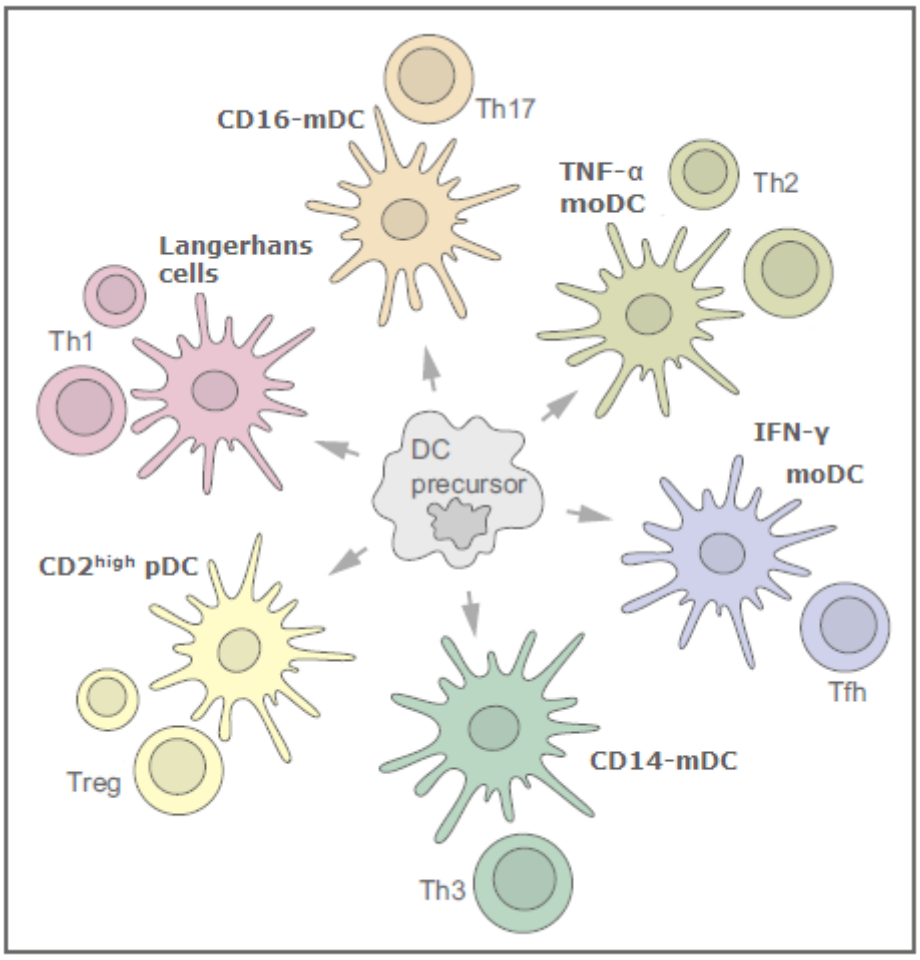
The Use of Human Dendritic Cell Subsets in Cancer Vaccines



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Abstract

The use of human dendritic cell subsets in cancer vaccines

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Dendritic cells (DC) have been used as therapeutic tools in cancer vaccines for more than a decade. The majority of clinical trials conducted in this field of immunotherapy have employed DC generated *ex vivo* from monocytes (moDC), as these DC can be easily propagated into large numbers. However, the clinical efficacy of moDC-based vaccines has remained unsatisfactory, as the laborious culturing protocols lead to exhaustion of their immunostimulatory capacity. Novel therapeutic strategies seek to capitalize on the distinct biological functions of naturally occurring DC subsets, i.e. myeloid DC (mDC) and plasmacytoid DC (pDC). Present in the skin, blood and secondary lymphoid organs, these subsets are capable of eliciting potent anti-tumour responses. Each subset has a specified effect on the immune system, and especially skin mDC and pDC are of interest for their capacity to induce tumour regression. Although natural DC subsets do not require extensive culturing periods, they are low in frequency and complicated isolation techniques are required to obtain them in sufficient number and purity. However, *in vivo* targeting techniques and identification of vaccine adjuvants that provide potent activation stimuli are promising developments. Combination therapies with conventional anti-cancer drugs can be considered to overcome the tolerogenic immune environment and stimulate efficient anti-tumour immunity.

Front page illustration: adapted from Palucka *et al*. 2010 [4]

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Introduction

Nowadays, cancer is considered a major health concern. Through the uncontrolled growth of abnormal cells into tumours that invade and destroy adjacent tissues, cancer can cause severe pain, neurological symptoms and organ damage. According to the GLOBOCAN project global estimates for 2008, the risk of dying from cancer before the age of 75 is 11.2% [5,6]. Hence, many strategies have been developed to reduce tumour mass in patients with established tumours. Conventional therapies include surgery, radio- and chemotherapy, but these established methods are often not capable of completely eliminating residual cancer cells and frequently fail to prevent disease recurrence [7-9]. Consequently, novel therapies that could efficiently combat cancer are advancing by making use of the body's natural response against malignant cells.

Immunotherapy and cancer vaccines

The immune system plays an essential role in the defence against cancer. Cells of the immune system continuously scan the body for the presence of mutated cells. Upon encountering an abnormality or tumour, the immune system usually responds by destroying the malignant cells. Consequently, patients receiving immunosuppressive drugs demonstrate an increased incidence in cancer [10]. However, not only medicine can hinder the immune system in clearing abnormalities. A number of cancer variants are themselves capable of deluding the immune system and become immunologically silent. Others can produce immunosuppressive substances that inactivate the immune cells responsible for their killing. As a result, malignant cells can escape destruction and develop into tumours [11,12]. The development of the cancer immune surveillance model has prompted many attempts to create a therapy that excites the suppressed immune system into eliminating remaining cancer cells [13].

Modulating the patient's own immune response to prevent or combat a disease, also called immunotherapy, has been in practice for quite some time in the form of vaccines. The biological principle behind vaccines is that they contain an agent that resembles a molecule typical of a disease-causing entity. The vaccine exposes this agent or antigen to cells of the immune system, thereby educating them to recognize the antigen as a foreign object. Consequently, upon the next encounter with the antigen the educated cells will immediately launch an attack to clear it from the body. In doing so, they render the patient immune to the disease-causing entity [14]. Many preventive and therapeutic vaccines have been developed to combat pathogenic agents such as smallpox, influenza and polio, indicating the success of immunotherapy against infectious diseases. Unfortunately, so far no such progress has been achieved in non-infectious settings [15]. In order to facilitate vaccine design, it is necessary to gain a more detailed understanding of the processes leading to a strong anti-cancer immunity.

Tumour immunology

Whether an anti-tumour vaccine will induce an immune response of therapeutic quality depends on four components: 1) the $CD8^+$ T effector lymphocytes, 2) the $CD4^+$ helper T

cells, 3) the regulatory T cells and 4) the immunosuppressive tumour environment. These four components are of importance in the stimulation or suppression of tumour-killing natural killer (NK) cells and the production of immunomodulatory agents called cytokines. They also influence the development of plasma B cells, which can produce antibodies that target tumour cells for destruction. Generating the correct immune response is of vital importance to the efficacy of a vaccine, as can be seen in Figure 1 [16].

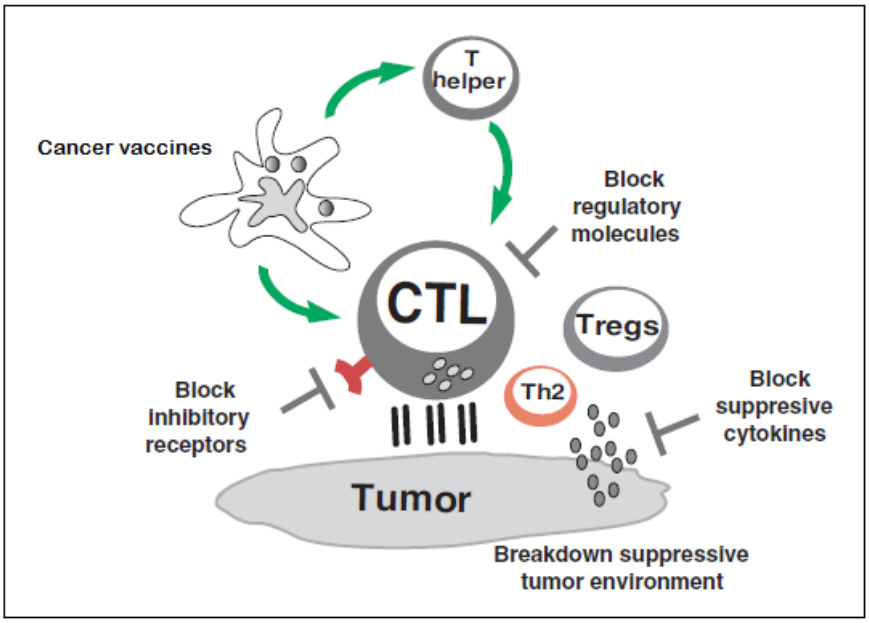

Fig.1) To improve the clinical efficacy of cancer vaccines and completely destroy all tumor cells, strong helper T and CTL responses need to be elicited by employing novel strategies. The regulatory and immuno-suppressive actions of regulatory T cells and T_H2 cells need to be blocked to allow full immunostimulatory function.
Picture adapted from Palucka *et al*. (2011) [1]

The cells responsible for the destruction of cancerous cells are the $CD8^+$ T effector cells, also called cytotoxic T lymphocytes (CTLs), and the NK cells. Memory T cells are a subset of $CD8^+$ T cells that have previously encountered cancer-specific antigens, and upon a second encounter can mount a stronger immune response with higher production of immunostimulatory cytokines and an increase in lytic activity. In the presence of tumours, the lytic activity of NK cells and $CD8^+$ T cells is activated through recognition of antigen associated with mutated or abnormal cells, also termed tumour-specific antigen. The malignant cells present these antigens in the context of major histocompatibility complex (MHC) class I molecules on the cellular surface. Upon encountering a dysfunctional cell, CTL and NK cells bring about apoptotic cell death. Apoptosis can be induced by the release of toxins, such as perforin and granzyme, or by interaction with death receptors on the surface of the target cell [14,17].

The effector responses of both $CD8^+$ T cells and NK cells are regulated by the function of $CD4^+$ T cells, also called helper T cells (T_H cells). The specific effect of T_H cell activity depends on the phenotype involved in immune regulation. For example, T_H1 cells are known to be involved in the response to intracellular microbes and cancer cells, whereas development of T_H2 cells is induced by extracellular pathogens. Conversely, T_H17 cells are a recently discovered type of helper T effector cells known to stimulate autoimmunity. A special type of $CD4^+$ T cells, called follicular helper T cells (T_{FH}), are specialized in eliciting B cell help [18]. Polarization of the immune system to T_H1 and T_{FH} responses is considered desirable for effective cancer therapy, whereas T_H2 responses are associated with tumour proliferation and poor prognosis in cancer patients. T_H1 and T_{FH} cells induce immunological responses against target cells by stimulating cytotoxicity

of tumour-killing NK and $CD8^+$ T cells and production of tumour-specific antibodies by plasma B cells [19].

The immunomodulatory actions of T cells are predominantly elicited through direct cell-cell interaction and cytokine secretion. For instance, the interleukin IL-12 is responsible for polarizing the immune response to a T_H1 and T_{FH} profile. In response to IL-12, NK and $CD8^+$ T cells become more efficient in tumour-specific killing and B cells produce more immunoglobulin IgM. In addition, it stimulates T_H-cell production of IL-21, which in turn also enhances immunoglobulin production [20,21]. Other interleukins of interest are IL-23 and IL-15. IL-23 is thought to stimulate T cell proliferation and to induce T_H17 and memory T cell activity [22]. Conversely, IL-15 is a T-cell growth factor that stimulates the functioning of $CD4^+$ and $CD8^+$ T cells, NK cells and mast cells [23]. Other cytokines considered important in anti-tumour immunity are interferon (IFN)-γ and tumour necrosis factor (TNF)-α [22].

To suppress the antitumor immune response of T_H cells and to maintain immunological tolerance, regulatory T cells (Treg cells) also patrol the body. Treg cells protect the body from autoimmunity and are necessary for the transition of one immunological response to the other. Activation of Treg cells leads to dampening of the tumour immune response by the production of immunosuppressive cytokines, like IL-10 and tumour growth factor (TGF)-β [24]. Therefore, it is important to deactivate or modulate Treg functioning, while simultaneously promoting proper T_H1 cell responses and anti-tumour CTL activity.

Antigen presentation and dendritic cells

$CD4^+$ or $CD8^+$ T cells need to encounter a foreign antigen before they can be activated. Antigens are presented to T cells through antigen-presenting cells (APC). These cells are specialized in antigen presentation, the mechanism of cells to take up, process and present antigen complexes. When a cell takes up and presents extracellular antigen, the process is called cross-presentation. Antigens are presented on the cellular surface of APC in the context of MHC classes I or II. These complexes can be recognized by T cells via the T cell receptor and their signalling induces T-cell activation [14].

The most important type of APC with the broadest range of antigen presentation are dendritic cells (DC). DC are immune cells that develop from myeloid and lymphoid precursors in the bone marrow, as depicted in Figure 2. Present in the skin, mucosal tissues and the blood, immature or inactivated DC scan these tissues for influences from the external environment. When they encounter and take up foreign antigen, they switch to an activated state and migrate to the lymph nodes to induce T-cell activation. By means of expressing costimulatory molecules, DC are two orders of magnitude more potent T_H cell activators than other immune cells, making them central players in the regulation of immunity and tolerance. Apart from presenting antigens, DC are also capable of scavenging pathogens and secreting cytokines. As a consequence, DC are considered an important link between the innate and adaptive immune system [25].

It is not surprising, therefore, that DC-based immunotherapy has been explored widely for tumour-clearing efficacy. Currently, human monocyte-derived dendritic cells (moDC) are the preferred type of dendritic cell used in DC-based vaccination trails. MoDC are DC differentiated *ex vivo* from blood-derived monocytes, which are readily available DC precursors [26,27]. After differentiation, moDC are manipulated into presenting tumour-specific antigen. In this way, they are able to stimulate protective and therapeutic

immunity against cancer in mice and humans [9,28]. Thus, moDC have been used as therapeutic tools in cancer vaccines for over a decade. Whereas most vaccinated patients display expanded antigen-specific immunity, so far only a small proportion of patients demonstrate objective durable tumour regressions [29]. Moreover, moDC require about 7 days and a considerable array of different compounds to mature, which may have a negative effect on their immunological qualities [30]. Therefore, it might be necessary to look at new sources of DC for cancer vaccines.

DC subsets

Although DC express a distinct set of cell surface markers, such as DC-SIGN and DEC-205, there is no unique cell-surface antigen that can be used to identify all DC. This can partly be explained by the fact that DC are a heterogeneous group of cells comprising functionally distinct sub-populations (Figure 2) [27]. The functional plasticity and diversity of these naturally occurring DC subsets can be exploited in the design of an effective cancer vaccine. DC subsets are found to circulate the peripheral blood and the epidermis and dermis of the skin in mice and humans [31,32]. Since the human equivalents to many mice DC subsets have been found already [33], this review will mainly focus on human DC subsets.

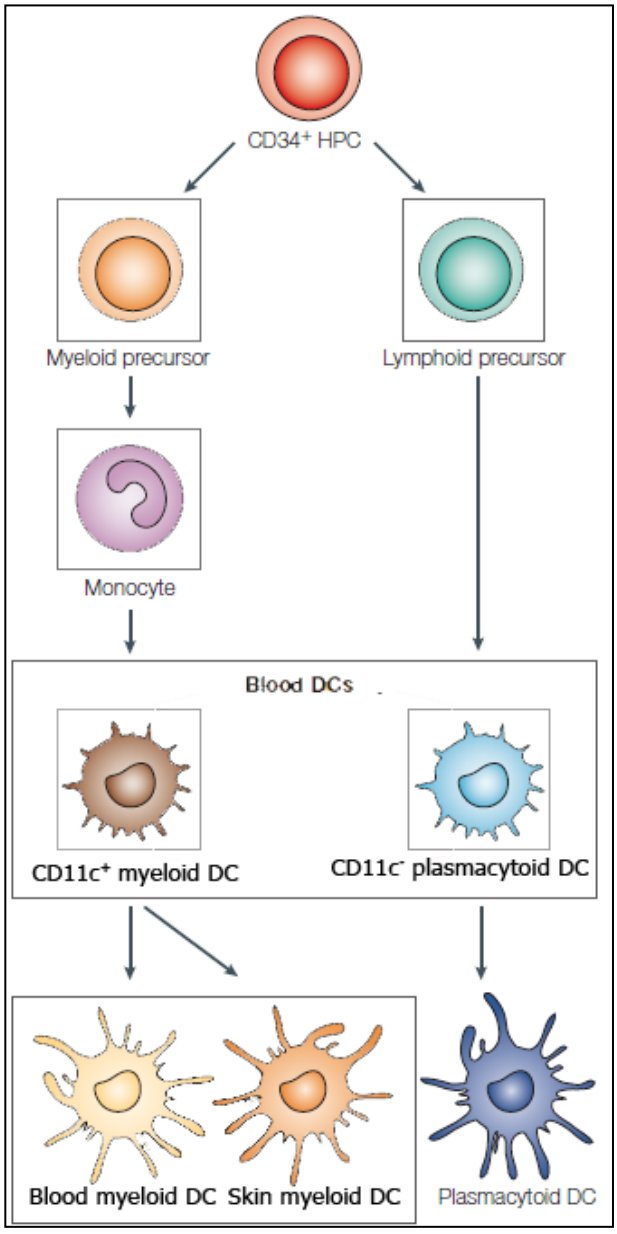

Fig. 2) Human DC subsets and their lineage. Hematopoietic stem cells (HPC) in the bone marrow can differentiate into myeloid and lymphoid precursors. Myeloid precursors develop through monocytes into blood myeloid DC, while lymphoid precursors end up as plasmacytoid DC. Whereas plasmacytoid DC stay circulating in the blood, a subset of myeloid DC migrate to the skin to become epidermal and dermal DC subtypes. Picture adapted from Banchereau and Palucka (2005) [3].

Apart from moDC, research in humans has mostly focused on myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC). Expressing diverse surface molecules and hailing from different sources, these subsets are proposed to have distinct functional adjustments in the innate and acquired immune system [32]. The specialized role of human DC subsets in governing the functioning and differentiation of CD4+ T_H1 cells, tumour-specific CTLs and Treg cells is a subject of active research. This review will discuss the latest findings in the research area of human DC subsets in the use of cancer therapy, and discuss the improvements needed to be made for the development of suitable anti-tumour vaccines. But first, it is necessary to understand the importance of DC functional plasticity and functional state in the

regulation of the activity of DC and to discuss how these features contribute to the specified role of dendritic cell subsets in the human immune response against cancer.

DC maturation, plasticity and functioning

The flexibility of the DC system does not only depend on the presence of specialized DC subsets in diverse locations. Functional plasticity and functional state of DC and their monocyte precursors are also key features of DC biology.

Circulating DC need to change functional state before they are capable of inducing a desired immune response. That is, DC have to switch from a resting state, also called immature state, into an activated or mature state which is more immunogenic. The functional and morphological modifications leading to the process of DC maturation are dependent on a combination of two signals. Firstly, DC have to recognize and take up foreign antigen. Secondly, DC require an activation signal from the environment, including microbial stimuli, tissue-derived factors and interaction with cells from the immune system [1]. However, DC are able to respond differentially to the various activation signals from the environment. To be precise, the phenotype and biological function of the mature DC are a direct consequence of the encountered activation stimuli. Hence, DC functional plasticity and maturation are important concepts in designing a DC-based vaccine that elicits anti-tumour immunity [27].

Immature and mature DC states

DC circulating peripheral tissues in steady-state conditions are usually considered immature. Although immature DC are exceedingly adept at taking up and sequestering antigen by endocytosis, they are incapable of effectively processing and presenting antigen or releasing immunostimulatory cytokines. In addition, immature DC demonstrate impaired migration *in vivo* [34] and may induce tolerance rather than CTL activation upon administration [35]. These poor therapeutic properties are due to low surface expression of costimulatory molecules and low amounts of receptors that stimulate migration through chemokine-binding. Moreover, the MHC class II molecules of immature DC are localized to the endosome-lysosome compartment. Thus, the immature DC is weakly immunogenic and poorly stimulates T-cell functioning [36].

The stable accumulation of peptide-MHC class II complexes needed for efficient antigen presentation is triggered by interaction with microbial pathogens and cells of the immune system. Maturation upregulates expression of adhesive molecules CD11 and ICAM-1, -2 and -3, as well as expression of MHC class II complexes and chemokine receptors such as CCR7. Also upregulated are the DC-characteristic B7.1, B7.2 and CD40 costimulatory molecules. B7 is meant to bind to CD28 on the surface of T cells, whereas the CD40 protein binds to the T cell-specific CD40L molecule [12,23]. The phenotypic changes associated with maturation promote DC migration to the draining lymph node. Furthermore, they enhance DC ability to interact with antigen-specific T cells through more efficient antigen presentation and cytokine production [35]. Hence, DC-based cancer vaccines usually employ mature DC.

DC activation signals and functional plasticity

Circulating steady-state immature DC continuously scan the body for various threats. Upon encountering a 'danger signal', DC are required to mature and elicit an immune response that adequately deals with the specific danger. However, as a wide range of microbial stimuli, tissue-derived factors and interaction with other immune cells all serve as danger signals, DC need to be endowed with functional plasticity to cope with each specified situation. Hence, depending on the nature of the encountered activation signal, DC are able to attain a range of immunostimulatory, immunoregulatory or tolerogenic characteristics in the mature state in response [37]. The concept of functional plasticity and its implication on DC activation are depicted in Figure 3.

A major group of 'danger signals' to activate DC are microbial stimuli [38]. Structurally-conserved pathogen-associated molecular patterns (PAMP), or pathogen-derived products present on or in microbial pathogens, are recognized by pattern recognition receptors (PRR) expressed by DC and other immune cells. Four major families of receptors comprise the PRR: 1) Toll-like receptors (TLR), 2) C-type lectins (CLR), 3) RIG-I-like receptors (RLR) and 4) NOD-like receptors (NLR). These receptor families each comprise many members which are differentially expressed by DC subsets. Consequently, DC PRR fulfil many biological functions [39]. For instance, members of the NLR family, including NALP1 and IPAF, are known to be involved in the maturation of proinflammatory cytokines such as pro-IL-1β [40,41]. Conversely, RLR like MDA5 and LGP-2 recognize RNA viruses in most nucleated cells and in response trigger the expression of IFN-α/β [42]. Stimulatory CLR are capable of enhancing the production of proinflammatory cytokines, whereas inhibitory CLR hinder the activity of TLR-mediated immune complexes. Well-studied CLR are dectin-1, MINCLE and CLEC9A [43].

Nevertheless, the best-studied PRR in anti-cancer immunity are TLR, which stimulate cytokine secretion and antigen presentation upon stimulation. Depending on the type of receptor, the family of TLR recognize various PAMPs and elicit different responses. For example, TLR2 recognizes lipoproteins and zymosan and induces an IL-10-mediated T_H2 immune profile. Contrastingly, TLR4 recognizes lipopolysaccharide (LPS) and elicits a T_H1 immune response through IL-12 production. Other important TRL expressed by DC subsets are TLR7 and TRL9, which recognize the microbial-specific genomes single-stranded RNA and CpG oligo-deoxynucleotides, respectively [44]. Interestingly, the type of pathogen also influences the response of DC to their PAMPS. Whereas LPS from *Escherichia coli* does induce a T_H1 immune response, exposure to LPS from *Porphyromonas gingivalis* does not [45,46].

The DC system is not only adapted to respond to various microbial stimuli, but also to recognize signals from the immune microenvironment. Differential factors during the development of DC subsets, such as cellular interactions and cytokine secretion, determine their functional potential. Especially cytokines are potent DC activation stimuli that have a large influence on the final phenotype of mature DC by stimulating various distinct signal transduction pathways [47,48]. For example, incubation with IL-15, TNF or IFN-α gives rise to immunostimulatory DC through the induction of STAT4 and IRF-8 signalling [49-51]. Conversely, activation of NF-κB and STAT6 signalling through incubation with TSLP, IL-10 or vitamin A yields DC that maintain tolerance [52,53]. Although it remains unclear whether these *in vitro*-cultured DC have an *in vivo* counterpart, it is known that tissue-localized DC can be polarized by interferons and interleukins produced by other cells from the environment, such as γδ-T cells, NK cells, stromal cells, lymphocytes and

mast cells. In theory, these differentially polarized DC will induce distinct responses from T cells, leading to stimulation or regulation of the immune system [54].

Apart from indirect interaction through cytokines secreted by other cells, DC also directly interact with cells from the innate and adaptive immune system. In the periphery and the secondary lymphoid organs, DC are capable of reciprocally interacting with NK, natural killer T (NKT) and γδ T cells [55]. The activation of NK is completely dependent on DC interaction, as mice studies suggest [56], and NKT cells and γδ T cells are also found to be activated by mature DC. Upon activation, these cells enhance their capacity to secrete IFN-γ, which in turn polarizes DC to induce T_H1 responses. In addition, γδ T cells secrete TNF-α and NKT cells secrete IL-4. NKT cells further acquire the capacity to kill tumour cells and to express CD40L, inducing strong DC activation [55]. DC are also known to directly interact with both T and B cells. DC are responsible for T-cell priming by inducing immune tolerance in several ways, e.g. by T-cell deletion and activating Treg cells [25]. Humoral immunity is also dependent on DC functioning by direct interaction with B cells and presentation of unprocessed antigen [57].

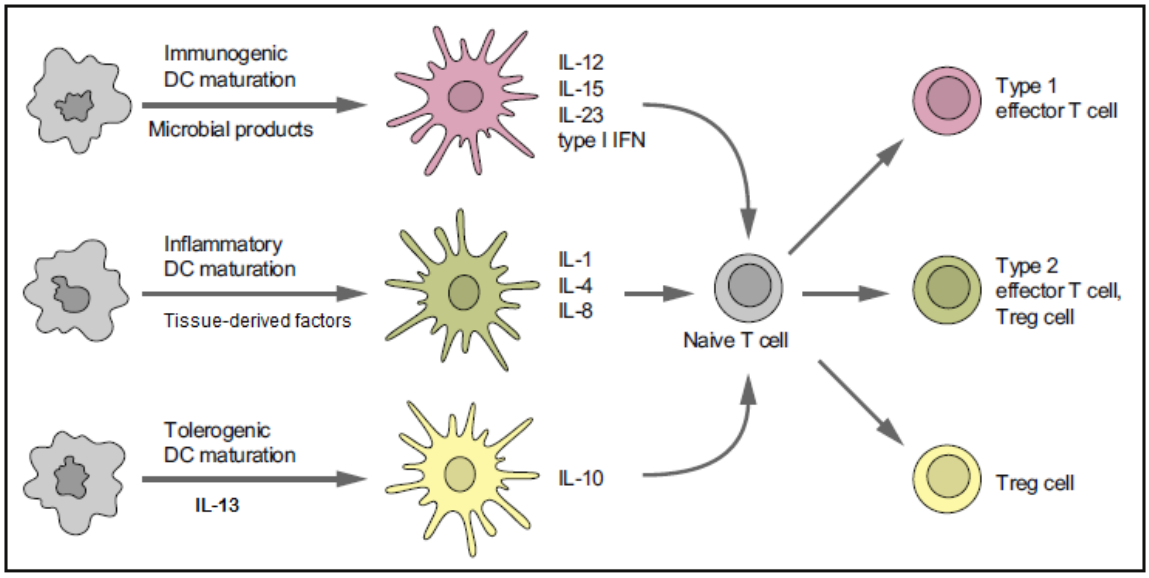

Fig. 3) DC plasticity and maturation. DC exist in different functional states: immature and mature. To become activated, DC can receive different activation stimuli. The final phenotype and function of the mature DC depends on the type of activation signal it received. Hence, the type of immune response elicited is dependent on the activation stimuli. Picture adapted from Palucka *et al*. 2010 [4].

DC plasticity and subsets

The presence of distinct DC subsets with the quality of functional plasticity allows the DC system to cope with both maintenance of tolerance to self-antigens and protection against microbial pathogens, by eliciting distinctive types of immunity. Cancer vaccines aim to generate mature DC that polarize the immune system towards a strong anti-tumour response. DC that express low levels of immunosuppressive cytokines (like IL-10) and high levels of cytokines that stimulate T-cell activation (like IL-12 and IL-15) are desirable for vaccine development [13,25].

However, virtually each DC subset expresses a unique pattern of PRR and cytokine receptors that causes qualitatively different effects upon activation [44]. Consequently, the

various cytokine combinations and PRR agonists used in research do not yield mature DC of equal immunogenicity. Thus, functional plasticity renders the concept of DC activation for vaccine-based cancer therapy quite complex [38,58,59]. An improved understanding of the concept of DC plasticity and maturation in DC subsets will be of key importance in the rational design of a cancer vaccine, as specifically the choice of adjuvant would be affected [60-62].

Monocyte-derived dendritic cells

Currently, most clinical studies utilize monocyte-derived dendritic cells (moDC) for cancer vaccines [26]. As the name implies, this type of DC are differentiated *in vitro* from human monocyte cultures. Monocytes are a type of $CD14^+$ pre-DC white blood cell that develop from myeloid progenitor cells in the bone marrow (see Figure 2) and circulate the bloodstream as a source of replenishment for resident macrophages and DC [14]. Consequently, monocytes present a readily-available source of easy to isolate DC progenitors that can propagate *in vitro* into vast numbers. Between three to eight percent of the leukocytes in the blood are comprised of monocytes, and to obtain ± 100-150 x 10^6 cells, one leukapheresis is sufficient. As a result, monocytes yield large amounts of moDC for research and DC-based cancer vaccines.

MoDC cultivation is a complex process in which every step can influence their eventual phenotype and immunostimulatory properties. Before moDC are ready for use in cancer vaccines, monocytes are isolated from the blood and differentiated into immature DC. Subsequently, tumour-specific antigen is loaded and maturation is induced. Thereafter, the moDC are studied for immunotherapeutic properties by *in vitro* or *in vivo* testing. Every single compound used has an effect on the total time of the cultivation process and the clinical features of the activated moDC, clearly illustrating the effect of functional plasticity. The most commonly used methods of moDC generation are summarized in this chapter and should be regarded as a review of the current standard proceedings in DC-based vaccine development. Nevertheless, the ideal moDC cultivation process remains to be discovered and optimizing the current methods is a field of active research.

Monocyte isolation, differentiation and antigen loading

To obtain moDC for research purpose, monocytes must be isolated from the human blood before they can be differentiated. Several techniques can be used for this, dependent on the research intention. For usage in preclinical trials, all peripheral blood mononuclear cells (PBMCs), including monocytes and lymphocytes, are usually isolated from the peripheral blood or buffy coat by gradient centrifugation. Subsequently, plastic adherence methods suffice to separate the monocytes from the lymphocytes [50]. For clinical studies, in which a higher monocyte yield and purity is desired, immunomagnetic separation or elutriation can be performed. Although immunomagnetic separation achieves monocyte isolation with very high purity, elutriation gives a much higher yield and would be the favoured method of isolation [63].

Isolated monocytes can be cultured in different media, including serum-free medium, autologous plasma or medium with human serum. Subsequently, immature DC are

generated by supplementing the medium with a cytokine cocktail. The two main ingredients of this cocktail are granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. GM-CSF is a white blood cell growth factor that serves to induce monocyte survival, proliferation and differentiation, while IL-4 is added to the medium to block the development of the monocytes into macrophages. Hence, the combination of GM-CSF and IL-4 is standard for DC generation, although incubation period (± 7 days) and cytokine concentration differ per research group [61]. The resulting immature DC express high levels of CD11c and are capable of efficient antigen uptake [27].

As immature DC are superior in antigen uptake compared to mature DC, tumour-specific antigen is loaded in the DC before maturation is induced [61]. The choice of tumour antigens is quite important, as it will determine the specificity of the elicited T-cell response. Tumour-specific antigens, usually mutated antigens or shared self-antigens, are conventionally loaded into moDC via plasmid transfection, electroporation or viral transduction. These methods provide consistent and efficient expression, although viability after transfection and electroporation is rather low [64]. Therefore, moDC are sometimes fused with tumour cells to create potent immunostimulatory APC [65]. Still, techniques for tumour cell fusion have been found to be inefficient and require improvement [66]. DC can be loaded with a complete tumour-specific molecule, only a short peptide or the part of the antigen recognized by the immune system, called the epitope. Other sources of antigen that can deliver multiple epitopes are mRNA, DNA or tumour proteins isolated from tumour cells [67].

MoDC maturation cocktails

Since mature DC are more immunogenic and are capable of eliciting tumour-specific T cell responses, maturation is induced in the antigen-loaded immature moDC before administration to the patient. The maturation process can last between 24 hours to 3 days depending on the used activation compounds. The contents of the maturation cocktail also determine the final characteristics and phenotype of the matured moDC, which corresponds to the concept of functional plasticity. Hence, several natural stimuli for DC activation have been tested, notably pro-inflammatory mediators, microbial and viral patterns and DC-costimulatory receptor ligands. An overview of various activation stimuli and their effects is depicted in Figure 4 [67].

Most maturation cocktails are composed of pro-inflammatory mediators. The "standard maturation cocktail" consists of combinations of TNF-α, IL-1β, IL-6 and prostaglandin E2 (PGE2), although many clinical studies only use TNF-α to induce activation [61]. Maturation of these "standard moDC" is generally induced 7 days after monocyte isolation, although it has been shown that earlier maturation can elicit improved protein expression and higher capacity for T-cell stimulation [68]. *In vitro*, usage of the standard maturation cocktail enhances the quality, migratory ability and yield of generated moDC by stimulating IL-12 production and CCR7 expression while downregulating IL-10 production. Proliferation of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells is also enhanced in the presence of this cocktail [69,70]. Addition of IFN-γ to the maturation cocktail is commonly employed to increase IL-12 secretion, even though studies have indicated it to have a negative effect on CCR7 expression and migration ability [71].

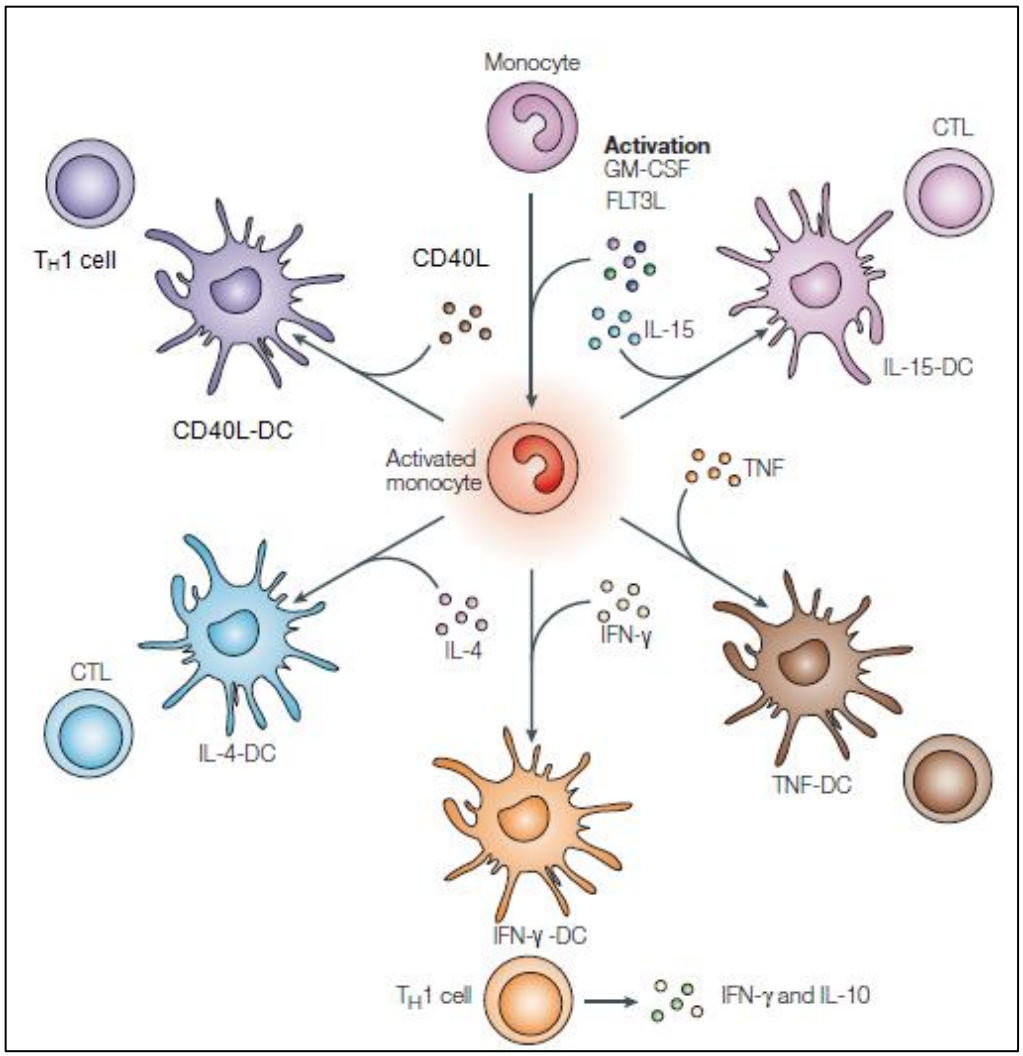

Fig. 4) MoDC and their plasticity. Monocytes can switch to an activated state through cultivation with Granulocyte-macrophage colony-stimulating factor (GM-CSF) and the hematopoietic growth factor FLT3L. To induce monocytes to differentiate into DC, they can be incubated with a variety of activation stimuli. The phenotype and immune response that the resulting DC elicit, depend on the compounds of the maturation cocktail. Picture adapted from Banchereau and Palucka (2005)[3]

TLR agonists are also commonly used as activation stimuli, as they are potent inducers of pro-inflammatory cytokine production. MoDC have been shown to express TLR1-8, with especially high concentrations of TLR2 and TLR4. All TLR stimulate production of IL-12, yet each family member can have a unique effect on moDC functioning [39,44]. TLR agonists commonly used in research are R848, polyinosinic:polycytidylic acid (poly I:C), peptidoglycan (PGN) and CL097. It is known that combinations of TLR agonists stimulate moDC function in a synergistic way. For instance, combination of poly I:C with TLR7/8 agonists R848 or CL097 enhances moDC migratory capability and improves immunostimulatory effects concerning NK cells, CTL functioning and $CD4^+$ T cell cytokine production better than poly I:C alone [70,72]. Although peptidoglycan (PGN) does not have great immunostimulatory effect as a TLR2 agonist, it can strengthen TLR-mediated immune responses by acting as an agonist of NLR family members NOD1/2 [73].

A popular compound often added to novel moDC maturation cocktails is CD40L, the ligand for the costimulatory receptor CD40. Activation of this DC receptor leads to

production of the T_H1-skewing cytokine IL-12 [20]. CD40 stimulation also increases expression of MHC classes and costimulatory molecules, which enhances DC capacity to activate T cells [74]. Furthermore, CD40L-activated moDC produce high amounts of IL-23, which sensitizes them to costimulation with various danger signals like single-stranded and double-stranded RNA [75].

MoDC-based clinical trials

The majority of vaccine-based phase I and phase I/II trials have employed moDC activated by the original standard maturation cocktail or a modification of it, although moDC activated solely by TNF-α have also been investigated. MoDC vaccines have been tested in patients with amongst others melanoma, multiple myeloma, prostate cancer, acute myeloid leukaemia, pancreatic and biliary tumours, metastatic renal cell carcinoma or glioblastoma multiforme [76]. MoDC-based vaccination in cancer patients was determined to be feasible, safe and without severe adverse events. Nevertheless, the *in vitro* immunological potential has not yet been achieved *in vivo*. T_H1/T_H2 balance generally remained unaffected after vaccination, nor did the concentration of antigen-specific antibodies increase. About 10-20% of the tested patients showed stabilization of disease for several months after vaccination, whereas about 40-50% demonstrated antigen-specific T-cell responses. Remarkably, clinical responses generally did not correlate with the measured *in vivo* immune response [77-85]. Thus, despite their therapeutic potential, vaccines employing moDC matured through standard maturation cocktails or TNF-α have not elicited immune responses of the quality required to achieve tumour rejection.

The reason for the limited immunological and clinical effects of current moDC-based vaccines can be attributable to DC exhaustion. The immunostimulatory effects of moDC maturation are temporally regulated. Upon removal from the maturation cocktail, fully matured DC quickly lose their ability to secrete sufficient levels of cytokines and their ability to prime naïve T cells to differentiate into CTL becomes attenuated [15,86]. As a result, upon moDC vaccination both $CD8^+$ and $CD4^+$ T cell are not sufficiently stimulated to elicit an anti-tumour response and remain in an anergic state [87]. The tumour microenvironment probably maintains this anergic state through induction of Treg cells or myeloid-derived suppressor cells [88]. Consequently, $CD4^+$ T cells are skewed to a T_H2 response, which promotes tumour development, polarizes tumour-associated macrophages and blocks stimulation of $CD8^+$ T cells [89,90]. The $CD8^+$ T cells that do get induced either lack the migratory capacity to travel into the tumour lesions, or are of such low avidity that they are not able to recognize tumour antigens presented through MHC class I on the cellular surface of target cells [91]. Thus, exhausted moDC are suboptimal in inducing T-cell effector and tumour-homing properties.

Although it remains unclear if the limited *in vivo* effects can be exclusively attributed to the selected compounds of the maturation cocktail, it has been argued that DC triggered through TLR agonists are more potent in the stimulation of the adaptive immune system than cytokine-driven DC. Maturation by solely proinflammatory cytokines yield exhausted DC, whereas TRL agonists yields mature moDC with sustained high IL-12 production that efficiently promote T and B cell help [92]. Interestingly, incubation with a combination of IFN-γ and a TLR4 agonist, such as LPS or FMKp, yield semi-mature 'polarized' moDC that are skewed towards eliciting a T_H1 immune response even after

removal from the maturation cocktail and administration to cancer patients [93]. Importantly, T cells stimulated by polarized moDC show the capacity to efficiently traffic into tumour sites and mediate therapeutic effects [15]. Phase I-II clinical trials with polarized moDC have achieved antigen-specific $CD8^+$ T-cell responses in 50-90% of vaccinated patients. Despite the improved immunological response, still only 10-30% of the patients demonstrated a complete or partial clinical response. These results suggest that DC exhaustion is probably not the exclusive reason for the current clinical failure of DC-based vaccines [94,95].

Concluding remarks on moDC

Since no definite objective clinical responses have been achieved with the current methods, research aims to find the optimal composition of maturation cocktails that allows sustained DC activation. In a large comparative study of various maturation cocktails by Zobywalksi *et al.* (2007), it was found that a combination of TNF, IL-1β, IFN-γ, R848 and PGE2, with the possible addition of poly I:C, yields the most suitable moDC for anti-cancer vaccines [96]. In a more recent *in vitro* study, it was demonstrated that a quadruple combination of activation stimuli (IFN-γ, LPS, R848, and CD40L) stimulated the generation of T_H1-polarizing DC better than combinations involving less or more components. Multiple activation stimuli promote moDC maturation not only by recruiting more IL-12 secreting cells, but also by stimulating those cells to produce higher levels of IL-12 [97]. While these novel maturation cocktails have only been tested *in vitro*, the discovery of novel immunostimulatory compounds, such as OK432 and pro-inflammatory kinin peptides, keeps the search for the optimal culturing conditions ongoing [98,99].

Currently, every author uses a different method of moDC generation, making it quite difficult to compare the results of published moDC clinical trials. A standardized protocol for the generation of moDC-based vaccines would allow reliable meta-analysis of clinical data and facilitate the search for the optimal maturation method [100]. Nevertheless, as active moDC-based clinical trials still seem to be using different methods of moDC generation [101], the current situation is not likely to change in the near future.

Myeloid dendritic cells

Monocytes are easy to isolate and can be propagated into large numbers *in vitro*. Conversely, circulating DC in human blood have very low frequency and are difficult to isolate, requiring several leukaphereses to obtain a sufficient yield. Hence, the majority of DC biology research has focused on *in vitro*-generated moDC instead of blood-derived DC. Nevertheless, the generation of moDC involves laborious protocols with compounds that can negatively affect immunotherapeutic capacity. Furthermore, as different moDC preparations yield obvious functional differences, it is questionable in how far moDC are comparable to natural DC. Thus, over the last few years research attention has shifted to characterizing and understanding naturally occurring human DC subsets, which do not require extensive culturing before administration.

A major DC type are myeloid DC (mDC), also called classical or conventional DC (cDC). Even though there are many differences in phenotype and effector functions, mDC

are thought of as the blood-counterpart of moDC [59]. All human mDC express CD13, CD33, MHC class II and CD11c, but lack lineage-specific markers CD3, CD14, CD19 and CD56. Deriving from myeloid- or lymphoid-committed precursors (see Figure 2), mDC are the main *in vivo* producers of IL-12. However, as tissue-localized mDC are functionally polarized by interaction with various cells and their products, mDC are capable of secreting a wide range of other inflammatory cytokines [54]. Thus, mDC can induce T_H1, T_H2 and antigen-specific CTL responses, making them interesting tools for immunotherapy [102].

In humans, mDC are divided into functionally distinct subsets. These subsets are present in peripheral blood, secondary lymphoid organs and skin and are defined by the expression of myeloid markers. Blood mDC are subdivided into three groups based on differential expression of the surface markers CD1c (BDCA1), CD16 and CD141 (BDCA3), whereas skin mDC subsets are identified by expression of langerin, CD1a or CD14. Not only do these mDC subsets differ in location and surface molecule expression, but also in cytokine secretion and capacity to stimulate T- and B-cell responses [32].

Blood mDC

Approximately 0.4% of all circulating PBMC are recognized as mDC [103-105]. Through reciprocal expression of CD1c (BDCA1), CD16 and CD141 (BDCA3), three types of mDC can be distinguished in the blood. Each subset can be isolated and separated by gradient centrifugation, followed by marker-specific immunomagnetic depletion and flow cytometry-based cell sorting [106]. CD16-mDC are the most prevalent type, covering 65-75% of blood mDC. CD1c-mDC comprise 10-20% of blood mDC, while CD141-mDC account for the remaining 3-5%. These last two subtypes share similar expression profiles and can also be found in lymph nodes, the tonsils and the bone marrow [107]. Although CD16-mDC have the highest frequency in peripheral blood, CD1c-mDC have been studied more extensively [32].

$CD1c^+$ mDC are the "classical DC" and highly potent APC [108]. They are characterized by the presence of the MHC class I-like antigen receptor molecule CD1c, also known as BDCA1, which recognizes mycobacterial cell wall lipids [109]. CD1c-mDC are considered to be the most potent T-cell inducers of the three blood mDC populations [32] and are especially potent in inducing lipid-associated T cells [110]. Through expression of CD150, they could play an additional role in NKT cell development [111]. Furthermore, CD1c-mDC appear to be important in inducing chemotaxis in response to inflammation, as they produce high levels of several chemoattractants and express various chemokine receptors [108,112]. Their TLR expression profile is very similar to that of moDC, but with elevated expression of TLR2 and TLR3 [44,112]. Correspondingly, TLR2 agonists stimulate proliferation of mature CD1c-mDC to high frequencies, which are associated with tumour regression and improved progression-free survival [103,113].

The second blood mDC subset, CD16-mDC, are inflammatory DC characterized by CD16, a receptor for the Fc region of IgG. They are capable of high pro-inflammatory cytokine secretion, especially TNF-α, GM-CSF and granulocyte colony-stimulating factor. CD16-mDC respond strongly to TRL agonists LPS and R848, but weakly to poly I:C as they lack TLR3 expression [44,112]. It is known that CD16-mDC are similar to $CD16^+$ monocytes and strongly express CXCR5 and dectin, an observation which further directs

the functional analysis of CD16-mDC in several disease processes [108]. CD16-mDC are considered the second most potent T cell inducers [32].

The CD141-mDC, or BDCA-3$^+$ mDC, are the third blood mDC subset. It is an unique subset that has recently been discovered to be the human homologue of the T_H1-polarizing mouse CD8$^+$ DC subset. Consequently, human CD141-mDC are capable of directly binding NK cells, NKT cells and activated CD8$^+$ T cells, while secreted signals from these cells induce CD141-mDC migration [114]. CD16-mDC do not express TLR4, 5, 7 and 9 [115]. Upon TLR agonist stimulation, human CD141-mDC produce IFN-β and IL-12. Compared to the other human mDC subtypes, CD141-mDC excel in cross-presenting soluble or cell-associated antigens for presentation to T_H1 and CD8$^+$ T cells on HLA class I molecules [116]. Thus, human CD141-mDC are professional APCs that are well-equipped to generate T_H1, CD8$^+$ T-cell and NK-cell immunity.

Skin mDC

The best studied human mDC subsets are the skin mDC subtypes. At least three skin mDC subsets can be identified, not only by differential expression of cell surface markers but also by localization. The epidermis is host to Langerhans cells (LC), whereas the dermis contains the two CD1a$^+$ mDC and CD14$^+$ mDC subtypes [117]. All these subtypes differ in their activated-state molecular profiles and cytokine secretion pattern, leading to specialized biological functions in immune regulation. For research purposes, skin mDC are often generated from blood CD34$^+$ hematopoietic progenitor cells (HPC) cultured with GM-CSF, Flt3-L and TNF-α. Subsequently, the cells are allowed to migrate through skin samples, where they acquire properties specific to cells associated with that location. Therefore, these cells can be perceived as moDC with skin mDC-like properties [118].

The 'classical' LC reside in the supra-basal layer of the epidermis where they are ideally situated to sample external antigen, for example from invading microorganisms. They can also be detected in stratified epithelia other than the epidermis. LC represent about 58% of all skin mDC [118] and can be recognized by their expression of human leukocyte antigen (HLA)-DR, CD1a and the lectins DCIR and langerin (CD207). The latter marker is unique for LC and allow for unequivocal identification [119]. TLR expression of LC is restricted to TLR1, 2, 6 and 10 [120].

LC have conventionally been perceived as a paradigm population in DC biology. Upon encounter and uptake of antigen in the epidermis, LC would undergo a complex maturation process that increases their migratory capacity. Subsequently, they would leave the skin and migrate to the draining lymph nodes. Once in the nodes, LC would stimulate T cells and consequently induce antigen-specific immunity. Although LC are suggested to have a role in mediating and maintaining peripheral tolerance, the capacity of LC to stimulate T-cell functioning through the secretion of the T-cell growth factor IL-15 has been proven *in vitro* and *in vivo* [121]. Accordingly, LC are extraordinarily efficient in cross-presenting peptide antigens to high-avidity CD8$^+$ T cells [122]. As a result, the CD8$^+$ T cells proliferate and acquire potent cytotoxicity towards tumour cell lines. In addition, LC induce robust proliferation of T_H cells [118]. These functional properties make them attractive targets for cancer vaccines.

CD14-mDC are present in the dermis. Recognized by a CD1a$^-$CD14$^+$HLA-DR$^+$ marker expression profile, they account for about 12% of all skin mDC [118]. At mRNA level, CD14-mDC express TLR2, 4- 6, 8 and 10 [120]. In contrast to LC, CD14-mDC seem to be

specialized in inducing the differentiation of naïve B cells into IgM-producing plasma cells through the production of a large set of soluble factors, including IL-6 and IL-12 [118]. Apart from directly stimulating B-cell differentiation [123], these factors induce the generation of IL-21-secreting T_{FH} cells that help naïve B cells to produce large amounts of IgM. Only CD14-mDC-activated T_{FH} cells induce immunoglobulin class switch to IgG and IgA [20]. A recent study demonstrated that $CD14^+$ monocytes and mDC are necessary for enhancing early CD40L expression on peripheral blood $CD4^+$ T cells, suggesting that CD14-mDC are also important in early-phase T-cell activation [124].

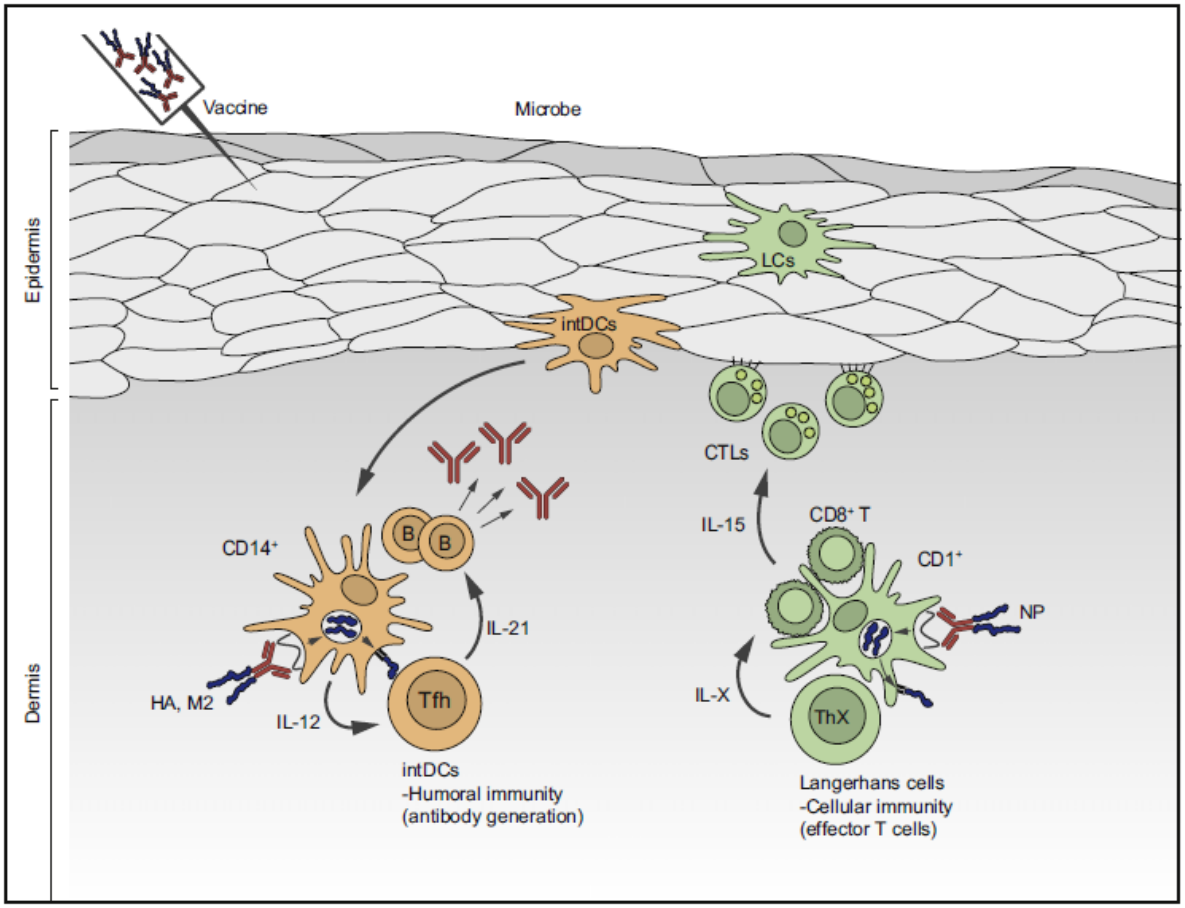

Fig. 5) Langerhans cells (LC) and dermal (interstitial, intDCs) DC in the regulation of T- and B-cell immunity. Skin mDC are positioned to receive foreign antigen in the shape of invading microbes or subcutaneous vaccine injection. Epidermal LC induce cellular immunity through CTL activation, mediated by IL-15. In contrast, $CD14^+$ mDC induce humoral immunity through activation of T_{FH}-mediated plasma B cell proliferation. Picture taken from Palucka *et al*. (2010) [4]

Taken together, these data suggest that skin mDC regulate two different components of the adaptive immune system, as is depicted in Figure 5. CD14-mDC are responsible for B-cell regulation, whereas LC are potent T-cell inducers. Similarly, epidermal LC are not capable of B-cell induction, while dermal mDC are inferior in cross-presenting antigen to $CD8^+$ T cells [125]. The last dermal mDC subset, CD1a-mDC, are considered to be functionally intermediate between the other two subsets [118]. They are capable of IL-12 secretion and the skewing of T_H1 responses [126]. Little else is known about them, so whether they represent a unique aspect in immune regulation needs to be addressed.

MDC in tumour immunity and vaccine trials

In a mature state, mDC are associated with tumour regression and good prognosis [103]. Conversely, immature mDC are associated with immune tolerance. Accordingly, accumulation of immature mDC in tumour sites suggest that neoplasmatic processes hamper mDC maturation [127] and polarize mDC to stimulate the development of IL-13 producing T_H2 cells. Apart from facilitating tumour development directly [89], IL-13 induces mDC to secrete tumour growth factor TGF-β which inhibits CTL functioning [128]. Apart from soluble factors, anergic mDC can also promote survival and clonogenicity of tumour cells through direct interactions [129,130].

Due to their fairly recent characterization and low frequency, mDC have not been regularly utilized in DC-based vaccines. Only a minority of phase I/II clinical trials report to have isolated dendritic cells from the blood of human donors by gradient centrifugation. These cells should be functionally and phenotypically similar to moDC, but have not undergone extensive maturation with exogenous cytokines. Blood-derived DC loaded with tumour antigen have been applied safely and without major adverse effects to patients with prostate cancer, multiple myeloma, B-cell lymphoma or various solid tumours. The results have been fairly positive so far, as about 70% of patients developed an antigen-specific response. Nevertheless, not all clinical responses correlated to the measured immune response and only few patients demonstrated complete or partial clinical responses [131-134].

Although skin mDC have not been actively isolated for use in clinical trials, $CD34^+$ DC precursors cultured with IL-15 *in vitro* differentiate into DC with remarkable LC-like properties [44,49]. Immunological and clinical responses have been observed in melanoma patients vaccinated with these DC. Patients that mounted a specific response against at least two melanoma antigens survived for a longer time than the patients who were unable to elicit such responses [135].

Concluding remarks on mDC

In the human body, mDC can be predominantly found in the blood and the skin, where they are comprised of functionally distinct subsets. Blood mDC are easy to identify, and the protocols required for vaccine preparation are not very different from those used in the preparation of moDC. Although $CD16^+$ mDC might be the most prevalent subtype, $CD1c^+$ and the newly characterized $CD141^+$ mDC demonstrate more immunostimulatory properties. Unfortunately, their extremely low frequency could be a limiting factor for practical use. A further problem are their tumour-stimulating properties when in the immature state, and administration of maturating vaccine adjuvants could be of crucial importance.

Notwithstanding the immunotherapeutic potential of blood mDC, current focus is at the skin mDC subsets as they more easily accessible through subcutaneous injections with activating cytokines. Both LC and CD14-mDC are quite well-characterized, and it might be interesting to develop a method that targets both these subsets simultaneously (see Figure 5). In theory, this would yield a strong T-cell and B-cell response against tumour-specific antigen. The identification of novel skin mDC subsets, such as $langerin^+$ DC and $CD1c^+$ DC in the dermis [121,136] continue to deliver new insights into the role of DC

subsets in immune regulation. How all these mDC subsets cooperate to shape the immune system remains to be discovered.

Plasmacytoid dendritic cells

Blood DC are not only composed of mDC. About 0.37% of circulating PBMC are accounted for by a unique DC subset called plasmacytoid DC (pDC) [104,105,137]. PDC are potent APC capable of efficient antigen uptake, processing and presentation. They are foremost considered as key players in antiviral immunity due to their capacity to secrete high amounts of type I interferons upon viral encounter [138]. A suggested role in tumour-specific immune responses has earned them much attention, although their specific position in anti-cancer immunity remains largely undefined [139].

PDC are biologically distinct from mDC in several aspects. First of all, pDC are devoid of myeloid lineage markers and do not express CD11c. Instead, they are identified by expression of BDCA2 (CD303), BDCA4 and CD123 [140]. Both peripheral blood and several lymphoid organs host pDC, but they are not found in the skin [127]. Just like mDC, pDC are divided into functionally different subsets by differential expression of the cell adhesion molecule CD2. $CD2^{high}$ pDC account for about 23% of all pDC and uniquely express lysozyme [141]. PDC also differ from mDC in TLR expression, immune function and cytokine secretion. For example, pDC are the only DC subset to express TLR9 [44] and secrete only negligible amounts of IL-12 when they are activated by IL-3 and CD40L [142]. Furthermore, PDC are considered the main producers of INF-α in the human body [143,144]. By secreting immunoregulatory cytokines in the mucosal tissues, pDC also play an important role in the maintenance of immunological tolerance [145].

Immunomodulatory properties of pDC

Research suggests that pDC are dedicated to recognizing viral antigens, thereby functioning as one of the first antiviral barriers. Upon viral encounter, peripheral blood pDC secrete considerable amounts of type I interferons [146] and numerous chemokines, which attract PBMC and induce inflammatory responses. Furthermore, viral encounters induce pDC maturation into CCR7-expressing DC capable of migrating to the lymph node. In the lymph node, pDC present antigen to T cells and thus initiate a specific immune response in an IL-12-independent manner [146-148]. PDC are unique in their role of initiating both innate and adaptive immune responses against viral infections through expression of TLR7 and TLR9. These TLR recognize viral-specific genomes, including single-stranded RNA and CpG-containing DNA motifs. Surprisingly, pDC lack expression of TLR3 and TLR8 that also recognize viral antigens [44].

In vitro studies have demonstrated that pDC are capable of eliciting other immune responses than those directed at viral infection. Antigen-pulsed pDC, especially the $CD2^{high}$ subset, can efficiently prime antigen-specific $CD8^+$ T cells [141,149,150]. In addition, activated pDC activate $CD4^+$ T cells and polarize them towards a T_H1 response [151,152]. Similar functional T-cell responses were obtained *in vivo* after vaccination with viral- or CpG-activated pDC [153,154]. PDC are not only important in T-cell activation, but are also

vital for the amplification of antibody responses. Secreted type I IFN mediate proliferation of non-Ig-secreting plasmablasts. Concomitant secretion of IL-6 induces their development into Ig-secreting plasma cells [155]. PDC and B cells can also directly interact via the tumour necrosis factor family ligand CD70. CpG-induced pDC activation upregulates expression of this molecule, which can bind to CD27 on the cellular surface of B cells. As a result, proliferation of both naïve and memory B cells is induced in an IFN-independent way [156].

As pDC have cytotoxic properties of their own, they can participate in the killing of target cells in T and B cell-independent way,. Through expression of the cytotoxic molecules granzyme B and TRAIL, pDC are capable of inducing apoptosis through direct cell-cell interaction [141]. Furthermore, their high type I IFN secretion may provide an endogenous adjuvant that promotes the maturation of other immunostimulatory DC populations [4]. Thus, pDC-priming strategies are promising for the next generation of vaccines, especially against viruses.

Notwithstanding their immunostimulatory properties, pDC are also important in immunological tolerance. Non-lymphoid tissue pDC play a significant role in the regulation of mucosal immunity and tolerance to inhaled or ingested antigens. They are also important in intrathymic Treg cell development and peripheral tolerance [145]. PDC mediate tolerance through expression of the immunoregulatory molecules IDO, ICOS ligand and granzyme B. IDO and granzyme B suppress $CD4^+$ and $CD8^+$ T cell responses, whereas ICOS ligand promotes proliferation of IL-10-producing Treg cells. Thus, alternatively stimulated pDC can inhibit protective immunogenic responses through the induction of Treg cells and suppression of effector T cells [2].

PDC in tumour immunity and vaccine trials

Human pDC have the ability to infiltrate solid tumours. In a wide variety of cancers, pDC have been found to be directly attracted to the tumour site, where they are maintained at an immature state that promotes tolerance and suppresses $CD8^+$ T-cell responses [157-160]. Homing of pDC to the tumour site and blockage of pDC maturation is probably achieved through the secretion of immunosuppressive factors by the tumour cells [161,162]. Immature tolerogenic pDC are also encountered in tumour-draining lymph nodes, where they promote proliferation of IL-10-producing Treg and suppress $CD8^+$ T-cell responses through expression of IDO, ICOS ligand and granzyme B [163-165]. Through direct interaction with tumour cells, pDC can even trigger growth, prolong survival, confer drug resistance and induce tumour cell migration. Soluble factors such as IL-3 and IL-10 contribute to these functions [166]. Finally, pDC may promote tumour angiogenesis through secretion of vascular endothelial growth factors [167]. Hence, the presence of immature pDC in tumours is associated with poor prognosis.

Even though pDC at the tumour site are blocked at an immature state that maintains an immunosuppressive environment, the presence of mature pDC is associated with potent anti-tumour responses [2]. Several studies have demonstrated that cultivation of pDC with a TLR7/9 agonist can restore $CD8^+$ T-cell responses and initiate tumour regression in a NK cell-dependent manner. Topical treatment with the TLR7 agonist Imiquimod enhances recruitment of mature pDC to the tumour site and increases their IFN-production, leading to an inflammatory environment and tumour regression [168,169]. Moreover, a phase I clinical trial has demonstrated that tumour-resident pDC can be stimulated with intratumoural injection of CpG-containing oligodeoxynucleotides. CpG-

mediated TLR9 ligation enhanced the immunostimulatory actions of pDC and blocked tumour cell growth [170]. The same effect can also be reached by isolating pDC and cultivating them with CpG before administration to the patient [166,171]. The latter method might be preferred, as tumour cells secrete immunoregulatory antigen that strongly inhibit IFN-production and proinflammatory cytokine secretion by pDC. In this way, tumour cells might inhibit TLR ligation-induced anticancer responses *in situ* [172,173].

Immunostimulatory properties of pDC might also depend on their anatomical location in the body [2]. Compared to pDC present in mesenteric lymph nodes, liver and other mucosal tissues, spleen-derived pDC are much less tolerogenic. Furthermore, they are more efficient in type I IFN production after TLR ligation [174]. Conversely, cultivation with mucosal tissue factors and tumour-derived immunosuppressive factors can induce immunoregulatory properties in spleen-derived pDC [173,175].

Schreibelt *et al*. (2010) report that they have recently completed a phase I/II clinical trial in stage IV melanoma patients. Treatment with tumour antigen-loaded, TLR ligand-matured pDC appeared feasible and safe. The majority of patients elicited responses against the monitoring protein, indicating that only a small number of naturally occurring pDC is sufficient in inducing immunological responses. This observation might be caused by the stimulatory effect pDC can have on other DC populations. At the moment, the manuscript is in preparation [44]. Aspard *et al*. have developed another pDC-based vaccine that elicited strong anti-tumour efficacy *in vitro* and in a humanized mice model, but that remains to be tested *in vivo* [176].

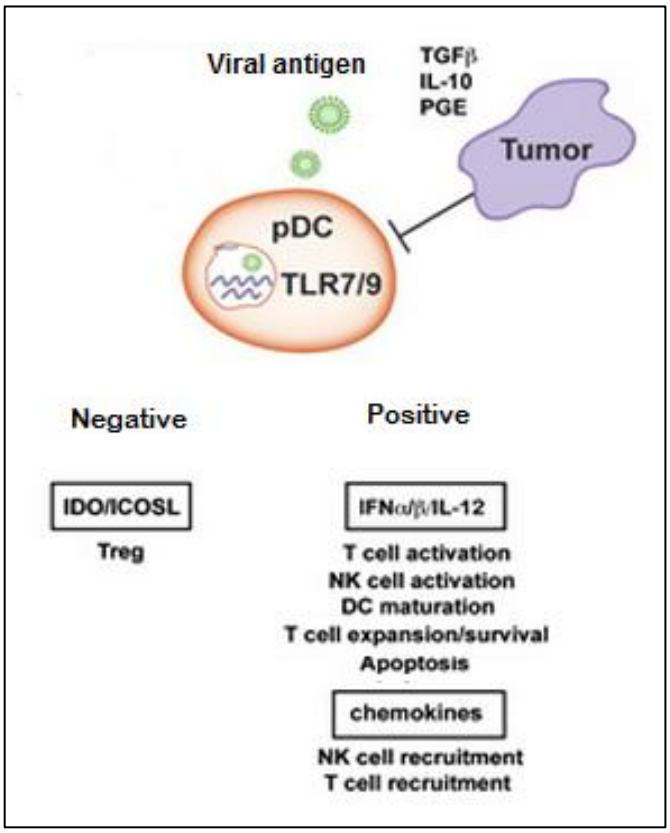

Fig. 6) The role of plasmacytoid DC (pDC) in tumour immunology.
Tumour cells secrete high amounts of immuno-regulatory cytokines that polarize pDC tolerogenic responses. As a result, pDC produce IDO and ICOSL that stimulate Treg functioning. However, under the influence of TLR7/9-mediated activation, pDC secrete a number of immune-stimulatory chemokines, type I interferon (IFN) and IL-12. Consequently, viral antigen can induce a matured pDC phenotype which stimulates T cell and NK cell activation and tumour cell apoptosis. Picture adapted from Swiecki *et al*. (2010) [2]

Concluding remarks on pDC

The immunological role of pDC is currently ambiguous. As they are capable of eliciting responses from both $CD8^+$ T cell and Treg cells, pDC seem to play an important role in both viral immunity and immune tolerance. Unfortunately, tumours can abuse the immunoregulatory capacities of circulating pDC by the secretion of modulatory factors, trapping them in an immature state that promotes immunological tolerance of the tumour. Nevertheless, when pDC hail from the correct anatomical location and receive appropriate activation stimuli, often in the form of TLR7 or TLR9 ligation, they switch functional states and generate effective tumour-specific immune responses. Thus, TLR-activated pDC-based approaches could be promising for the treatment of human cancer and are currently under investigation.

Dendritic cells subsets in therapeutic vaccines

MoDC-based vaccines have been used as therapeutic vaccines in cancer patients for more than a decade, and so far three different patient groups can be distinguished based on treatment outcomes. Firstly, the majority of moDC-treated patients fail to mount an immune response to tumour-specific antigens, and hence show no clinical improvements. The second patient group is capable of mounting tumour antigen-specific immunity, but do not undergo objective lasting tumour regression. That is, clinical responses remain absent in spite of the induction of the correct immune responses. Thirdly, in a minority of patients both an immune response and tumour regression can be detected [60]. Although the third group of patients is the smallest, these patients are essential in understanding the immune responses needed to control tumour growth and to obtain objective clinical responses. Therefore, the challenge remains to identify approaches to increase the number of this third group of patients. This objective could be achieved by improving on moDC-based vaccines, but also by utilizing the distinct biological functions of naturally occurring DC subsets.

Targeting DC subsets for immunotherapy

The major issue with employing natural DC subsets in DC cancer vaccines is that they are extremely low in frequency, making them difficult to isolate in sufficient numbers. Although moDC can be propagated *in vitro* to large numbers, the current methods lead to generation of exhausted moDC incapable of eliciting objective clinical responses. Nevertheless, novel techniques that allow antigen to be delivered directly to DC *in vivo* would obviate the need for laborious DC isolation protocols and circumvent the problem of DC exhaustion (Figure 7) [177]. *In vivo* delivery of tumour-specific antigen can be achieved by using fusion proteins, composed of one or multiple antigens coupled to an antibody that recognizes an extracellular DC-specific receptor. These techniques have been proven to be feasible by targeting DC-SIGN and DEC-205 [178,179]. Studies in mouse models have demonstrated that *in vivo*-DC targeting can lead to efficient antigen presentation and generation of antigen-specific $CD4^+$ and $CD8^+$ T cell anti-tumour

immunity. However, a DC maturation signal needed to be provided to prevent tolerance [180-182]. Nevertheless, vaccinations in mice have elicited strong T_H1 responses in the absence of an activating adjuvant. This was achieved by targeting certain DC-specific lectins, such as Clec9A, Dectin-1 and DCIR, which are capable of delivering an intracellular DC activation signal [183-185]. The absence of an adjuvant is also suspected to enhance T_{FH} and B cell activation, although the precise mechanisms are unclear and based on contradictory results [186].

Other antigen delivery systems to target DC *in vivo* are under investigation. An example of a different strategy is to make use of genetically manipulated probiotic lactic acid bacteria. Upon oral administration, these bacteria would target mucosal DC in the gut and couple antigen expression with the required adjuvant effect of microbial activation stimuli [187]. Utilizing viral vector-based systems also seem to be a promising development in immunotherapy. Nevertheless, it is not clearly understood how such vectors are capable of antigen and adjuvant delivery to DC *in vivo* [188,189].

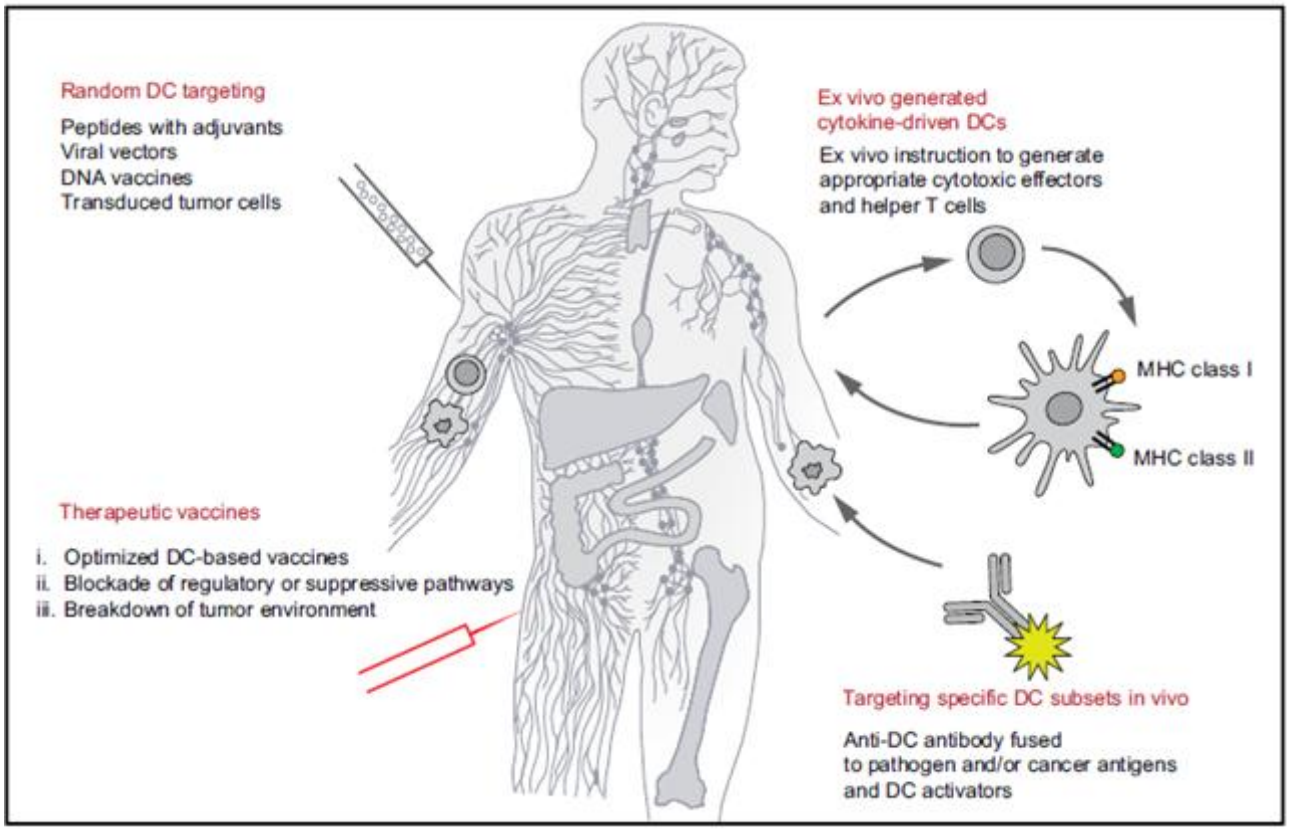

Fig. 7) Current trends in DC-based vaccines. Methods that target DC randomly, such as adjuvant peptides, deliver strong activation signals. However, they might elicit tolerance by being taken up by the wrong DC subtype. *Ex vivo*-generated moDC have not yet achieved objective clinical responses and require laborious culturing procedures. Targeting specific DC subsets *in vivo* is a relatively novel step towards obtaining a very specific immune response. Eventually, DC-based therapeutic vaccines will block suppressive pathways by breaking down the tumour environment and only activating desired anti-tumour immune cells. Picture adapted from Palucka *et al*. (2010) [4]

The biological function, tissue distribution and cellular surface receptors of several DC subsets have been sufficiently characterized to warrant feasible identification and *in vivo* targeting [4]. Since DC originating from a particular tissue can provide detailed instructions to T cells to home back to that specific tissue [190], targeting skin mDC would be the optimal treatment strategy for melanoma patients. Epidermal LC induce potent antigen-

specific CTL responses and can instruct T cells to home to the tumour sites in the skin. Topical application of antibodies targeting the LC-specific molecule langerin has successfully delivered tumour-associated antigen to LC *in situ* and induced potent proliferative responses of $CD4^+$ and $CD8^+$ T cells *in vivo* [191]. Dermal CD14-mDC could be targeted in a similar way to induce efficient humoral responses (Fig. 5). Thus, targeting skin mDC could significantly improve intradermal vaccination [192]. In addition, blood mDC subsets have been repeatedly targeted by means of anti-CD11c antibodies, a method which has induced robust $CD4^+$ and $CD8^+$ T cell responses [193]. Another interesting aspect of *in vivo* DC targeting would be to modulate the function of tolerogenic pDC. Targeting the C-type lectin DCIR on human pDC has been demonstrated to modulate IFN-α production [194]. Alternatively, the pDC-specific molecule BDCA2 provides an attractive target for antibody-mediated depletion of tolerogenic pDC [2,195].

Accumulating evidence from human and mice studies indicate the presence of considerable crosstalk between the different DC subsets. For example, murine pDC enhance the antigen presentation capacity of mDC, making mDC more efficient in presenting tumour-associated antigen to T cells [196]. Likewise, human mDC activated by LPS are able to effectively stimulate pDC maturation [197]. Thus, the pDC and mDC subsets seem to act synergistically and cooperate in order to enhance the plasticity of the DC network, thereby handling the various demands of the immune system [198]. Consequently, it might be beneficial to harness both subsets in vaccination strategies to strengthen elicited anti-tumour responses. The immunotherapeutic potential of the novel DC-targeting strategies remains to be proven in clinical trials.

Vaccine adjuvants directed at DC *in vivo*

A major challenge of *in vivo* DC subset targeting is to induce robust and lasting T-cell responses that yield sustained clinical responses. Accomplishment of this aim is hindered by immunosuppressive factors secreted by tumour cells. Release of these factors, such as IL-10, TGF-β and IL-6, can lead to a substantial reduction of circulating pDC and mDC [199]. In addition, endogenous DC that develop in the presence of these tumour-derived suppressive factors demonstrate impaired maturation and hampered ability to support effector T-cell functions [200-202]. Intratumoural DC can be targeted by repeated treatments with adjuvant combinations, such as activation signals and chemokines, in order to expand the number of DC *in situ* and to stimulate their anti-tumour effector functions [203]. The choice of adjuvant should be determined based on the expression pattern of DC activation receptors. Since DC adjuvant activation can influence the migratory properties of T cells, knowledge of the stimuli required for DC activation is of critical value for vaccine design [4].

Flt3L is a hematopoietic growth factor capable of inducing expansion and differentiation of almost all DC precursors, especially pDC [204]. Systemic administration of this ligand increases the number of circulating DC, as wells as DC present in lymphoid tissues and tumours [205]. Recently, it was suggested that Flt3L is necessary to enable DC to support NK cell effector function, an important component of anti-tumour immunity [206]. Furthermore, murine DC cultured with Flt3L promote $CD8^+$ T cell survival and expression of apoptosis regulator proteins Bcl-2 and MCL-2, even though they are less efficient in inducing $CD8^+$ T cell proliferation [207]. Although phase I/II studies have reported that Flt3L can induce anti-tumour activity by itself, synergistic action between

Flt3L and DC activators, such as CD40L, has been demonstrated [208]. Flt3L administration is already frequently in use to expand *in vivo* DC for research purposes [3].

The exceptionally potent immunostimulatory properties of IL-12 have made it a popular tool for immunotherapy. Systemic IL-12 infusion has only reached modest clinical efficacy, although it can result in enhanced migration of $CD8^+$ T cells to the tumour site [209-211]. Furthermore, IL-12 injection into tumour sites of head and neck squamous cell carcinoma patients leads to activation of B cells in the draining lymph nodes and subsequent tumour regression [212]. A newly developed strategy to introduce IL-12 to tumour sites is intratumoural injection of syngeneic DC transduced to express high levels of IL-12. In this model, therapeutic benefit is achieved through crosspriming of $CD8^+$ T effector cells originally reactive against tumour-associated antigen expressed by stromal cells [213]. Despite this novel method, IL-12 does not achieve sufficient clinical efficacy as a stand-alone drug. However, local IL-12 injection by viral or plasmid vectors can make this interleukin an exceedingly potent vaccine adjuvant.

Another very promising interleukin for cancer therapy is the T-cell growth factor IL-15. *In vitro* and *in vivo* studies have indicated a role for this interleukin in NK cell development and inhibition of antigen-induced T-cell apoptosis [214]. IL-15 further promotes the survival of antigen-specific $CD8^+$ T cells and can induce functionally efficient CTL in the absence of T_H help [215,216]. DC precursors stimulated with GM-CSF and IL-15 develop into LC-like DC with efficient *in vitro* priming capacity of tumour antigen-specific $CD8^+$ T cells. It has been argued that these LC-like DC are more efficient than moDC generated from a GM-CSF/IL-4 cocktail [49,217]. In addition, IL-15 is able to reverse T cell anergy and induce tumour reduction [218]. Although clinical data is lacking, IL-15 shows much promise as a therapeutic immunostimulatory molecule.

Flt3L, IL-12 and IL-15 do not target specific DC subsets. Contrastingly, the potent TLR9 agonist CpG should only have an effect on pDC, since they are the only DC subset expressing TLR9 [44]. Intratumoural injection of CpG is able to reverse the functional inhibition of pDC in cancer patients and increase their recruitment and activation in lymph nodes. Subsequently, the rescued pDC promote the proliferation of tumour-specific $CD8^+$ T cells [219,220]. In humans, TLR9 is also expressed by B cells. Consequently, administration of CpG can lead to B-cell proliferation and differentiation, antibody isotype switching, antibody secretion and activation of NK cells with strong antibody-dependent cell-mediated cytotoxic activity [221,222]. Unfortunately, CpG is also associated with IDO expression, IL-10 secretion and Treg cell generation [223].

Thus, the above immunotherapeutic drugs could improve vaccines by functioning as adjuvants stimulating DC-mediated immunity and improving clinical responses. Many TLR agonists are also popular in activating DC subsets, such as R848 and poly I:C. However, TRL ligands need to be carefully selected to stimulate maturation of both mDC and pDC, or at least to avoid interference with the development of either subset. The clinical use of TLR ligands in DC activation can be facilitated by using commonly used prophylactic vaccines as an alternative source for synthetic TLR agonists [224]. Combinations of several TLR ligands and inflammatory cytokines could be even more efficient in reactivating suppressed DC [225]. Finding the optimal vaccine adjuvant that favours the induction of anti-tumour immune responses can only be achieved by conducting clinical trials. So far, clinical data is insufficient to define the optimal vaccine adjuvant.

Conclusion and future prospects in DC vaccines

Many therapeutic vaccine approaches have been developed to engage the DC system into effectively combating malignant tumour cells (Fig. 7). Through the presence of functionally distinct DC subsets endowed with functional plasticity, the mature DC system can elicit an immune response appropriate to virtually any situation through interaction with $CD4^+$ and $CD8^+$ effector T cells, plasma B cells, NK cells, NKT cells, Treg cells and many others. From randomly targeting DC by peptide adjuvants to specific targeting of characterized DC subsets by anti-DC antibodies, DC-based vaccination is a rapidly-advancing branch in immunotherapy that holds much promise.

Nevertheless, at the present moment most DC-based vaccine are not capable of eliciting objective clinical responses (Table 1). Human clinical trials and studies in mouse models indicate that not all vaccinated subjects mount tumour antigen-specific immune responses upon vaccination. Moreover, in only a minority of the cancer patients vaccination leads to some clinical effect, which is often not correlated to the observed immune response and short-lasting.

Table 1 DC vaccination studies

Study	Type of DC	BR	$CD4^+$ T	IR	CR	Correlation IR-CR	Reference
Clinical trials							
I-II-III	"Standard"	-	T_H1/T_H2	50%	20%	No	76-80
I-II	TNF-α	-	T_H1/T_H2	40%	10%	No	76,81-84
I+II	IFN-γ +TLR4	-	T_H1	50-90%	10-30%	No	94,95
I/II	Blood DC	-	T_H2	70%	15%	No	131-134
I/II	LC-like DC	-	x	70%	20%	Yes	135
I/II	PDC+TLR9 agonist	x	T_H1	60-80%	30%	x	44,170
Mouse models							
	In vivo mDC	+	T_H1	50-80%	80%	Yes	181,185,191
Human models							
	In vivo mDC	+	T_H1	80%	x	x	178,182,183

BR: B-cell response; IR: immunological response; CR: clinical response; X: no available data. Percentages refer to total number of vaccinated subjects and are taken as average of all related study results. Clinical trials are indicated in phase numbers.

The reasons for the discrepancy between observed immune responses and elicited clinical effects are unclear. Most clinical trials have been performed using *ex vivo*-generated DC derived from monocytes or other DC precursors, as they are easy to isolate and can be propagated into huge numbers. Furthermore, a few clinical trials have employed matured blood-derived DC, despite their natural low frequencies. Notwithstanding the practical benefits of *in vitro* cultivation, the generation of DC involves numerous laborious protocols with compounds that can negatively affect immunotherapeutic capacity. Correspondingly, many DC demonstrate functional exhaustion *in vivo* and are unable to sufficiently stimulate anti-tumour immunity.

Novel immunotherapeutic strategies aim to target naturally occurring DC subsets *in vivo* through specific antibodies or by utilizing activating vaccine adjuvants. These methods should stimulate DC into polarizing the immune system towards a T_H1-mediated response while stimulating anti-tumour antibody production (Table 1). As DC subsets are activated *in vivo* and do not require extensive culturing in maturation cocktails, the problem of DC exhaustion should be circumvented. Each subtype has its unique biological function related to its location and cytokine secretion profile. So far, the most promising immunotherapeutic DC-based approach seems to target intradermal DC and tolerogenic pDC. T-cell stimulatory LC and B-cell enhancing CD14-mDC can be easily accessed through subcutaneous injection with antigen-antibody complexes. By harnessing both these subsets, both the cellular and humoral branches of immunity can be stimulated to combat malignant tumour cells. Intratumoural injection with CpG seems to be a suitable strategy for activating tumour-resident pDC, and can easily be combined with skin mDC-targeting immunotherapies. Although there are many other DC subsets that show promise for use in cancer vaccines, such as blood mDC, they are currently not sufficiently characterized to warrant their therapeutic potential in humans.

Future considerations

The efficacy of a DC vaccine does not depend solely on the choice of maturation procedure or the type of activated DC. Other factors that are of importance in the design of a vaccine are amongst others route of administration, antigen formulation and combination therapies. For example, direct intranodal injection of DC facilitates migration to the T cell-areas in the lymph node compared to injecting DC intradermally [226]. Likewise, electroporation of mature moDC with mRNA encoding tumour-associated antigen provides a continuous maturation signal, enhancing antigen expression in the lymph nodes compared to antigen-loading through conventional methods [227]. DC vaccines can also be combined with therapies that offset immunosuppressive pathways, such as Treg-depleting drugs or antibodies that block immunosuppressive cytokine (Fig. 1). However, these methods appear less effective and can lead to significant side effects [228,229]. These and other studies are equally important in innovating DC vaccine design as studies that characterize the immunological properties of DC subsets, put the subject falls outside the scope of this review.

DC generation methods have low reproducibility, as DC phenotype can somehow differ despite utilizing the exact same laboratory protocol. Therefore, it is essential that research will move toward a standardized protocol for DC cultivation [99,274]. Reproducibility is not only an issue in DC generation. Clinical trials have monitored *in vivo* immune responses by employing a wide variety of bioassays. A surplus of distinct laboratory protocols are used to perform these methods, generating highly variable results [230]. In

addition, the conventional phase I-III clinical trial paradigm dictated by the World Health Organisation or the Response Evaluation Criteria in Solid Tumours (RECIST) program [231,232], might not be optimal for assessing novel DC vaccines. Hence, the Cancer Immunotherapy Consortium of the Cancer Research Institute aim to develop new protocols for multicenter trials that utilize harmonized assay protocols. Furthermore, the new clinical endpoints should be clearly defined while allowing for long-term surveillance of survival and decrease in tumour burden under the presence of antigen-specific effector T cells. These novel measures will facilitate investigation into the correlation of immune response and clinical effect [233].

Conclusion

An improved understanding of the DC network and its role in tumorigenesis, tolerance and anti-tumour immunity will lead to novel methods to involve the DC subsets in combating cancer and improving disease outcome. Research into immunotherapeutic DC vaccines are giving promising results. In 2010, the first DC-based vaccine was approved by the Food and Drug Administration. Provenge is a moDC-based therapy that has been demonstrated to significantly increase survival in vaccinated prostate cancer patients correspondingly to measured immune responses [234]. This novel drug proves that development of moDC-based vaccines should not be ignored in favour of research into naturally occurring DC subsets. Even so, capitalizing on the functional characteristics of DC subsets have yielded good results (Table 1) with potent anti-tumour immune responses in human subjects. Therefore, DC vaccines are an advancing branch of immunotherapy that holds much promise.

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