of recognizing the heterologous cancer cell populations present in the tumor environment.

2.2.2. Antigen loading

Several methods have been used to enhance DC loading with TAAs, in order to increase DC vaccine efficacy. DCs can be loaded with peptides, DNA, RNA, or tumor cells to provide them with the TAAs. An overview of the advantages and disadvantages of each method is given in **Table 1**.

2.2.2.1. DC pulsed with peptides

Loading DCs with short peptides (between 8 and 11 amino acids long), predicted to bind with MHC I and resulting in CD8+ T cell responses, is the most common method to load DCs with antigens in the field of DC vaccination. Since DCs loaded with these peptides can only activate CD8+ T cells, an alternative method is to load DCs with long peptides (between 28 and 35 amino acids long). Due to their length, these peptides are presented by DCs through cross-presentation and are thus capable of targeting both CD4+ and CD8+ T cells. A phase II study is currently being conducted assessing the immunogenicity of a vaccine consisting of DCs matured with poly-ICLC and loaded with NY-ESO and MelanA peptides coupled to an adjuvant called Montanide ISA-51 (NCT023334735).

2.2.2.2. DCs pulsed with DNA/RNA

DCs can be genetically modified to produce and express tumor-associated antigens, by introducing DNA or RNA encoding for these TAAs through nonpathogenic viruses, such as adenoviruses, or through electroporation.

mRNA has a short half-life and can be loaded directly on DCs without using vectors or requiring knowledge of the patient's haplotypes. The electroporation method has been shown to be the most efficient way to introduce mRNA into DCs, by temporarily increasing cell permeability

Antigen-loading method	Advantages	Disadvantages
Peptides	• Easy to manufacture • Easy for immune monitoring	• Limited to tumor types with known/identified TAAs • MHC restriction
RNA	• No integration in host genome • Encoding for specific antigen • Different antigens can be encoded by one construct • Possible to encode for maturation agents	• Variable expression • Vulnerable to degradation • Limited to tumor types with known/identified TAAs
DNA	• Antigen presentation in high levels	• Integration in host genome • Limited to tumor types with known/identified TAAs
Tumor lysates	• No need to identify the TAAs in advance • Presentation of multiple epitopes resulting in both CD4+ and CD8+ responses • Prolonged antigen presentation	• The presence of immunologically irrelevant antigens/self-antigens can induce T cell tolerance • Co-delivery of immunosuppressive factors (IL-10, TGF-β) hampering DC maturation and T cell-stimulating capacity • Labor intensive
DC/tumor cell fusion	• No need to identify the TAAs in advance • Presentation of multiple epitopes resulting in both CD4+ and CD8+ responses • Prolonged antigen presentation	• Low fusion efficiency • Limited availability of autologous tumor cells

Table 1. Overview of the advantages and disadvantages of different antigen loading methods used to generate DC-based vaccines.

through application of an electric field, thus facilitating mRNA entry into the cells, without the need for additional agents [28]. A transfection with mRNA enables presentation of multiple antigens, as well as the loading with maturation agents and cytokines simultaneously. Transfection of tumor-derived RNA in DCs was used to treat melanoma patients, and antitumor T cell responses were reported in about 50% of the patients [29]. Besides delivering TAAs to DCs, RNA transfection can also deliver maturation agents to DCs in order to enhance their T cell stimulatory capacity [30], thereby overcoming the need to mature the DCs in vitro through the addition of different maturation stimuli and shortening the time needed to generate the vaccine.

Plasmid DNA transfection provides a more stable gene material, thus offering longer expression time. One of the most efficient ways to transfer DNA into DCs is through nucleofection, a nonviral, electroporation-based method that allows the DNA to directly enter the nucleus [31]. A study showed transfection of tumor DNA into DCs to be feasible and to result in DCs capable of presenting antigens to T cells resulting in the activation of antigen-specific T cells [32]. However, a major drawback to use DNA in the generation of a DC-based vaccine is the risk of genome integration of the DNA. This risk can be circumvented by using mRNA to load the DCs with the desired antigens.

2.2.2.3. DC pulsed with tumor lysates

Generating tumor lysates can be done through multiple freeze–thaw cycles or by irradiating tumor cells with UV rays [33]. These methods have been shown to induce both apoptosis and necroptosis of the tumor cells and result in the release of DAMPs, such as heat-shock proteins (HSP) and high-mobility group box 1 (HMGB1). These factors are known to induce the so-called immunogenic cell death that results in subsequent DC maturation, thereby promoting effective immune responses. DCs, matured with LPS and IFN-γ and subsequently loaded with tumor lysates, were clinically tested in ovarian cancer patients. Vaccination with these DCs resulted in robust IL-12 production and potent CD4$^+$ and CD8$^+$ T cell responses [34]. A phase III study in glioblastoma using DCs loaded with autologous tumor lysates (DC-VAX-L) is currently ongoing (NCT00045968). A pilot clinical trial testing a personalized vaccine was conducted using autologous DCs pulsed with oxidized autologous whole tumor cell lysate. Vaccination induced T cell responses to autologous TAAs and previously unrecognized neo-epitopes, associated with prolonged survival [35].

2.2.2.4. DC/tumor cell fusions

DCs can be fused to tumor cells using polyethylene glycol as a fusogenic agent or by electrofusion. The advantage of this technique is that all the TAAs that are expressed by the tumor can be processed by the DCs and presented in the context of both MHC classes I and II, resulting in the activation of CD4$^+$ and CD8$^+$ T cells [36, 37]. A clinical study in multiple myeloma patients has shown this approach to be safe and feasible and to result in effective T cell responses and disease stabilization in a majority of the patients [38].

The optimal antigen loading method has not yet been identified, and all of the current methods have advantages and disadvantages (see **Table 1**), but it is clear that delivering antigens in the context of both MHC I and MHC II is of critical importance in order to induce a sustained antitumor response.

2.3. DC maturation

In order to induce a potent antigen-specific T cell response, immature ex vivo generated DCs need to be fully matured before their readministration to the patients. The readministration of immature DCs has been shown to induce tolerance instead of immunity against the target antigen(s) [39, 40]. Studies comparing the immunogenicity of immature and mature DCs show that maturation is necessary for the induction of immune responses in patients [41, 42]. Mature DCs are characterized by a high expression of MHC class I and II molecules on their surface, expression of different costimulatory molecules, secretion of pro-inflammatory cytokines and chemokines necessary for T cell activation, and migration toward the draining lymph nodes. The production of inflammatory cytokines by DCs is essential in order to activate CD8$^+$ T cells. The amount of IL-12 produced by DCs was shown to correlate with the induction of strong antitumor responses. Two clinical trials, one in melanoma [33] and one in malignant glioma [42], have shown that high IL-12 concentration derived from DCs was predictive for a favorable clinical outcome. Initially, DC vaccines consisted of DCs generated ex vivo that were loaded with tumor antigens but without the inclusion of a maturation stimulus to activate the DCs. Although safety and feasibility were established, encouraging different research

groups to further explore this strategy, this first generation of DC vaccines actually showed limited success [43]. The lack of maturation was thought to be a major reason for failure. These observations led to great efforts put into developing different types of maturation stimuli that would increase DC-based vaccine efficiency resulting in profound clinical effects. Moreover, the timing of maturation of the DCs seems to be an important factor to consider. Several studies have described "DC exhaustion," characterized by a loss of IL-12 secretion capacity and T cell activation capacity after gaining full maturation [44, 45].

2.3.1. TNF-α, IL-1β, IL-6, and PGE₂ cocktail

In early years the golden standard to maturate DCs ex vivo in the vaccine field was the addition of a cocktail of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, with prostaglandin E_2 to cultures of immature DCs [46]. This cocktail has been shown to increase the expression of MHC I and II molecules, CD40, and CCR7 but on the other hand also failed to stimulate IL-12p70 production. Although PGE_2 has been shown to promote migration of DCs toward lymphoid tissues through the upregulation of CCR7 expression on DCs, some studies have suggested that PGE_2 might be responsible for the lack of IL-12 production and for inducing Tregs and MDSCs, leading to suboptimal immune responses [47, 48]. This prompted a search for alternative maturation cocktails.

A combination of TNF-α, IL-1β, INF-α, IFN-γ, and poly-IC creates so-called α-type 1 polarized DCs, characterized by an improved IL-12p70 production. DCs matured with this cocktail and loaded with glioma-associated antigen were used to treat patients with malignant glioma and resulted in an upregulation of type 1 cytokines and chemokines, including interferon-α and CXCL10. Trials assessing α-type 1 polarized DCs are currently ongoing in several cancer types (NCT02151448, NCT00970203, NCT01876212).

2.3.2. Toll-like receptors

Immune cells are capable of sensing pathogens via pattern recognition receptors such as the Toll-like receptors (TLRs). Activation of TLRs, by different pathogens or danger signals, on DCs induces the activation of different signaling pathways responsible for the upregulation of costimulatory molecules and production of pro-inflammatory cytokines. Many vaccine adjuvants, targeting these receptors, have been developed in the last two decades. Poly-IC is a synthetic TLR3 ligand analog, widely used in clinical trials, including trials with DC vaccines. TLR3 ligand used as an adjuvant increased DC maturation, interferon secretion, and tumor suppression [49–51]. TLR4 recognizes lipopolysaccharide (LPS) structures present on the membrane of Gram-negative bacteria. LPS combined with IFN-γ results in the activation of highly immunogenic DCs, eliciting strong antigen-specific CTL responses and a high IL-12/IL-10 ratio in vitro [52, 53]. A nontoxic derivative of LPS called monophosphoryl lipid A (MPL) is a TLR4 agonist used in many vaccines as a cancer vaccine adjuvant [53–56].

Simultaneous activation of different TLRs has also been shown to mediate synergistic effects on DCs resulting in strong upregulation of IL-12 production, thus enhancing and sustaining a Th1-polarizing capacity. It was shown that DCs matured with poly-IC, a synthetic TLR3 ligand analog and/or R848, are able to produce high levels of IL-12p70. The addition of PGE_2 improved

the migratory capacity of the DCs while maintaining their IL-12p70 production capacity upon T cell encounter in vitro [57]. Synergy induced by TLR combinations is further discussed in [58].

The use of TLR agonists to mature DCs is discussed in detail further in this chapter.

2.3.3. CD40L

CD40L, a member of the TNF superfamily, is expressed on activated T cells and interacts with CD40 present on activated DCs and other APCs. The CD40/CD40L pathway plays a role in T cell priming and differentiation. Furthermore, CD40 engagement with CD40L is necessary for DC activation and results in an upregulation of costimulatory molecules and secretion of cytokines such as IL-12. Delivering CD40L to DCs mimics T cell help and allows DC licensing in order to induce CD8+ cytotoxic T cells. A phase II study using DCs loaded with tumor RNA and CD40L RNA combined with sunitinib (a tyrosine kinase inhibitor) in metastatic renal cell cancer patients showed the vaccine to be well tolerated, and clinical benefits were experienced in 62% of the patients [59]. A phase III trial assessing the overall survival benefit of using this approach is currently ongoing (NCT01582672). DCs matured with a combination of CD40L and IFN-γ, resulting in an enhanced IL-12p70 production, and loaded with gp100 were used to vaccinate melanoma patients. The amount of IL-12p70 was positively correlated with the generation of gp100-specific CD8+ T cells [42].

2.3.4. TriMix

TriMix is a maturation cocktail consisting of mRNA encoding for CD40L and CD70 and a constitutive active form of TLR4. The aim of this mix is to mimic the physiological activation of antigen-presenting cells (APCs). It facilitates (i) DC activation, (ii) DC maturation, and (iii) activation of cytotoxic CD8+ T cells by DCs. The introduction of CD70 provides a costimulatory signal to naïve T cells expressing CD27, thus supporting T cell proliferation. This formula has been shown to result in maturation and increased T cell stimulatory capacity of DCs. The additional benefit is the use of RNA technology to deliver these components simultaneously with the antigen-encoding mRNA, through electroporation, thus avoiding incubation of DCs with different cytokines that can result in exhausted DCs. This mix has been shown to enhance secretion of IL-12 and several other pro-inflammatory cytokines by DCs and to stimulate differentiation of naïve CD4+ T cells into effector Th1 cells [30, 60–62]. A phase I trial in advanced pretreated melanoma patients showed these TriMix DCs to be safe and immunogenic, resulting in a 27% objective response rate [63]. The addition of ipilimumab (an anti-CTLA-4 monoclonal antibody) to the TriMix DCs in a phase II trial in advanced pretreated melanoma patients resulted in a 6-month disease control rate of 51% and overall tumor response rate of 38% [64].

2.4. Optimal delivery route

Importance of the delivery route lays in the ability of the injected DCs to travel to lymph nodes. The lymph nodes are the rendezvous point for DCs and T cells to interact. Several delivery routes have been tested including intradermal, subcutaneous, intravenous, intraperitoneal, intranodal, and intratumoral delivery of ex vivo generated DCs. When injected intradermally,

a majority of the DCs seem to remain at the injection site, with only less than a few percentages of injected cells reaching the draining lymph nodes. Nevertheless, this small amount of DCs was sufficient to induce antigen-specific responses [65]. DCs delivered intratumorally show retention at the tumor site with little migration to the lymph nodes [66]. In a phase I trial with metastatic melanoma patients, autologous peptide-pulsed DC vaccines were injected via different routes (intranodal, intravenous, and intradermal). This study concluded that the intranodal route seems to be superior for T cell sensitization [67]. Furthermore, according to a meta-analysis of clinical trials of DC-based vaccines in pancreatic cancer and renal cell carcinoma, the choice of the administration route seems to influence therapeutic efficacy. Indeed, lymph node-targeting routes (subcutaneous, intradermal, and intranodal) were shown to be superior over the intravenous route [68]. Nevertheless, the intravenous route has been shown to be an effective administration method for DC vaccines, in the context of some pancreatic carcinoma and melanoma trials. Combining several administration routes is an alternative strategy. The combination of intradermal with intravenous delivery of TriMix DCs has shown to elicit durable clinical responses in advanced melanoma patients [63].

2.5. Combination therapy

2.5.1. Checkpoint inhibitors

The expression of different inhibitory immune checkpoints can affect the efficacy of a DC vaccine by hampering tumor-specific T lymphocytes to exert their function. In recent years, several monoclonal antibodies against checkpoint inhibitors have been developed, with the aim to recover T cell cytotoxicity. The best characterized checkpoint receptors are CTLA-4 and PD-1/PD-L1. Clinical evidence in patients with melanoma suggests that combining anti-CTLA-4 monoclonal antibodies with DC therapy is more effective than either agent alone [69]. It was shown that treatment of patients with advanced melanoma with DCs electroporated with mRNA encoding for TriMix and loaded with four melanoma-associated antigens combined with ipilimumab resulted in an overall response rate of 38% and a 6-month disease control rate of 51% [64]. Treatment with DCs loaded with neo-antigens of patients who underwent ipilimumab treatment showed to elicit antigen-specific CD8^{+} T cells [70].

Although this approach showed promising results, anti-CTLA-4 monoclonal antibodies are associated with a high percentage of immune-related toxicity. Alternatively, PD-1 monoclonal antibodies, which inhibit PD-1/PD-L1 interactions on tumor cells, seem to have a more favorable toxicity profile. The combination of PD-1 inhibitors with DC therapy is currently being tested in several clinical trials for several cancer types (NCT01067287, NCT02528682, NCT03152565, NCT03014804, etc.) [8].

2.5.2. Chemotherapy

Antitumor immune responses induced by DC therapy can be further enhanced by endogenous production of immune stimulatory cytokines. Lymphodepleting chemotherapy can create an optimal cytokine environment for expansion of antitumor immune cells (T cells and NK cells), by eliminating negative immune cell populations and lowering tumor burden. The recovery phase following lymphodepletion creates an opportunity to use DC vaccines combined with adoptive T cell therapy. Furthermore, some chemotherapeutic agents have been

shown to induce immunogenic cell death of tumor cells, which can further potentiate anti-tumor immune responses elicited by DC therapy [71]. AML patients treated with autologous AML/DC fusion cells after chemotherapy resulted in a lack of relapse in 72% of the patients, at a median follow-up of 57 months [72]. Other examples of synergy between chemotherapy and DC therapy can be found in [73, 74]. Several clinical trials assessing the combination of DC vaccine with chemotherapy (with or without other interventions) are currently ongoing in several cancer types (NCT00082641, NCT00338377, NCT00617409, NCT02649582, etc.) [8].

2.5.3. Targeted therapy

Increasing the effectiveness of DC vaccines can be achieved by modulating the tumor micro-environment through a decrease in immune suppressive cells at the tumor site (**Figure 1**).

Regulatory T cells (Tregs) play a role in the induction of peripheral tolerance, by downregulating activation of autoreactive T cells. In most tumor types, the presence of Tregs correlates with a poor clinical outcome [75]. Depletion of Tregs can be achieved by using monoclonal antibodies targeting CD25 (IL-2 receptor α chain). The use of daclizumab combined with antigen-pulsed DCs not only in a trial in metastatic cancer patients resulted in the depletion of Tregs but also in a suppression of tumor-specific CTLs (due to the expression of CD25 on both Treg and effector T cells) [76]. Another CD25-targeting strategy is the use of denileukin diftitox (also known as ONTAK), a recombinant IL-2-diphtheria toxin antigen. Although a study showed ONTAK to be able to deplete Tregs, while maintaining antigen-specific CTLs in renal cell carcinoma patients [77], contradictory evidence demonstrates the induction of tolerogenic DCs and depletion of NK cells when using ONTAK [78]. A study assessing the combination of ONTAK with DC therapy has recently been completed in stage III and IV melanoma (NCT00056134), and one trial is currently ongoing in ovarian cancer (NCT00703105). A non-CD25 targeting therapy is the inhibition of indoleamine 2,3-dioxygenase (IDO), an immune regulatory enzyme that supports Treg function. A trial assessing the combination of 1-methyl-D-tryptophan (an IDO inhibitor) with DC therapy in metastatic breast cancer patients has recently been completed (NCT01042535). Other non-CD25 targeting drugs such as sunitinib and dasatinib (tyrosine kinase inhibitors) are known to inhibit Treg activity. The combination of sunitinib with DC therapy is currently being tested in a phase III trial in patients with advanced renal cell carcinoma (NCT01582672).

MDSCs are a heterogeneous population of immature progenitor cells, known to suppress T cell function. MDSCs have been shown to negatively regulate immune responses in cancer [79]. It was shown that MDSCs can impair the activity of DC vaccines, by decreasing the ability of DCs to mature, take up antigens, migrate, and induce IFN-γ production by T cells [80]. The targeting of MDSCs can be done through different ways. Beside the effect of sunitinib on Treg, this tyrosine kinase receptor can also deplete MDSCs. Cyclooxygenase-2 (COX-2) inhibitors were shown to decrease expression of MDSC-attracting chemokine CCL2 and increase expression of CXCL10, thereby promoting attraction of CTLs. A phase II trial is currently assessing the safety and feasibility of α-type 1 polarized DCs with the addition of a COX-2 inhibitor called celecoxib in patients with peritoneal surface malignancies (NCT02151448). Many other therapies can decrease MDSC-mediated immune suppression such as VEGF inhibitors, all-trans retinoic acid, lenalidomide and chemotherapeutic drugs such as gemcitabine. Clinical trials combining these agents with DC based vaccination are ongoing.

2.6. Conclusions

Although it is shown that ex vivo DC vaccination strategies are safe, well tolerated, and capable of inducing tumor antigen-specific immune responses in a substantial number of vaccinated patients, a lot of challenges remain. Generating autologous ex vivo DCs is a time-consuming and labor-intensive process with significant logistic challenges and high production costs [8]. In addition, only specific types of tumor have been studied in the ex vivo setting, mainly due to practical limitations. These include the lack of appropriate tumor antigens or the absence of sufficient tumor material when tumor lysates are used for antigen loading of DCs [81]. Therefore, the in situ modification of DCs represents an attractive alternative strategy.

3. In situ DC vaccination

To overcome the limitations associated with the ex vivo DC manipulation, different strategies to manipulate DC in vivo are currently under investigation. For example, by targeting in vivo
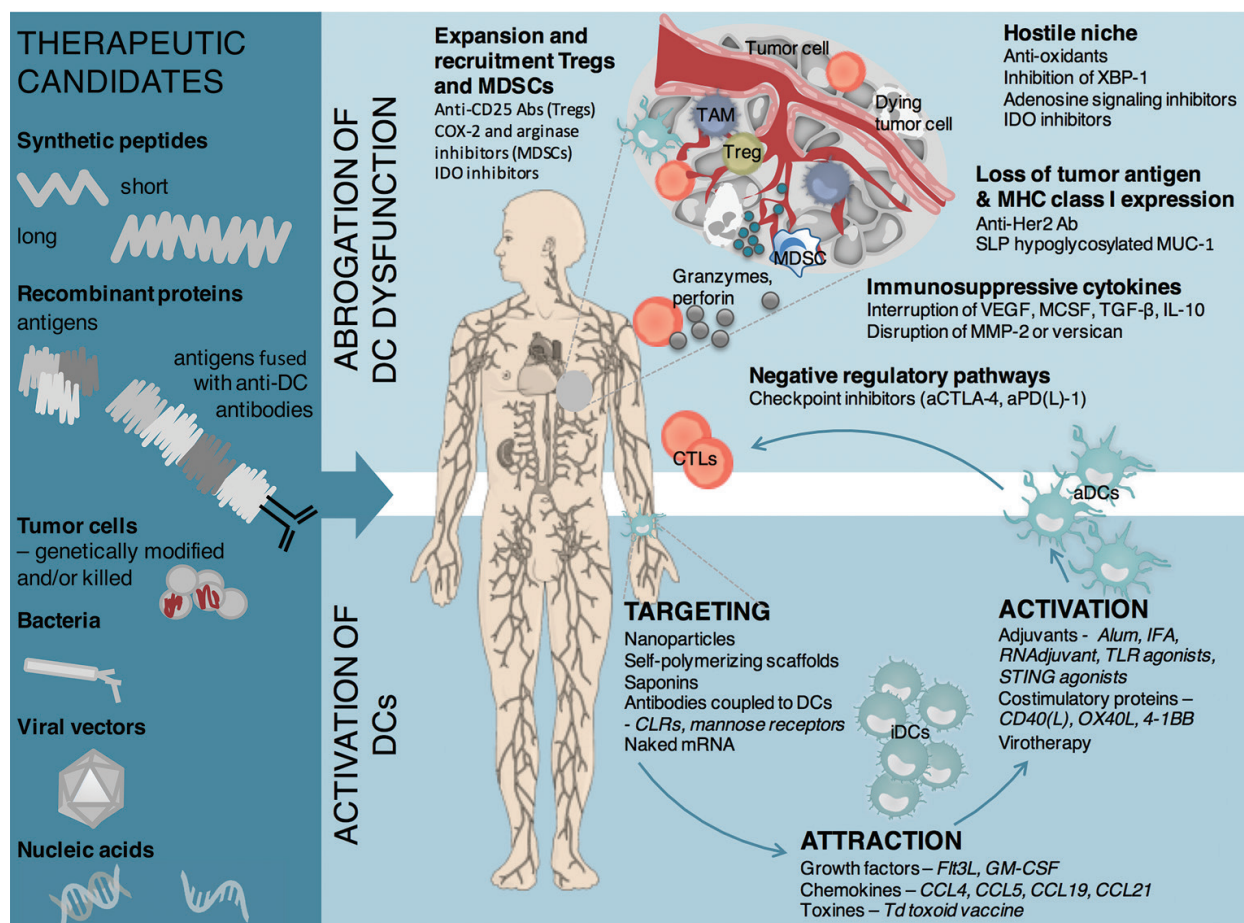

Figure 1. Overview of different therapeutic agents and targets to modulate dendritic cell function in the tumor microenvironment. To abrogate DC dysfunction (upper panel), different therapies can be envisaged to inhibit tumor-promoting characteristics, thereby remodeling the TME, to restore the functionality of the DCs. In the lower panel, it is shown how DCs can be targeted in vivo to deliver DC-activating cargo, to increase the number of DCs, and to promote their capacity to activate effector T cells, leading to the eradication of the tumor nodule (s). Various therapeutic formats can be used to achieve this, and these are listed in the left panel. DC = dendritic cell, aDC = activated DC, iDC = immature DC, Treg = regulatory T cell, MDSC = myeloid-derived suppressor cell, TAM = tumor-associated macrophage.

DCs and exploiting their natural sentinel functions, the need to identify tumor-specific antigens is circumvented. Therefore, this strategy can take advantage of the complete antigenic repertoire of the tumor and is not limited to predefined TAAs. In this way, it is feasible to develop an off-the-shelf product that can elicit strong antitumor immune responses against tumor-specific neo-antigens in a wide variety of cancer types. Different strategies to attract, expand, activate, and target DCs in situ will be discussed here.

3.1. Attraction of dendritic cells

In order to elicit an effector T cell response, appropriate numbers of DCs are required at the tumor site to take up the antigens and present these to the T cells. Indeed, Lavin et al. showed a decrease in CD141$^+$ DCs accompanied by a low number of activated CD8$^+$ T cells in the tumors of patients with early-stage lung adenocarcinoma [8]. Rather than focusing on attempts to deliver high numbers of ex vivo generated DCs to patients, new strategies are being developed to attract DCs to the site of interest in vivo through the administration of different growth factors or chemokines [82]. The most relevant approaches to expand and/or attract DC subsets in vivo are highlighted in this section.

3.1.1. Growth factors

One approach is the use of Fms-related tyrosine kinase 3 ligand (Flt3L), a key growth factor in the generation of DCs from hematopoietic progenitors present in the bone marrow. Systemic administration of recombinant human Flt3L (CDX-301, Celldex Therapeutics) to healthy donors increases the frequency of different types of DC subsets. Treating cancer patients with Flt3L can facilitate on the one hand the isolation of different subsets in sufficient numbers for multiple rounds of ex vivo DC vaccination. On the other hand, the increased frequency of DC subsets may improve uptake of TAAs and increase migration toward lymph nodes to induce successful immune responses [83]. The latter has been tested in a clinical trial (NCT02129075), where stage IIB–IV melanoma patients were vaccinated with a combination of systemic recombinant hFlt3L (CDX-301), to mobilize DCs, and a fusion protein of a human monoclonal antibody with specificity for a DC receptor, DEC-205, linked to the tumor-associated antigen NY-ESO-1 (CDX-1401) together with adjuvant poly(IC:LC). It has been shown that CDX-301 greatly expanded peripheral blood DCs and evidence of priming T cell immunity to the vaccine antigen was demonstrated [8].

Other trials are evaluating the effect of Flt3L administration in combination with stereotactic radiotherapy in a phase II clinical trial in patients with advanced NSCLC (NCT02839265) or in combination with adenoviral TAA expression in a phase I study in glioblastoma patients (NCT01811992) [9].

Multiple vaccine platforms include granulocyte-macrophage colony-stimulating factor (GM-CSF) in their formulations [9]. GM-CSF is known for its function in DC recruitment and maturation and also facilitates the homing of CTLs to the tumor site. Treatment with GM-CSF-secreting genetically modified tumor cells (GVAX-Pancreas NCT00084383 or Melanoma-GVAX-NCT01435499) resulted in promising antigen-specific protective immune responses, particularly when administered together with supporting drugs such as cyclophosphamide or innate immune ligands (STINGVAX) [8]. This is discussed in a later section of this chapter.

3.1.2. Chemokines

Chemokines participate in the antitumor immune response by regulating the trafficking and positioning of lymphocytes as well as by regulating different effector functions. Moreover, chemokines can act as a natural adjuvant in vaccination protocols for the treatment of various malignancies and infectious diseases [12].

CCL4 (macrophage inflammatory protein-1β, MIP-1β) is a potent chemoattractant for T lymphocytes, NK cells, as well as immature DCs. Spranger et al. have demonstrated the migratory capacity of DCs in response to recombinant murine CCL4 [84]. Complementary, Luo X et al. proved the chemotactic activity to CD4$^+$ T cells, CD8$^+$ T cells, NK cells, and immature DCs by transfecting CT26 cells with the MIP-1β gene. When AdhMIP-1β was injected in situ, lymphocytes were recruited to the tumor site. This intratumoral administration of AdhMIP-1β elicited a striking increase in tumor-specific CTL activity [85]. In metastatic melanoma patients, it was shown that an increased CD8$^+$ T cell infiltration is associated with enhanced expression of CCL3, CCL4, CCL5, CXCL9, and CXCL10 and an increased likelihood to respond to ipilimumab [86].

CCL5 (regulated upon activation, normal T cell expressed and presumably secreted, RANTES) has a strong chemotactic activity toward multiple immune cells, including DCs, macrophages, monocytes, NK cells, leukocytes, and T cells by binding on CCL5 receptors (CCR1, CCR3, CCR4, and CCR5).

Several preclinical studies have shown that co-immunization strategies, combining CCL5 as a vaccine adjuvant with DC growth factors or TAA, potently induce antitumor immune responses. Moreover, it was shown that ectopic expression of CCL5 at the tumor site attracts and activates different types of immune cells, such as DCs, CD4$^+$ Th1 cells, CD8$^+$ T cells, and NK cells [87]. However, therapies based on the induction of immune responses by CCL5 should also consider surface expression of CCL5 receptors on the tumor. The level of CCL5 expression by tumor or tumor stromal cells is critical for determination of its beneficial or detrimental activities. The goal of CCL5-based tumor vaccines is to maximally activate immune effector cells and minimally mobilize tumor cells [87].

CCL19 (Epstein–Barr virus-induced molecule 1 ligand chemokine, ELC) is produced by a subset of DCs and possibly by other nonlymphoid cells, in T cell areas of the lymphoid tissue. CCL19 is produced by fibroblastic reticular cells and is essential for the formation and maintenance of the T cell zone in lymphoid organs, where both T cells and DCs are recruited from the periphery and meet each other. The ability of CCL19 to attract T cells, B cells, DC, macrophage progenitor cells, and NK cells is mediated through the CCR7 receptor.

In an advanced lung carcinoma model (CC-10Tag), it was shown that intranodal administration of recombinant CCL19 led to a significant reduction in tumor burden coupled with extensive mononuclear infiltration [88]. Using CAR-T cells engineered to express IL-7 and CCL19, complete regression of preestablished solid tumors and prolonged survival in different mouse tumor models was observed. In addition, an increased infiltration of DCs and T cells into the tumor tissues was established by treating the animals with CAR-T cells expressing IL-7 and CCL19 [89].

CCL21 (secondary lymphoid tissue chemokine, SLC, Exodus-2, thymus-derived chemotactic agent 4, 6CKine) is evaluated intratumorally in preclinical models of lung, melanoma, and prostate cancer, leading to a significant increase in CD4+ and CD8+ T lymphocytes and DCs infiltrating both the tumor and the draining lymph nodes resulting in T cell-dependent anti-tumor responses [82].

3.1.3. Toxins

Alternatively, tetanus/diphtheria (Td) toxoid vaccine can be used to mobilize DCs. Most people have received Td toxoid vaccines in their childhood, so by treating them with Td toxoid vaccine a CD4+ T cell memory response is initiated, promoting the migration of DCs to the lymph nodes and activating an immune response. Indeed, preconditioning of tumors with Td toxoid vaccine in glioblastoma patients, receiving autologous monocyte-derived DC vaccines loaded with GBM antigen pp65, significantly improved the survival and antigen-specific T cell responses [8].

3.2. Activation of dendritic cells

Besides the observation that there is a decrease in the number of cross-presenting DCs accompanied by a low number of activated CD8+ T cells in tumors of patients with early-stage lung adenocarcinoma, DCs isolated from cancer patients often lack the expression of maturation markers, have an immature phenotype, and fail to activate T cells [8]. Therefore, a successful cancer vaccine still requires a powerful adjuvant in order to properly activate the DCs and to meet the minimal criteria for engaging the immune system [9]. Choosing a suitable adjuvant is important, as it can potentially override immunosuppression and allow the vaccine to maximize its therapeutic potential [9]. In the following sections, we will discuss some of the most used and powerful adjuvants able to activate DCs.

3.2.1. Aluminum salts (alum)

Alum, the first adjuvant to be used in human vaccines, is thought to function by adsorbing and then slowly releasing antigens in vivo to enhance the immune response. Alum has been reported to activate the inflammasome pathway particularly in DCs. Supplementing alum vaccines with other adjuvants and cytokines like Montanide (NCT00031733) and IL-12 in patients who have undergone surgery for stage II/III/IV melanoma has been reported to elicit Th1 antitumor responses [9].

3.2.2. Incomplete Freund's adjuvant (IFA)

Montanide adjuvants are an iteration of IFA that function by forming depots to concentrate vaccines at the injection site and facilitate slow release of antigens to enhance uptake by APCs. Different phase I/II studies are ongoing to assess the benefit of using Montanide in combination with different TLR agonists and standard chemotherapy in a variety of cancer types including melanoma, prostate cancer, and glioma (NCT02425306, NCT01079741, NCT02126579, NCT02293707, NCT02193347, NCT02795988) [9].

3.2.3. RNAdjuvant

CureVac AG, a biopharmaceutical company focusing on mRNA-based drugs, developed RNAdjuvant. This is a noncoding synthetic RNA (CV8102) that activates a set of pattern recognition receptors and induces upregulation of IFN-inducible genes at the injection site. Intratumoral therapy with CV8102 led to a dose-dependent tumor growth inhibition resulting in complete tumor eradication in nearly 50% of CT26 tumor-bearing mice. Moreover, combination of intratumoral CV8102 and systemic anti-PD-1 treatment led to significantly enhanced antitumoral responses compared to monotherapy. At the moment, a phase I, dose-escalation study of intratumoral CV8102 is ongoing to evaluate safety and tolerability. A second phase I trial is ongoing in patients with melanoma and squamous cell carcinoma of the head and neck combining intratumoral CV8102 with anti-PD-1 blockade.

3.2.4. TLR agonists

TLR agonists activate and mature DCs and have the potential to reverse T cell anergy, thereby overcoming immune suppression.

Hiltonol (poly-ICLC) is a stabilized dsRNA therapeutic viral mimic or "danger signal" that activates multiple elements of innate and adaptive immunity via signaling through TLR3. It is a stand-alone immunomodulator, but when properly combined with antigen, it generates a comprehensive Th1 immune response. Hiltonol is tested in numerous clinical trials, in patients with nonmelanoma skin cancer, glioma, lymphoma, ovarian cancer, prostate cancer, B and T cell lymphomas, etc. (NCT02423863, NCT01188096, NCT01976585, NCT03162562, NCT03262103, NCT00880867). Another TLR3 agonist is rintatolimod. Besides its use as therapeutic adjuvant in clinical trials for HIV-1 infection and chronic fatigue syndrome, rintatolimod is also tested in phase I/II studies in Her2+ breast cancer patients which are treated with Her2 peptides and a combination of GM-CSF and rintatolimod (NCT01355393). It is also used in combination with IFN-α to treat patients with metastatic colorectal cancer (NCT01545141, NCT03403634), and a phase I/II trial in patients with recurrent ovarian, fallopian tube, or primary peritoneal cancer receiving an autologous vaccine composed of autologous oxidized tumor cell lysate (OC-L) administered in combination with rintatolimod (NCT01312389) is ongoing.

Bacillus Calmette-Guérin (BCG) activates TLR2 and TLR4 in macrophages and DCs. This vaccine was primarily developed for the prevention of tuberculosis and is nowadays the standard treatment for patients with in situ or non-muscle invasive bladder cancer. Currently, successful trials using BCG in combination with topical treatment of 5% imiquimod in melanoma patients are ongoing. Glucopyranosyl lipid A(G100), a synthetic TLR4 ligand, has showed success in early clinical trials in eliciting Th1-polarized antitumor immunity (NCT02501473) [90]. Picibanil (OK-432) is a lyophilized preparation of *Streptococcus pyogenes* and is approved in Japan for the treatment of cervical, gastric, and oral cancer [9, 90]. eTheRNA immunotherapies are evaluating the use of intranodally administered TriMix mRNA in a phase Ib clinical study in melanoma patients.

The only known natural ligand to activate TLR5 is flagellin, a constituent protein of bacterial flagella. Preclinical in vitro and in vivo data indicate that formulations using liposomal engrafted synthetic peptide containing flagellin fragments can induce DC maturation.

One phase I clinical trial has been completed using entolimod (CBLB502, a pharmacologically optimized flagellin derivative) in patients with late-stage solid tumors (CT01527136). The treatment was well tolerated with only common adverse events such as fever, transient hypotension, and hyperglycemia. Another TLR5 agonist, M-VM3 (Mobilan), a recombinant non-replicating adenovirus encoding human TLR5 and its ligand flagellin, is currently in two clinical trials (NCT02654938, NCT0284499) for prostate cancer [90].

Stimulation of TLR7/TLR8, receptors for single-stranded RNA, significantly augments DC maturation, Th1 cellular immunity, cross-presentation of antigens, and humoral immune responses. One of the three FDA-approved commercialized small-molecule TLR7/TLR8 agonists is imiquimod, formulated as a dermal cream, for HPV-mediated external genital warts, superficial basal cell carcinoma, and actinic keratosis. The imiquimod 5% cream was tested in a randomized controlled trial (NCT0066872) in patients with nodular and superficial basal cell carcinoma and demonstrated to be superior to excision surgery. Another promising lipid-modified imidazoquinoline is 3 M-052. It is evaluated as an adjuvant in many vaccine models and showed promising preclinical results in mouse melanoma and prostate tumor models.

Unmethylated CpG oligodinucleotides, a TLR9 agonist, used in peptide vaccines has been shown to boost antitumorigenic T cell responses. MGN1703, a covalently closed natural DNA molecule, is a TLR9 agonist which elicits significant IFN-α induction and broad activation of human immune cells in vitro. This molecule is taken into a phase II study (NCT01208194) to treat patients with metastatic colorectal cancer, in combination with standard chemotherapy and bevacizumab (a monoclonal anti-VEGFA antibody). Patients who received MGN1703 showed a superior progression-free survival compared to placebo. The compound is also tested in a phase I clinical trial to determine the highest tolerable dose in combination with ipilimumab (anti-CTLA-4) in patients with advanced solid tumors (NCT02668770) and in patients with small-cell lung cancer (NCT02200081).

Another pattern recognition receptor is stimulator of interferon genes (STING), and upon activation of this pathway, an interferon response is induced. STING ligands are cytosolic double-stranded DNA molecules, host signaling second messenger cGAMP, and pathogen-derived cyclic dinucleotides (CDNs) [9]. Corrales et al. showed a regression of local as well as distal tumors in different mouse models, and the induction of lasting memory responses against tumor rechallenges could be achieved upon treatment with CDNs [91]. STING agonists are also combined with other therapeutic agents such as the STINGVAX vaccine platform, which uses GM-CSF-secreting cells along with modified STING agonists. Hanson et al. showed that nanoparticulate STING agonists are potent lymph node-targeted vaccine adjuvants. In addition, synthetic STING agonists, such as ADU-S100, are already tested in patients with advanced solid tumors (NCT02675439). Besides the specificity of the STING agonists, DNA vaccines, such as VGX-3100, may also have the inherent potential to activate the STING pathway.

Another ligand of the PRR is *Listeria monocytogenes* (Lm), and attenuated Lm strains promote antigen cross-presentation and antitumoral immune responses. Overall, att-Lm strains have the capacity to deliver TAAs and also act as adjuvants for boosting DC vaccines by neutralizing immunosuppressive factors (NCT02575807), recruiting DCs by expressing GM-CSF (NCT01417000), or inducing DC activation through TLR and STING signaling [9].

3.2.5. Costimulatory proteins

Several vaccine strategies have been designed to leverage CD40-mediated DC activation, including vaccination with tumor cell lines modulated to overexpress CD40L (NCT00458679, NCT02719015, NCT02466568), anti-CD40 antibody (NCT02376699, NCT02482168, NCT01103635), and recombinant CD40L protein (NCT00001145) [9]. Preclinical data showed that the intranodal and intratumoral delivery of TriMix mRNA results in the activation and maturation of DCs, leading to robust antitumor responses and prolonged survival in different murine tumor models. Currently, a phase I clinical trial (NCT03394937) is ongoing in melanoma patients to assess safety and tolerability of the intranodal delivery of TriMix mRNA. Another phase I clinical trial is planned in early-stage breast cancer patients, who will be treated intratumorally with TriMix mRNA prior to surgery. Other costimulatory proteins involved in the expansion, function, and survival of T cells are OX40L and 4-1BB. Preclinical results in a variety of induced and spontaneous tumor models suggest that targeting 4-1BB with agonist antibodies can lead to tumor clearance and durable antitumor immunity. Clinical trials of two agonist antibodies, urelumab and utomilumab, are ongoing. Both antibodies have demonstrated promising results in patients with lymphoma and are being tested in combination therapy trials with other immunomodulatory agents. However, important considerations should be given to 4-1BB-mediated toxicities.

OX40 ligand (classically expressed on activated APCs) is a costimulatory membrane-bound protein, and OX40 signaling can be induced by different technologies such as OX40-specific agonistic antibodies (phase I clinical trial NCT01644968 in metastatic cancer patients), OX40L-Fc fusion proteins (NCT02221960), RNA aptamers and transfected tumor, and DCs. In analogy to the mouse tumor models, anti-OX40 monoclonal antibodies (mAb) may further benefit from combination strategies. There is one combination trial combining anti-OX40 mAbs with radiation and cyclophosphamide (NCT01303705) in cancer patients with metastatic prostate cancer. Other combination trials analyzing the potential of combining anti-OX40 with anti-CTLA4, anti-PDL1, and anti-CD20 mAbs are ongoing (NCT02205333). Currently, a first-in-human, phase I dose-escalation study is ongoing evaluating the safety and tolerability of escalating intratumoral doses of mRNA-2416 (encoding OX40L) in patients with relapsed/refractory solid tumor malignancies or lymphomas.

3.2.6. Virotherapy

Oncolytic viruses (OVs) are attenuated, mutated, or naturally benign viruses that preferentially target and lyse cancer cells while leaving normal, non-transformed cells relatively unharmed. The antiviral immune response that follows OV infection occurs within the vicinity of the tumor; overturns tumor-associated immune evasion mechanisms; enhances DC activation, maturation, and TAA uptake and presentation; and thus has the potential to establish a robust antitumor specific immune response. Currently, there are numerous examples of these OVs including reovirus, vesicular stomatitis virus, vaccinia virus, Newcastle disease virus, measles virus, poliovirus, herpes simplex virus, coxsackievirus, adenovirus, and Maraba virus. Interestingly, the therapeutic administration of OVs drives two contrasting immunities, namely, antiviral and antitumor [92]. It has been demonstrated that a "prime boost" immunization strategy—sequential immunization with different strains of oncoviruses expressing the same TAA—negates the risk of generating "distracting" antiviral immunity in mice and is now being

evaluated in clinical trials (NCT02285816) [9].OVs have the natural capabilities to alter the maturation status of DCs, but other studies on engineered OVs (e.g., adenovirus, HSV, arbovirus, poxvirus) have also focused on enhancing the interaction of OVs with DCs by encoding growth factors (GM-CSF and Flt3L), chemokines (CCL2), cytokines (IL-12, RANTES, and IFN-β), and defensins (β-defensin-2) within the viral genome. For example, an oncolytic adenovirus encoding MIP-1α and Flt3L has been constructed to promote DC recruitment and expansion in vivo, which ultimately had a strong synergistic effect on the infiltration of tumors by DCs and T cells. The administration of IL-12 and GM-CSF-expressing adenovirus (Ad-ΔB7/IL12/GMCSF) in combination with DCs in B16-F10 melanoma tumor-bearing mice also showed increased DC migration to draining lymph nodes due to the upregulation of CCL21$^+$ lymphatic vessels around tumor tissues. In addition, GM-CSF-expressing adenovirus ONCOS-102 is used in a phase I clinical trial (NCT01598129) in patients with advanced solid tumors. Another example is JX-594, also known as Pexa-Vec. Intravenous delivery of this GM-CSF-expressing vaccinia poxvirus with a deletion of the thymidine kinase gene resulted in increased tumor-infiltrating CD8$^+$ T cells and reduced metastasis in hepatocellular carcinoma (NCT00554372). Intratumoral injections of HSV-1 expressing GM-CSF, also known as talimogene laherparepvec (T-VEC), have been shown to trigger the development of antitumor immunity in metastatic melanoma patients (NCT00769704). This is achieved through DC stimulation (attraction and maturation) via GM-CSF, resulting in enhanced priming of antigen-specific T cells [92]. Currently, T-VEC is being evaluated in combination with checkpoint blockade drugs (NCT01740297) [9].

3.3. Targeting DCs in vivo

In order to directly target DCs in vivo, different strategies can be followed to deliver maturation agents or antigens to the DCs. Nanoparticles can be loaded with adjuvants and antigens, enabling them to activate lymph node-resident DCs and induce potent immune responses. Examples of biologics that can be incorporated in nanoparticles are immune-activating cytokines and growth factors (IL-12, IL-2, GM-CSF), neutralizing antibodies against immunosuppressive cytokines (TGFβ), stimulatory ligands (TLR agonists), and factors selectively targeting specific DC (subsets) along with antigens [93–95].

Another approach to deliver your DC modulatory agent is to use self-polymerizing scaffolds, which drain to the lymph node and become immunogenic particles in vivo. Saponins, plant-derived glycosides that form stable immune-stimulating complexes along with cholesterol and phospholipids, are also a safe candidate for delivering cargo to the DCs in vivo. They are being explored for their antitumorigenic potency in combination with novel platforms such as nanoparticles, oncolytic viruses, and chemotherapy agents [9]. Another option to target the DCs and activate them is the use of antibodies specifically binding to DCs. The antigen is bound to antibodies directed against surface receptors of DCs leading to uptake of the antigen. Endocytic antigen uptake through C-type lectin receptors like DEC-205 has been shown to induce cross-presentation. CDX-1401, a vaccine comprising of DEC-205 fused with tumor antigen NY-ESO-1, has been proven therapeutic and safe against advanced malignancies and is being evaluated in patients with ovarian carcinoma, leukemias, and melanoma (NCT02166905, NCT01834248, and NCT02129075). An example of another DC targeting antibody is a vaccine developed by Celldex targeting mannose receptors expressed on APCs in combination with TLR agonists [9]. However, if these antibody–antigen conjugates are not

accompanied by adjuvant to stimulate the immune system, tolerance rather than immunity might occur [81]. The challenge of this approach will be to match the DC surface target and the selected adjuvant with the desired immune outcome, all in the context of an altered immune system [96]. Besides the abovementioned approaches, Van Lint et al. already demonstrated the feasibility of injecting "naked" (non-packaged) mRNA in vivo (either intranodally or intratumorally). It has been shown that naked mRNA is preferentially taken up by cross-presenting CD8α+ DCs [97, 98]. This approach circumvents the necessity of using different kinds of delivery vectors or targeting molecules.

4. Conclusion

In conclusion, it is of utmost importance that DCs used for vaccination are equipped to stimulate effector T cells, and this can only be achieved when these DCs are fully matured and activated. Many challenges remain that must be considered to improve the efficiency and clinical outcome of DC-based immunotherapy in cancer. These include the choice of the different synergistic immunotherapies, selection of ideal adjuvants, administration route, and timing of the different treatments. It is likely that the future of cancer immunotherapy will be a combination of different immunotherapy platforms. One arm will focus on inhibiting tumor-induced immunosuppression, while the second arm will aim to activate antitumor immunity.

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