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REVIEW

Dendritic cell-based immunotherapy: a basic review and recent advances

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Abstract Dendritic cells (DCs) are considered a very promising arm to activate the immune system in immunotherapeutic strategies against cancer. DCs are the most powerful antigen-presenting cells (APCs), being highly efficient at generating robust immune responses. They are also considered the center of the immune system, since they provide a crucial link between both innate and adaptive immune responses. Thus, DC-based cancer immunotherapy aims to take advantage of these unique characteristics of DCs to better fight cancer. During the last decade, they have been the subject of numerous studies intending to develop immunotherapeutic strategies against cancer through vaccination. For this purpose, it is essential to gain a better insight into DC immunobiology, regulation of innate and adaptive immune systems, and tumor microenvironment, as well as applying the latest advances in science in order to boost their enormous anti-tumor immunotherapeutic potential. In this review, we will hold focus on DC immunobiology (from their origin, location, and special properties and distinct subsets to the innate and adaptive immunity), on the new concept of cancer immunoediting, and on the knowledge given by clinical trials using DC vaccines. Finally, future perspectives for this emerging field are highlighted.

Keywords Dendritic cells · Immunotherapy · Vaccines · Cancer

Introduction

Immunity results from a complex interplay between the innate immune system (which is antigen-nonspecific) and the adaptive immune system (which is antigen-specific) [1], and dendritic cells (DCs) are an essential link between both the innate and adaptive immunity [1, 2]. These cells that were primarily described by Steinman and Cohn are the center of the immune system. DCs exhibit unique immunoregulatory ability and act as sentinel members of the innate immune system. Although DCs represent a very small population of leukocytes, they are the most powerful antigen-presenting cells (APCs), known as "professional APCs." Furthermore, DCs are highly efficient at generating robust immune responses, having the unique ability to activate naive T cells [1, 3]. They are able to recognize, capture, process, and present antigens (AGs) and are capable of producing cytokines in the presence of AGs and pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs)—also known as danger signals [3].

Before going any further, we would like to pay tribute to Ralph Steinman for his priceless contribution and tireless work in the field of DCs, culminating in the award of the Nobel Prize in Physiology or Medicine (2011) that he won for his discovery of DCs and its role in adaptive immunity.

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Dendritic cell immunobiology

Origin and location

DCs originated from $CD34^+$ hematopoietic stem cells (HSCs) in the bone marrow and consist of developmentally and functionally distinct subsets that differentially regulate T cell function [3]. Briefly, the generation of most DC subsets is controlled, under steady state, by the cytokine Fms-like tyrosine kinase 3 ligand (Flt3L), whereas during inflammation and infection, another cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), mobilizes and stimulates the production of monocyte-derived DCs [4]. Immature DCs take residence at sites of potential antigen entry and are specialized in antigen capturing and processing. They are abundant at the skin and internal and mucosal surfaces, such as the respiratory, genitourinary, and gastrointestinal systems [3, 4]. During maturation, DCs migrate into the lymphoid tissue where specific immune response can effectively be activated [1, 3].

DC subsets

Despite the fact of being a small population of leukocytes, DCs do not represent a homogeneous population. In fact, there are several types of DCs, each one of them with distinct markers and functions.

Firstly, it is crucial to establish the difference between plasmacytoid DCs (pDCs) and conventional DCs (cDCs) [3, 4]. pDCs are key effectors in the innate immune system because of their extraordinary capacity to produce type I interferons (IFN) upon viral infection. Also, pDCs can be involved in tolerance (immune suppression) in their immature state.

The cDCs can be further subdivided according to their location: lymphoid organ-resident DCs, peripheral tissue-resident DCs—such as Langerhans cells (in the epidermis) and interstitial DCs—and circulating DCs. cDCs recognize bacterial components and produce proinflammatory cytokines such as TNF-α, interleukin-6 (IL-6), and IL-12p70 to activate proinflammatory T cell subsets (Th1 and Th17) and consequently recruit cytotoxic T lymphocytes (CTLs) [3, 4].

Antigen capture, processing, and presentation

Immature DCs recognize PAMPs—which are evolutionary conserved structures, namely microbial lipids, carbohydrates, nucleic acids, and intermediates of viral replication—via pattern recognition receptors (PRRs). There are several PRRs that can be involved in innate recognition of pathogens, including Toll-like receptors (TLR), nucleotide-binding-oligomerization-domain (NOD-like) receptors, C-type lectin receptors (CLRs), active protein kinase (PKR), and RIG-I-like helicases [3].

Following DC recognition, antigen capture can occur by different mechanisms, including macropinocytosis, endocytosis, and receptor-medicated phagocytosis [1, 4, 5]. Regarding the last one, phagocytosis is defined as the engulfment of large particulate antigens, such as bacterial pathogens, in a process requiring actin remodeling to form a cup around the particle that eventually closes to form a phagosome. Those processes of antigen capture by DC are mediated by a large number of receptors that deliver the antigen to processing compartments [1, 4, 5].

DCs process proteins into antigenic peptides that are presented on major histocompatibility complex (MHC)—MHC class I and II molecules [1, 4]. Lipid AGs, on their turn, are processed differently and are loaded onto non-classical MHC molecules of the CD1 family [1].

After antigen uptake and processing, DCs present them:

1. Via MHC-II to $CD4^+$ T lymphocytes (exogenous via): generally, this via occurs when DCs present exogenous peptides via MHC-II molecules derived from proteins that have been endocytosed and degraded by acid-dependent proteases in endosomes [4, 6].
2. Via MHC-I to $CD8^+$ T lymphocytes (endogenous via): this via is used to present intracellular antigens (endogenous peptides) associated with MHC class I molecules on the cell surface (e.g., in a viral response—when a virus infects a cell, viral peptides can be presented by DCs which allow the immune system to recognize and $CD8^+$ T lymphocytes are directly activated to kill the infected cell) [4, 6].
3. Via cross-presentation—consisting of presenting exogenous AGs on MHC-I molecules that culminate in stimulation of $CD8^+$ T lymphocytes T killer/cytotoxic cells. The process of phagocytosis is crucial for this mechanism of cross-presentation. It must be emphasized that cross-presentation is a special capacity of DCs [4, 6], particularly a capacity of specific subsets, namely $CD8^+$ DCs and migratory $CD103^+$ DCs [7].

Pathogen recognition receptor triggering and DC activation

DCs have the capacity to sense their environment via surface and intracellular receptors, which include several abovementioned families such as CLRs, surface and intracellular TLRs, NOD-like receptors (NLRs), and intracellular helicases. Some of the CLRs (receptors for both PAMPs and endogenous ligands) contain signaling motifs in their cytoplasmic regions that deliver either activation or suppression signals. Helicases can be activated by recognition of nucleic acids and differentially modulate DC functions, resulting in distinct immune responses. Indeed, DCs are equipped with a

complex set of molecules that helps them to respond to the complexity of the external molecular and cellular world. Particularly, receptors that recognize PAMPs can be targeted for immunotherapy, as the activation of DCs is likely to contribute to beneficial therapeutic effects related to nonspecific activation of anti-tumor immunity by PAMPs (approach pioneered with Coley's toxin). Also, a Bacillus Calmette-Guerin vaccine was used as a bladder carcinoma's treatment. Imiquimod (ligand for TLR7 and TLR8) is applied as a superficial basal cell carcinoma's treatment. Multiple other TLR ligands—CpG oligodeoxynucleotides (ODN) and polyinosinic-polycytidylic acid (polyI:C)—have been tested in clinical trials. In conclusion, DC maturation alone does not result in a unique DC phenotype. Different signals provided by different PAMPs (derived from microbes) induce distinct DC molecular sensors; therefore, DCs acquire different phenotypes that result in different immune responses [1].

T cell activation

DCs play a critical role in directing effector T cell responses towards a Th1, Th2, Th17, or regulatory T cell (Treg) response. Upon maturation, DCs upregulate the expression of specific molecules, providing three types of signals to T lymphocytes that will determine their activation status and respective final phenotype [3]:

- *Signal 1* Antigen-specific signaling via T cell receptor (TCR) mediated by binding of MHC-peptide complexes to the TCR. This is the initial interaction between DCs and T cells [3].
- *Signal 2* Co-stimulation by surface molecules on DCs, which is responsible for amplifying or regulating their interaction with T cells [3]. For instance, co-stimulatory molecules CD80 and CD86 are surface relevant markers of DCs that have undergone maturation, being a ligand for proteins on T cells essential for its activation and survival [2].
- *Signal 3* Secretion of pro- or anti-inflammatory cytokines that stimulate the differentiation from naive T cells to effector T cells [3]. Depending on the cytokine release profiles (e.g., IL-12, IL-13, IL-6, TGF-β, or IL-10), DCs can polarize naive T cells into Th1, Th2, Treg, or Th17 phenotype [8]. Because of its relevant role in inducing Th1 and CTL responses, we highlight the IL-12p70 which is a very well-studied multifunctional proinflammatory cytokine. IL-12p70 promotes the activation of natural killer (NK) and T cells to produce mainly IFN-γ, which favors the generation of CTLs and enhances the activation of cytotoxic NK cells. Additionally, besides the activation of innate and adaptive immunity against tumor cells, IL-12p70 also reveals anti-tumor effects associated with the inhibition of angiogenesis through IFN-γ [3].

Briefly, distinct T cell responses are generated depending on whether antigen capture occurs either in peripheral tissues or lymph nodes directly [1, 4]. In peripheral tissues (Fig. 1), DCs capture antigens through several complementary mechanisms [1]. After antigen capture, DCs undergo a highly regulated process of maturation and remodeling into fully activated APCs capable of eliciting effective immune responses [3]. The process of maturation is associated with downregulation of antigen-capture activity, increased expression of MHC molecules, and enhanced expression of co-stimulatory molecules on DC surface, such as CD80 and CD86. Furthermore, an increased production of chemokines that are responsible for attracting naive and memory T cells also occurs. During this process, DCs loaded with AGs enhance their levels of C–C chemokine receptor type 7 (CCR7). This receptor allows them to exit peripheral tissues (non-lymphoid) and migrate into the lymph nodes (lymphoid tissue) [1, 3]. AGs can also be captured directly by lymph node-resident DCs (Fig. 1). When it happens, DCs are the first ones responsible for presenting peptides to naive $CD4^+$ T cells. A maturation stimulus results in T cell priming and IL-2 production, which in turn facilitates T cell proliferation and clonal expansion [1]. In the absence of maturation stimuli, immature DCs are kept in a steady state and present self-antigens to T cells, which leads to immune tolerance either through T cell deletion or through the differentiation of Treg cells (or suppressor T cells) [1, 4].

Basically, the interaction either between DCs and $CD4^+$ T cells or DCs and $CD8^+$ T cells stimulates the differentiation into effector T cells with distinct functions. $CD4^+$ T cells can be polarized in Th1 cells, Th2 cells, Th17 cells, or T follicular helper (Tfh) cells that help B cells to differentiate into antibody-secreting cells, as well as Treg cells that downregulate the functions of other lymphocytes. On the other hand, $CD8^+$ T cells can give rise to CTLs [1].

Interaction between DCs and other immune cells

DC maturation and the presence of different DC subsets allow to control the diverse response repertoire of T cells, and other classes of lymphocytes, such as B cells and NK cells [4]. In the lymphoid tissue, DCs are responsible for activating not only T cells but also B lymphocytes, through AG presentation in the form of peptide-MHC complexes on the surface of the DCs (Fig. 1). As mentioned before, the co-stimulation and secretion of various cytokines additionally strengthen antigen presentation, which is critical to induce proper immune responses [3]. Lastly, it is worth emphasizing that DCs may also interact with cells of the innate immune system, including NK cells, phagocytes, and mast cells, which modulate the final immune response [1]. Particularly, NK cells are engaged in

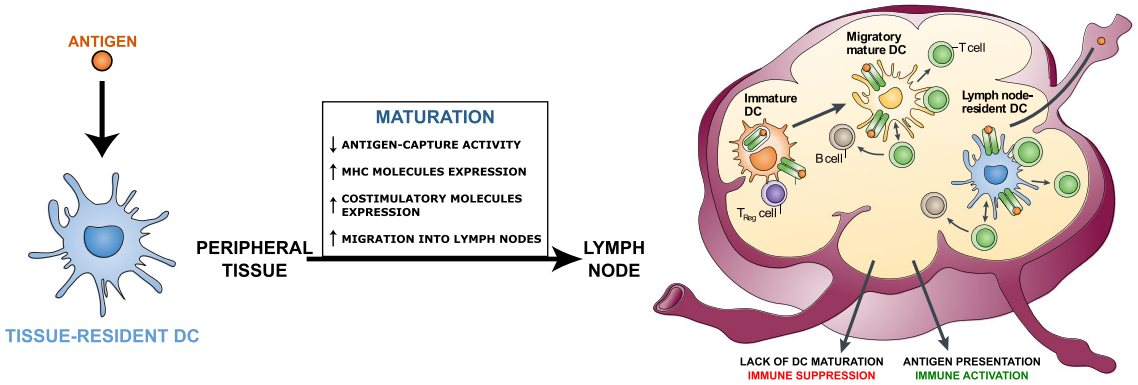

Fig. 1 Launching the immune response: DCs can capture AGs in peripheral tissues via several mechanisms. After AG capture and processing, DCs go through a process of maturation where several transformations take place, becoming able to migrate into the lymph nodes. Mature DCs are fully activated antigen-presenting cells capable of eliciting effective immune responses. Also, AGs can also be captured directly by lymph node-resident DCs which are responsible for presenting peptides to naive $CD4^+$ T cells and develop an immune response. Lastly, in the absence of maturation stimuli, immature DCs are kept in a steady state and present self-antigens to T cells, leading to immune tolerance (either via T cell deletion or via the Treg cell differentiation). Adapted from: Palucka, K. and Banchereau, J. [1] (*DC* dendritic cell, *Treg cell* regulatory T cell, *MHC* major histocompatibility complex)

the eradication of tumor cells and seen as cytotoxic lymphocytes. After activation, NK cells exocytose cytotoxic granules containing perforin and various granzymes, leading to the perforation of target cells and subsequent apoptotic death induced by permeated granzymes. There are several studies where anti-tumor immune responses were shown to be significantly less efficient when NK cells are absent [9].

Cancer: elimination, equilibrium, and escape

Tumor AGs are produced by cancer cells and have the potential to trigger an immune response, being useful not only as cancer biomarkers but also in cancer immunotherapy. Within the cancer immunotherapy field, it is particularly relevant to understand which ones have the ability to elicit an immune response, which results in tumor elimination. There are three types of antigens of high tumoral specificity: viral antigens, antigens that result from a mutation or rearrangement of a gene-coding sequence, and antigens that are encoded by cancer-germline genes. More in-depth understanding of different types of tumor AGs is reviewed by Couliet et al. [10].

Despite of a competent action of the immune system, tumors acquire the ability to develop and grow in three sequential steps (elimination, equilibrium, and escape), according to the currently accepted model of cancer immunoediting (Fig. 2).

In the initial phase—elimination phase—there is an efficient control of the cancer cell growth due to the induction of robust tumor-specific immune responses [11]. However, some cancer cell variants can resist to eradication in this initial phase. This resistance establishes the beginning of the equilibrium phase. This second phase, normally the longest one, can last decades, in which tumor outgrowth is prevented by immunologic mechanisms: T cells, IL-12, and INF-γ that are required to maintain tumor cells in a state of functional latency/dormancy. However, certain changes can occur as a consequence of genetic instability of tumor cells held in equilibrium, including (i) lack of recognition by adaptive immunity (antigen loss or development of defects in antigen processing or presentation), (ii) insensitivity to immune effector mechanisms, or (iii) induction of an immunosuppressive state within the tumor microenvironment. Therefore, such changes are translated into immunogenicity declining or increased resistance to cytotoxic actions promoted by the immune system. After this, there is uncontrolled tumor growth related to the third phase—escape phase [11, 12].

Ex vivo and in vivo methods of DC-based immunotherapy

DC vaccines are a very powerful anti-tumor immunotherapeutic strategy. The main goal includes the activation of an immune response able to eliminate cancer cells and to produce long-lasting immunity. DC vaccines make use of DC precursors, differentiation into DCs and loading with tumor AGs. The administration via different possible routes into patients aims to achieve an effective and directed anti-tumor immune response. The absence of a standardized procedure for ex vivo manipulation results in a plethora of methods differing in the source of DCs, the nature and procedure for antigen loading, the maturation stimulus, and, finally, the route of administration. The most used methods are described in detail below; however, advantages and drawbacks of the different methodologies used for production of DC-based anti-tumor vaccines could be further explored in the paper of Constantino and colleagues [13].

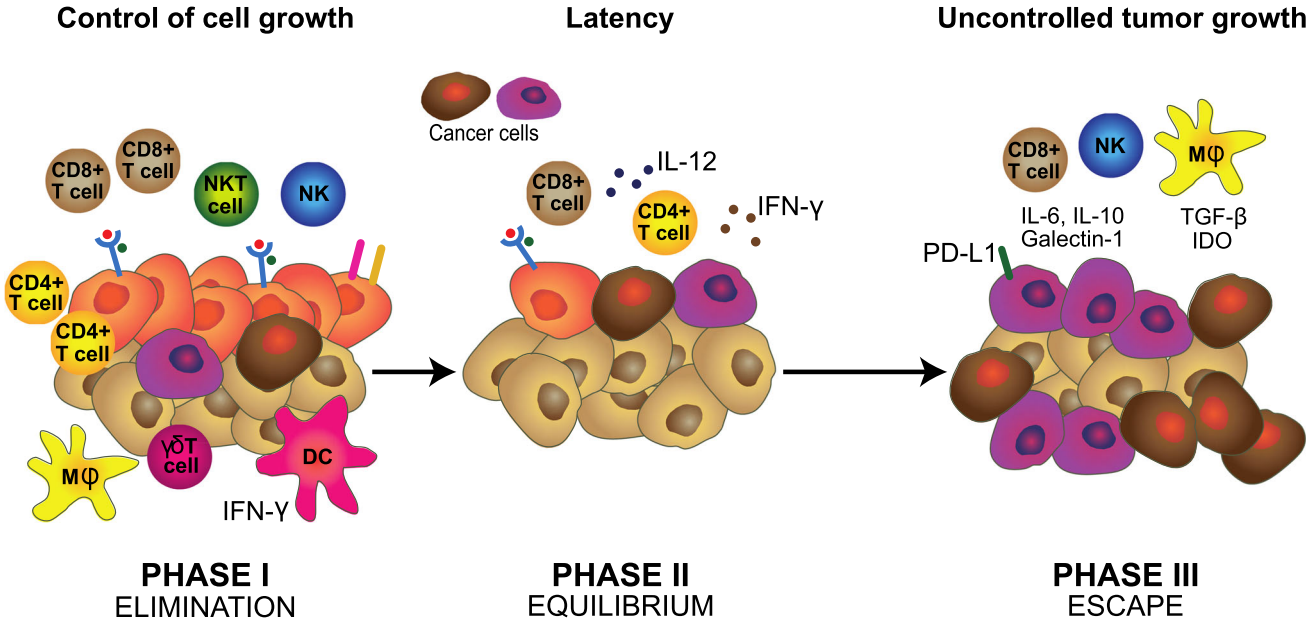

Fig. 2 The cancer immunoediting concept. Three sequential steps of cancer development: phase 1: elimination: control of cell growth associated with induction of tumor-specific immune responses; phase 2: equilibrium: latency where cancer growth is prevented by several immunologic mechanisms; and phase 3: escape: uncontrolled tumor growth, due to genetic instability related to cancer. Adapted from Schreiber, R. D.; Old, L. J.; and Smyth, M. J. [11] (*DC* dendritic cell, *NK* natural killer cell, *Mϕ* macrophage, *NKT* natural killer T cell, *CTL* cytotoxic T lymphocyte, *Th1* T helper 1, *PD-L1* programmed death-ligand 1, *IDO* indoleamine 2,3-dioxygenase, *IL* interleukin, *IFN* interferon, *TGF* transforming growth factor)

Methods of generating DCs

Ex vivo methods have been developed for generating DCs. There is no current consensus on the optimal ex vivo method, being possible to make use of differentiation from monocyte-derived DCs or $CD34^+$ hematopoietic precursors, as well as in vivo expansion of circulating DCs [14].

Monocyte-derived DCs

The differentiation of DCs from peripheral blood mononuclear cells (PBMCs) isolated from a patient is the most commonly used technique. The preference for monocyte-derived DCs (moDCs) instead of $CD34^+$-derived DCs is not related to their superior clinical efficacy, but rather to the limited number of $CD34^+$ precursors that can be isolated from apheresis products. Monocytes are differentiated into immature DCs by culturing cells, during 5 to 7 days, in the presence of GM-CSF and IL-4. Subsequently, the immature DCs are stimulated with a cytokine maturation cocktail for the development of a mature phenotype [14–16]. While this method can last 5–7 days, another novel and faster method allows to reduce this period of time for 2 days, obtaining DCs termed FastDCs [14]. Basically, this faster method (2-day protocol) makes use of human monocytes in vitro to generate DCs by culturing cells in the presence of GM-CSF and IL-4 for 24 h, followed by the addition of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and prostaglandin E2 (PGE2) for an additional period of 24 h. This method was well-described by Obermaier B et al. [17].

DCs derived from $CD34^+$ hematopoietic precursors

Another possibility for generating DCs involves the use of $CD34^+$ precursors. Through this method, $CD34^+$ precursors are mobilized from the bone marrow by treatment of patients with GM-CSF prior to leukapheresis procedures. The harvested cells are expanded in culture for 1 week in the presence of GM-CSF, Flt3L, and TNF-α, resulting in a mixture of moDCs, DCs that are phenotypically similar to epidermal Langerhans cells (LCs) and a large proportion of myeloid cells at different stages of differentiation. It should be noted that LCs may have important impact on stimulating T cell responses. Indeed, $CD34^+$-derived DCs, and particularly their LC subset, were shown to stimulate more effective CTL responses in vitro than their counterpart moDCs [14, 18, 19].

In vivo expansion of circulating DCs

The expansion of circulating DCs, that represent less than 1% of PBMCs, is achieved by administration of hematopoietic growth factors (such as Flt3L). In vivo expansion of circulating DCs leads to the upregulation of maturation markers,

production of cytokines upon stimulation, and induction of T cell responses [14, 20].

Strategies for AG loading (Fig. 3)

Loading DCs with peptides, proteins, and tumor cells

Pulsing DCs with peptides, proteins, or tumor cells is the most common approach by which DCs are loaded, mostly performed before maturation. Within this strategy, peptides are loaded directly onto MHC-I and MHC-II molecules on the surface of the DCs, whereas proteins and tumor cells require to be initially processed and presented by the DCs to stimulate $CD4^+$ and $CD8^+$ T cells. The major drawback of using peptides is the need to know the patient's haplotype and the defined peptides that would bind these specific haplotypes. On this point, using proteins and tumor cells is advantageous due to the fact that it is not limited to a particular haplotype. In the case of proteins and tumor cells, the main disadvantage is that MHC-I pathway is not specifically targeted, although studies have noted activation of $CD8^+$ T cells which indicates the occurrence of cross-presentation [14, 21, 22].

Viral vectors

The use of viral vectors is an attractive alternative for AG loading on DCs, since it allows the insertion of genes encoding tumor AGs or whole proteins, as well as the elimination of genes encoding virulence or replication factors. In some cases, the vector may induce DC maturation, bypassing the need for implementing a separate maturation procedure. Another advantage is the possibility of adding genes encoding cytokines or co-stimulatory molecules, to enhance the immunogenicity of DCs. Pre-existing immunity against the vector may, however, reduce the patients' ability to induce in vivo responses, which makes their safety the primary concern. It is worth noting that lentivirus-based vectors are typically less immunogenic due to the removal of the majority of genes encoding viral proteins. Therefore, their repeated use without inducing immune responses against the vector is possible. Additionally, lentivirus-based vectors have particular advantages, such as (1) retaining the potential to trigger the activation of the innate immune system via endosomal or cytoplasmic sensors (i.e., TLRs, RIG-I, PKR, etc.), (2) additional capacity to transduce quiescent and non-dividing cells, and (3) representing a potential vehicle for direct therapeutic vaccination, since they may be pseudotyped with glycoproteins to target specific cell types, and directly target DC subtypes in vivo [14, 23–26].

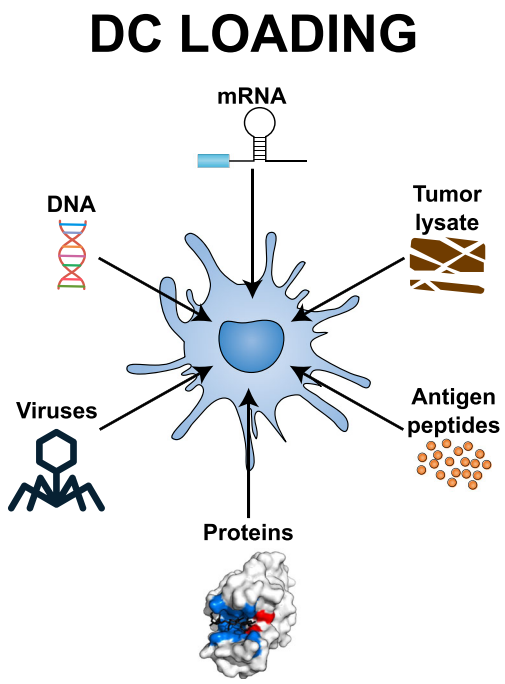

Fig. 3 Distinct DC vaccine loading approaches have been tested in clinical trials: loading of DCs with peptides, proteins, and tumor lysates; mRNA transfection; and delivery of DNA and the use of viral vectors. Adapted from Butterfield, L. H. [35] (*DC* dendritic cell)

mRNA transfection

The loading of DCs with messenger RNA (mRNA) encoding tumor-associated AGs (TAAs) is another attractive option that has been demonstrated to induce $CD4^+$ and $CD8^+$ T cell responses. The mRNA, with short half-life and not being part of the host genome, can be loaded directly on DCs without requiring the use of vectors or knowledge of patient's haplotypes [3, 14]. Also, mRNA transfection enables presentation of multiple antigenic epitopes, as well as the loading with stimulus maturation (such as CD40L) or cytokines. It has been shown that electroporation is the most efficient method to introduce mRNA into DCs, promoting a temporary increase in cell permeability that facilitates the entry of the mRNA without the need for additional reagents [14, 27–29].

DC maturation

DC maturation is a particularly relevant process to be done prior to vaccination. There is yet no consensus on an appropriate maturation stimulus for generating DCs with strong immunostimulatory activity. Multiple combinations of maturation stimulus have been tested, including proinflammatory cytokines, CD40 ligand (CD40L or CD154), and TLR agonists [14–16]. The use of TLR agonists results in increased levels of IL-12, which is translated into a powerful activation of DCs and subsequently effective immune responses. Maturation of DCs is key for efficient vaccine production as mature DCs show generally enhanced expression of co-stimulatory molecules and increased production of cytokines and chemokines, while immature DCs fail to induce antigen-

specific responses and have the potential to induce differentiation of regulatory T cells. More detailed review of distinct studies on DC maturation stimuli is well-presented by Sabado R. et al. [14].

In vivo administration of AG coupled to antibodies: in vivo DC targeting

There are many strategies to perform antigen loading and maturation of DCs in vivo. One of them is the in vivo administration of AGs coupled to specific antibodies (Abs), which represents a recent approach of DC-based vaccines, bypassing the whole expensive and intensive ex vivo DC generation process. Before going any further, we would like to mention that this article will mainly hold focus on ex vivo-produced vaccines. However, this in vivo appealing strategy allows the production of vaccines in a large scale, since it is not based on the commonly used ex vivo approach of customized vaccine for each patient. More specifically, the administration of these AG-Ab complexes allows stimulating natural DC subtypes at multiple sites in vivo [14, 30, 31]. To develop these DC-targeting vaccines, there are numerous factors that need to be considered, including the biological function of target DC subsets (e.g., induction of humoral and/or cellular immunity), the selection of proper AGs to control the disease, the characteristic receptors expressed by a particular DC subset, the choice of vaccine adjuvants, and also the tissue distribution of DC subsets [32]. Due to its encouraging results suggesting strong anti-tumor immunity [33], this immunization strategy will briefly be discussed below regarding its suitability for clinical trials and lastly as a new and promising strategy.

Clinical trials: progress in DC-based anti-tumor immunotherapy

During the last decade, multiple methods, both ex vivo and in vivo, have been developed for loading DCs with TAAs (Fig. 4) and thereby creating anti-cancer vaccines capable of eliciting immunological response with clinical relevance [34, 35].

This section intends to summarize the results regarding recently completed clinical trials (CTs), aiming to highlight progresses made on DC-based vaccines for cancer therapy.

DCs loaded ex vivo with tumor cell lysates

Numerous phase I/II CTs have been developed using DCs loaded with tumor cell lysates in several distinct tumor types and, overall, the results were encouraging in terms of safety and efficacy. Particularly, they showed no toxicity and an efficient activation of the immune response in a large proportion of cases [34].

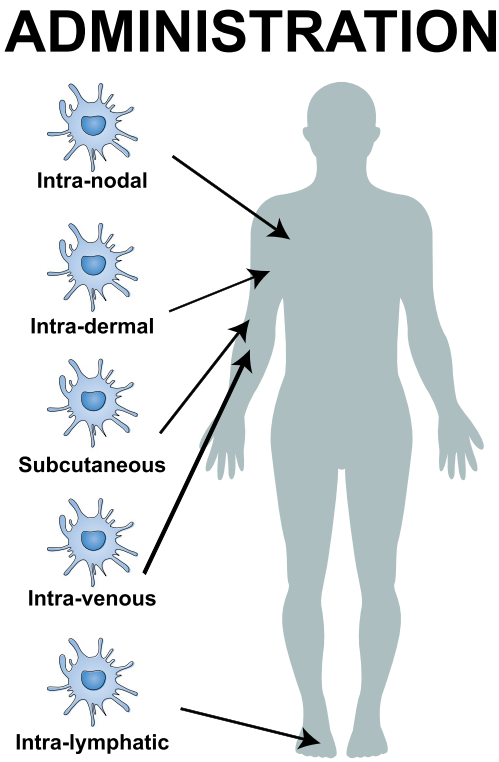

Fig. 4 Different administration routes for dendritic cell vaccines: (1) intranodal, (2) intradermal, (3) subcutaneous, (4) intravenous, and (5) intralymphatic. Adapted from Butterfield, L. H. [35]

As an example, patients with melanoma (43 of them in stage IV and 7 in stage III) were vaccinated with autologous DC pulsed with allogeneic cell lysates (TRIMEL) derived from three melanoma cell lines. In this study, more than 60% of patients showed delayed hypersensitivity reaction type IV (DTH) which was positively associated with increased survival and augmented stability of disease [36]. It is worth emphasizing that other similar CTs were able to correlate the development of anti-tumor immune responses (as assessed by the appearance of DTH) with improved clinical outcomes [34]. Thus, high interest must be kept in this immunotherapeutic strategy, especially against melanoma.

DCs pulsed ex vivo with tumor AGs

The ex vivo-pulsed DCs with tumor AGs (full-length proteins or short peptides) can elicit both protective and therapeutic anti-cancer immune responses [34]. Taking as an example, in the first pilot study testing the safety of this approach, three of four follicular B cell lymphoma patients showed clear and favorable clinical outcomes (one of them showed complete regression, another showed partial regression, and the third presented complete resolution of disease) [37]. Since this first study, multiple phase I/II CTs focused on this approach have been tested in various types of neoplasms [34]. Altogether, CT

results suggest that this strategy is safe, although only few studies reached phase III [34].

DCs pulsed with mRNA

The use of RNA (both RNA extracted from tumor or mRNA coding for specific TAAs) has been tested during the last decade. Several reports provided unequivocal proof that DCs ex vivo pulsed with tumor-derived RNA are capable of eliciting both protective and therapeutic immune responses. Also, phase I/II CTs showed the safety of using DCs loaded with tumor-derived RNA or DCs engineered for the endogenous expression of tumor AGs [34]. An example of loading DCs with mRNA is the TriMixDC-MEL vaccine administered to melanoma patients. This vaccine involves the co-electroporation of DCs with four types of mRNA, of which one encodes a tumor AG (one of the four possible tumor AGs tyrosinase, MAGE-A3, MAGE-C2, or gp100) and the other three so-called TriMix mRNAs—mRNA encoding ligand CD40L, CD70, and constitutively active TLR4. Vaccination with TriMixDC-MEL resulted in TAA-specific $CD8^+$ T cells' entrance at the sites of intradermal injection and also in patient's circulation. Moreover, a detailed characterization of the antigenic specificity of $CD8^+$ T cell populations allowed to conclude that there was a certain degree of compartmentalization of $CD8^+$ T cells, due to its distribution by the skin and peripheral blood [38].

DCs fused ex vivo with tumor cells

The fusion of DCs with tumor cells results in cell hybrids, also known as "dendritomes." Phase I/II CTs that took place in the last decade have showed that dendritomes are not harmful. Additionally, development of DHT response was observed that indicates immune system activation in a very large proportion of cases [34]. For example, the vaccination with dendritomes—in this case, dendritomes resulted from autologous DC fusion with myeloma cells derived from multiple patients in combination with GM-CSF—has shown induction of humoral and cellular anti-tumor immune responses. Still, most patients with advanced disease revealed stabilization of disease [39].

Other DC-based approaches

In addition to those previously described, other strategies have been explored focusing on the immunogenic potential of DCs with varying success rates. Among these strategies, it is worth highlighting the intratumoral administration of ex vivo-expanded and not-loaded-with-AG DCs, the DC-based exosomes, and the direct administration of AGs coupled to Abs (in vivo DC targeting) already described above [12].

Regarding intratumoral administration of ex vivo-expanded DCs, this approach is not applicable to a large range of tumors because of their location, which makes the administration a dangerous process with high morbidity rates. This strategy has demonstrated positive clinical outcomes in some phase I/II CTs [34].

DC-based exosomes—that are lipid nanovesicles secreted by DCs carrying multiple molecules—were also tested in patients with advanced non-small cell lung cancer in a phase I CT. The exosomes, derived from autologous DCs, were loaded with tumor-associated AGs, and the results suggest that DC-based exosomes represent a viable and well-tolerable strategy. Additionally, some patients revealed stabilization of the disease, activation of long-term specific T cell response, and increase in lytic activity of NK cells [40].

Regarding in vivo DC targeting, very recent insights were conquered in the first clinical trial using CDX-1401—fully humanized anti-DEC-205 monoclonal Ab conjugated to the NY-ESO-1 tumor AG. This first-in-human study demonstrates that this new strategy is feasible, safe, and immunogenic. Moreover, this study provided simultaneously rationale for combination immunotherapy strategies including immune checkpoint blockade in a total of 45 patients with advanced malignancies refractory to available therapies: 30 patients experienced disease stabilization (median duration of 6.7 months), two patients had tumor regression (approximately 20% shrinkage in target lesions), and six of eight patients who received immune checkpoint inhibitors within 3 months after CDX-141 administration showed objective tumor regression [41].

Conclusions for the next generation of CTs

What was possible to learn about DC-based cancer vaccines from the numerous CTs? First of all, there is a unanimous and very important lesson over the last decades of CTs: DC-based cancer vaccines are relatively safe and non-toxic. Based on this, its application must be initiated in patients with less advanced cancer stages, rather than patients who do not respond to the standard therapies, as it may result in better clinical outcomes [42]. In fact, a patient with less advanced cancer will consequently show a less debilitated immune system and a superior immune response promoted by DC vaccination can be expected.

A relevant aspect associated with the selection of tumor AGs is the identification of AGs related to cancer growth. Indeed, a limited fraction of tumor AGs (approximately 10%) seems to be immunogenic, and among these, only a few are considered as consistent tumor-rejection AGs (TRAs), i.e., AGs that elicit an immune response resulting in tumor eradication. Therefore, additional efforts should be exerted to identify authentic TRAs, which involve a highly personalized process including exome sequencing followed

by functional validation assays [34, 43]. In fact, exome sequencing seems to be an extraordinary new technology to identify new tumor-specific mutant AGs [44]. Additionally, predicting the binding affinity of tumor mutant peptides to MHC I molecules can be done by computer algorithms. Strong MHCI binding affinity is one potential indicator of potent immunogenicity, and major TRAs have been discovered taking that into account [45]. Regarding AG loading, each vaccine should carry a great variety of AGs, aiming at activating more distinct effectors of the immune system. Therefore, for obtaining a vaccination with complete antigenic content related to the specific tumor, DCs may be either loaded with whole tumor cells (or tumor cell lysates) or transfected with whole RNA extracted from the tumor [14, 43].

Another important aspect concerns the most appropriate administration route (Fig. 4). The intradermal route demonstrated a reduced level of migration to the lymph nodes (less than 2% of the DCs injected). In its turn, ultrasound-guided intranodal delivery route has a risk of injecting the vaccine into fat instead of a cellular area [35]. On balance, optimizing the administration routes can contribute to obtain more potent vaccination, besides monitoring the in vivo migration of DCs (for example, through magnetic resonance using DCs labeled with perfluoropolyether (PFPE)[46]) [35].

DCs that are administered might not efficiently reach the tumor site. Thus, even though extratumoral DCs may also provide therapeutic benefits, strategies to direct the migration of DCs towards tumor nests are being developed [34].

DC-based vaccines may have limitations as stand-alone therapeutics. However, its combination with other therapies may increase the overall effectiveness, as will be discussed in more detail below. Given its huge potential, combining immunotherapy with conventional therapies (surgery, chemotherapy, and radiotherapy) is a current strategy in which multiple CTs are focused on [35].

Ultimately, we can claim that there is a rising progress in the field of DC vaccines. We are now facing increasingly defined, focused, and coherent approaches that are being created. A next generation of vaccines on the basis of personalized medicine is also expected. This is a novel field involving selective vaccines for each individual patient based on their specific tumor biology (personalized for each patient) [35].

Future perspectives

DCs are expected to be of great promise in the future. Examples of future perspectives are the development of new adjuvants, the in vivo targeting of DCs, the DC-derived exosomes, and the use of "combination therapy" (immunotherapy combined not only with standard therapies such as chemotherapy and radiotherapy but also with other immune therapies involving checkpoint blockers).

Development of new adjuvants

Adjuvants are defined as compounds that help or enhance the immune response against a vaccine AG, increasing or modulating the humoral or cellular immune response against an AG. It happens that some AGs are weakly immunogenic; thus, adjuvants are key to intensify the immune response. Also, more immunogenic AGs may benefit from having a specific delivery vehicle that eases targeting and/or controlled release of the AG to DCs [47].

The use of TLR ligands has been associated with sustained activation of DC, enhancement of CTLs' functions, increase in levels of IL-2, improved migration ability in response to CCR7 ligand, stimulation of humoral response, and finally induction of $CD4^+$ T cells. Among some examples of effective TLR ligands for activating immune response, imiquimod (which is a ligand for TLR7 and TLR8) is used for the treatment of superficial basal cell carcinoma [14, 48]. Moreover, several other TLR ligands are currently being tested in CTs, including CpG oligodeoxynucleotides, which target TLR9, and polyinosinic-polycytidylic acid (polyI:C), which targets either TLR3 or helicases, depending on the size of the polyI:C [1, 14].

Also, 4-1BB/CD137 receptor (member of the TNF receptor family) is expressed by activated T cells and recognizes ligands on APCs [49]. It is assumed that 4-1BB has a significant role in the survival of memory $CD8^+$ T cell. An anti-CD137 Ab (with agonist action) showed to increase the tumor rejection by promoting the survival of $CD8^+$ T cells, and this anti-tumor effect was mediated by DCs [50, 51].

Another promising target includes the CD40 receptor. CD40 expression on DCs and the increased connection to its ligand—CD40L—on $CD4^+$ T cells lead to enhancement of co-stimulatory molecules and cytokine production, and subsequent activation of $CD8^+$ T cells. This interaction between CD40 and CD40L can also increase the cross-presentation mediated by DCs [52]. A new approach for extending the activated state of DCs is related to the use of a recombinant receptor containing the cytoplasmic domain of CD40 fused to the ligand-binding domains and a membrane-targeting sequence. In this approach, activation of DCs with this recombinant receptor resulted in prolonged activation of DCs, more potent induction of $CD8^+$ T cells, and tumor eradication [53].

Cytokines, such as IL-15, IL-7, and IL-12, have been shown to enlarge T cell survival and function [14].

Moreover, adjuvants have also been shown to protect AGs from degradation, albeit this depends on the nature of adjuvant [47]. Regarding this, a wide variety of nanoparticles (NPs) can be synthetized to be used as vehicles for specific DC targeting, including (1) AGs encapsulated within NPs, which protected them from degradation, (2) NPs conjugated to AGs, which are recognized by specific receptors, and (3) labeled NPs that are recognized by certain receptors and allow an effective tracking

of migration. Further information on nanotechnology-based manipulation of DC for enhanced immunotherapy strategies, including explanation of different approaches of nanotechnology and some studies on them, can be found in a review developed by Klippstein R. et al. [54].

In vivo targeting of DCs

Alternative to ex vivo methods, in vivo DC targeting represents a novel approach that involves targeting specific molecules to DCs, as stated before. The candidate receptors include Fc receptors, CD40, and CLRs. The CLR receptors are the most attractive, as different DC subsets express different CLRs (such as DEC205, DC-SIGN, mannose receptors (MR), or Dectin-1). Furthermore, CLRs are involved in the capture of numerous glycosylated self-AGs and pathogens for antigen presentation, DC trafficking, DC-T cell interactions, and consequently activation of immune response [14]. Furthermore, targeting of AGs to CLRs also resulted in the enhancement of Ab responses [55].

Additionally, we would like to mention the inCVAX (in situ autologous cancer vaccine) as a novel approach involving photoimmunotherapy (combination of phototherapy and immunotherapy), being an emerging strategy of therapy. It consists of two-injection procedure: firstly, photothermal laser application on a selected tumor (intending to promote the release of the whole tumor AGs), and secondly, vaccination with immunoadjuvant (that may activate DCs and facilitate an increased uptake of tumor AGs previously released) [56]. The immunoadjuvant—*N*-dihydro-galacto-chitosan (GC), a semi-synthetic functionalized glucosamine polymer—plays a relevant role in changing the immunosuppressive tumor microenvironment. GC converts macrophages from the M2 phenotype back to the classically M1 phenotype, known to augment Th1 response and facilitate CTL effector functions, which ultimately favors cancer elimination [57]. Also, inCVAX cured 40% of animals inoculated with metastatic mammary tumor and provided long-term protection [58]. Moreover, clinical response rate of inCVAX was up to 70% in an early non-randomized human metastatic breast cancer, and some patients remained tumor-free with all metastases eliminated for 4 years [59]. These results are indeed encouraging, and efforts are currently being made to enhance this strategy and combine with other therapies to improve its efficacy.

DC-derived exosomes

DC-derived exosomes (DC-Exo) may be a valuable tool to use in cancer immunotherapy, since they have shown to turn cancer cells into more immunogenic targets. DC-Exo carry many molecules of DCs and the incorporation of these vesicles by human breast adenocarcinoma cells increased their ability to activate T cells. Hence, it is possible that this strategy can contribute to the effectiveness of DC vaccines, and that possibility must be further investigated [60].

Synergy of therapeutic combinations

Combination with conventional chemotherapy or radiotherapy

Cytotoxic treatment may have multiple positive effects on the immune system, from simple release of tumor AGs as a result of cancer cell destruction to the immunological effects. Indeed, the release of tumor AGs leads to enhanced uptake and presentation of a broad array of AGs for T cell activation. The immunological effects promoted by the cytotoxic action include upregulation of immunostimulatory molecules (e.g., DAMPs), increased tumor AG expression, reduction of suppressor cells, and increased CTL proliferation and activation [35].

For example, 26 patients affected by different types of advanced cancer refractory to treatment were subjected to a combined therapy of radiation, immature DCs, keyhole limpet hemocyanin (KLH), and T cells. Twenty-one out of 26 patients had a successful elimination of metastatic and recurrent tumors in the initial treatment, and half of them showed complete response with no evidence of disease recurrence. These promising results help to encourage the development of research around combination of the conventional therapies with DC-based anti-tumor immunotherapy [61].

Also, cyclophosphamide and paclitaxel were shown to stimulate DC maturation at low doses, therefore being used in combination with DC vaccines [62, 63].

Combination of DC-based vaccines with suppression of inhibitory signals

Tumors can express negative co-stimulatory molecules that suppress the immune response and secret a wide range of factors that inhibit innate and adaptive immunity. There is a strong immunosuppressive environment associated with tumors, mediating several mechanisms prone to evade immune surveillance: (i) loss of tumor AG expression, (ii) change of MHC molecules, (iii) lack of co-stimulation, (iv) expression of inhibitory ligands, (v) induction of Treg cells, (vi) expression of indoleamine 2,3-dioxygenase (IDO), which is an enzyme involved in the generation of Treg cells, and (vii) production of immunosuppressive cytokines (transforming growth factor β (TGF-β), IL-6, and IL-10). Thus, new approaches for overcoming the tolerance/suppression induced by tumor microenvironment may enhance the immunogenicity and potentiate the efficacy of DC vaccines in vivo [14].

The suppression of inhibitory signals in combination with DC-based vaccines has great potential for eliciting better immune response against cancer.

Blocking the interaction between the programmed death-1 (PD-1) receptor on activated T cells and its respective ligand (PD-LI) that is overexpressed on a variety of tumors and DCs has been shown to enhance the in vitro immune response. There are different strategies to interfere with this mechanism such as anti-PD-1 Abs [14] or small interfering RNA (siRNA) used to silence the PD-L1 and PD-L2 ligands on DCs [42, 64].

The cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which is involved in the inhibition of T cell activation, represents another promising target to hinder. Thus, using anti-CTLA-4-blocking Abs makes possible to maintain immune responses mediated by CTLs [14, 42]. Note that an anti-CTLA-4-blocking Ab, the ipilimumab, showed satisfactory therapeutic effects and has been approved by the FDA for the treatment of metastatic melanoma in 2011 [42].

Another possibility is targeting components of signaling pathways involved in the inhibition of DC functions, namely suppressors of cytokine signaling (SOCS) family and glucocorticoid-induced leucine zipper (GILZ).

SOCS have been shown to inhibit JAK/STAT signaling, which is important for DC function in response to TLR agonists. SOCS inhibition can also be performed using siRNA [65].

The inhibition of GILZ may also result in therapeutic benefits, given that GILZ inhibition has been demonstrated to alter the maturation of DCs in response to TLR agonists and CD40L [66]. Furthermore, GILZ expression in macrophages infiltrating Burkitt lymphomas contributed to the failure of the immune system to reject the tumor [67].

Also, immunotherapeutic strategies targeting the immunosuppressive cytokines (IL-10 and TNF-β) are currently being explored in order to augment the vaccines' effectiveness [14].

IL-10-receptor Abs demonstrated to enhance specific immune response and IL-12 production [68]. Additionally, the inhibition of TGF-β suppressed the proliferation of Treg cells and increased the number of TAA-specific T cells [69].

Advantage of DC-based immunotherapy over other cancer immunotherapy strategies

Other cancer immunotherapies with some promising results include adoptive cell transfer of tumor-infiltrating lymphocytes (TILs), genetically engineered T cells, and the previously referred immune checkpoint inhibitor antibodies. Adoptive cellular therapy (ACT) is a form of immunotherapy that involves the ex vivo isolation and expansion of antigen-specific T cells for adoptive transfer back to patients. Even though clinical benefits have been achieved in the treatment of hematologic malignancies and melanoma, the efficacy of ACT in the management of utmost solid tumors is thus far limited since transferred T cells fail to function and persist in vivo. Besides the need of tumor resection, another obstacle in ACT for solid tumors is the failure to successfully isolate or expand TILs to enough numbers. Furthermore, in most medical centers, lymphodepletion before transfer is a standard procedure of the treatment; nonetheless, ~50% of the patients experience side effects of this pre-treatment, which are commonly infection related, i.e., neutropenia and bacteremia. Another alternative approach to obtain T cells with anti-tumor reactivity is genetic engineering of T cells to express chimeric antigen receptors (CARs). CARs are constructed by linking an antigen-binding domain, usually a single-chain variable fragment (scFv), to an intracellular T cell signaling domain such as CD3-ζ (first-generation CAR), and currently also including one or two co-stimulatory domains (second/third-generation CAR). The most remarkable clinical results so far have been obtained using CAR T cells targeting CD19 in patients suffering from B cell malignancies. Conversely, CAR T cell therapy has been associated with significant toxicity, such as cytokine-release syndrome and neurotoxicity. Taking together, an important argument favoring DC-based immunotherapies is their safety profile. Twenty years of observations in numerous phase I and II trials demonstrated that DC vaccines are generally well-tolerated and induce minimal toxicity. The most common manifestations are local reactions at the injection sites, such as rash and pruritus, and occasionally, systemic effects occur, including fever and malaise. Despite the initial concerns regarding the possibility of inducing autoimmunity, the DC-based therapies are rarely associated with severe immunotoxicity reactions. Indeed, adverse events related to immune checkpoint blockade have included autoimmune complications. For instance, the effect of CTLA4 blockade, regrettably, is not restricted to tumor-specific T cells. The proper inhibition of autoreactive T cells can be disrupted, leading to undesirable immune-mediated toxicities, including immune-related hostile events most commonly affecting the liver (hepatitis and transaminitis), skin (rash, vitiligo, and pruritus), endocrine glands (hormonal imbalance), and bowel (diarrhea and colitis) [70].

Overall, immunotherapy has the potential to improve outcomes for patients with tumors. DC vaccines, monoclonal antibodies, immune checkpoint blockade, and adoptive and genetically engineered T cell therapy have each proved promising results. Forthcoming directions lie in understanding how to join each of these approaches with standard chemo- and radiotherapy, defining the timing to initiate immunotherapy, and learning how to combine immunotherapeutic strategies to maximize effectiveness and limit toxicity [71].

Conclusion

Forty-one years after their discovery, the importance of DCs on immunity modulation has been greatly recognized by the award to Ralph Steinman of the Nobel Prize for Medicine in 2011, "for his discovery of the DCs and its role in adaptive

immunity." Although the use of DCs in immunotherapy has initially been target of skepticism by the medical community, it remains a promising therapeutic approach supported by their wide use in CTs with the purpose of originating or amplifying the anti-tumor immune responses. Basically, DC-based immunotherapy focuses on the use of own patient's DCs to activate the immune system towards cancer cell elimination. However, some aspects have contributed to the limited success of DC-based therapy, such as the need to optimize the conditions to generate immunostimulatory DCs and the effect of the tumor microenvironment associated with tolerance and immune suppression. That being said, new strategies can be the key to achieve effective and long-term anti-tumor immune responses that translate into excellent clinical outcomes. Particularly, it is relevant to combine DC-based vaccines with new approaches that overcome the immunosuppressive mechanisms related to the tumor microenvironment, increase the immunogenicity (achieve a stronger anti-tumor immunity), and promote the activation and interaction of multiple arms of the immune system that were recently identified as critical for anti-tumor response. It is therefore hopeful that immunotherapy, already with some cases of success and strong potential to eliminate metastases and fight against cancer relapse, can become a major treatment for cancer patients.

Finally, if research in immunotherapy and other forms of cancer therapy keeps evolving as it is doing at this moment, cancer can become a more controlled disease in our lifetime, and it must be kept in mind that: "Just as immunotherapy is moving to the forefront of cancer therapy, DC-based therapy is moving to the forefront of cancer immunotherapy," as stated by Karolina Palucka and Jacques Banchereau.

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