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REVIEW

Current approaches in dendritic cell generation and future implications for cancer immunotherapy

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Abstract The discovery of tumor-associated antigens, which are either selectively or preferentially expressed by tumors, together with an improved insight in dendritic cell biology illustrating their key function in the immune system, have provided a rationale to initiate dendritic cell-based cancer immunotherapy trials. Nevertheless, dendritic cell vaccination is in an early stage, as methods for preparing tumor antigen presenting dendritic cells and improving their immunostimulatory function are continuously being optimized. In addition, recent improvements in immunomonitoring have emphasized the need for careful design of this part of the trials. Still, valuable proofs-of-principle have been obtained, which favor the use of dendritic cells in subsequent, more standardized clinical trials. Here, we review the recent developments in clinical DC generation, antigen loading methods and immunomonitoring approaches for DC-based trials.

Keywords Clinical applicability · Dendritic cell · Cancer immunotherapy

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Introduction

The introduction, over a century ago, of the concept of "immune surveillance" led to the quest for ways to initiate de novo and enhance existing immune responses against tumors, thereby aiming at the specific eradication of cancer cells, whilst leaving normal tissues untouched [129]. This concept was initially supported by the detection of spontaneously developing tumor infiltrating lymphocytes (TIL) with the capacity to kill malignant cells in a HLA-restricted fashion and was later on substantiated by the discovery of tumor-associated antigens (TAA) against which anti-tumor immune responses can be directed [67, 128]. However, malignant tumor cells develop mechanisms to escape elimination by these immune responses and possess mechanisms to tolerize the immune system, leading to tumor establishment. Tumor cells that escape elimination can persist in equilibrium with the immune system until the balance between the immune response and the tumor tilts towards tumor growth due to the outgrowth of poorly immunogenic tumor cells (immunoediting) and suppression of the immune system [53, 205].

The field of cancer immunotherapy has grown very rapidly in the past few decades. In order to initiate an immune response, induce memory and break immunological tolerance against the tumor, dendritic cells (DC) have emerged as what appears to be the ideal cellular tools [13, 140, 158]. Since tumor cells can express a whole array of TAA, the ideal anti-cancer vaccine may consist of DC loaded with TAA expressed by the tumor of that particular patient [23, 119]. In recent years, strategies have been developed for the large-scale generation of DC, yielding sufficient numbers of cells for use in clinical trials. Meanwhile, many different protocols have been designed to load antigens onto DC. Together, these findings made it possible

to start clinical studies with antigen-loaded DC in cancer patients. This review will focus on recent advances made in the procedures to generate large numbers of clinical-grade DC from various types of progenitor cells with a special focus on differentially isolated monocyte-derived DC and will discuss the problems associated with DC generated from cancer patients. After a brief description of the currently used strategies to load DC with antigens (Ag) and the possible methods for monitoring the induced immune response, a short overview will be given of DC-based clinical trials that have been carried out in cancer patients so far and their outcomes. Finally, we will focus on the problems arising in these first trials and point out new insights which should be taken into account to improve DC vaccinations in the future. Figure 1 gives an overview of DC-based immunotherapy and highlights the aspects discussed in this review which could have significant impact on the efficacy of DC-based immunotherapy.

Dendritic cell characteristics

The properties of DC that make them unique APC have been the subject of recent reviews and will only be briefly discussed here [12, 169].

The DC population is highly heterogeneous but their life cycle can be roughly divided in two stages: the immature and mature stage [12, 169]. In the immature stage, DC capture Ag through various mechanisms: macropinocytosis, receptor-mediated endocytosis and phagocytosis. After uptake, iDC start to process Ags into peptides for subsequent presentation to T cells as mDC. The phenotype of iDC is characterized by a low expression of MHC molecules, co-stimulatory molecules, adhesion molecules and DC markers. In contrast, they express a large amount of inflammatory chemokine receptors, allowing them to extravasate into inflamed tissues [12, 148]. The encounter of a so-called "danger signal" initiates maturation, whereby DC become highly motile, veiled cells and lose their ability for Ag uptake by down-regulating endocytic and phagocytic receptors. mDC optimize Ag processing through the up-regulation of components of the Ag-processing machinery and acquire the capacity to present antigens to and stimulate T cells by up-regulation of MHC molecules, adhesion/co-stimulatory molecules (CD40, CD54, CD58, CD80, CD83, CD86) and the DC marker DC-LAMP. Whereas most of these markers are already present at low levels on iDC, CD83 is absent on iDC and hence, CD83 expression allows discrimination between iDC and mDC. However, recently it has been shown that CD83 can be absent on monocyte-derived mDC generated in IL-3 and IFNβ and matured with poly(I:C), which nevertheless show a mature phenotype based on expression of other maturation markers. Maturation also induces acquisition of chemokine receptors such as CCR7 on the DC surface, which enable traYcking to lymphoid organs in response to chemokines secreted by stromal cells in the lymph nodes. mDC, in turn, secrete large amounts of chemokines to attract various cell types of the immune system and, depending on the maturation stimulus, they secrete particular cytokines to skew the immune response in a speciWc direction [12, 104, 141, 169].

Effective T-cell priming requires three consecutive signals between DC and T cells. DC can activate both $CD4^+$ T cells and $CD8^+$ T cells through Ag presentation via MHC class II and MHC class I, respectively (signal 1). Signal 2 consists of the interaction between CD80 and CD86 on the DC and CD28 on the T-cell surface. In the absence of co-stimulation, T cells recognizing the Ag presented by the DC are tolerized. Although the effector cells in tumor immunology consist mainly of TAA specific CTL, it has

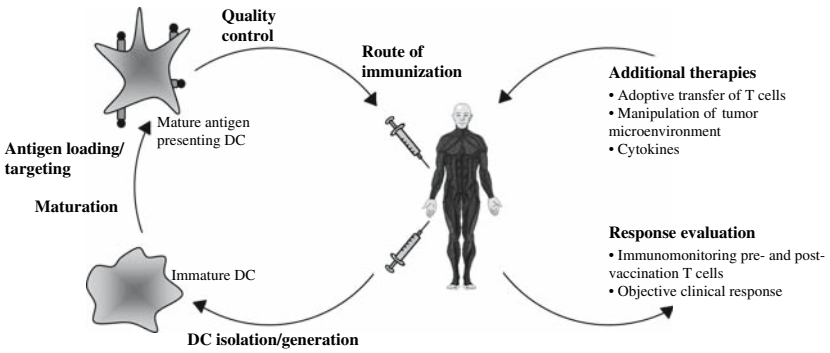

Fig. 1 Advances in DC-based immunotherapy. DC-based therapy has been shown to be very promising but several variables still need to be optimized. Further research is needed to determine the optimal procedure for DC isolation and/or generation, the most efficient maturation stimulus to activate the DC and the optimal method to load DC with antigens. The DC preparation needs to be extensively controlled before administration to the patient and the optimal route of immunization still needs to be defined. An important issue is also the standardization of techniques used for monitoring of the vaccine-induced immune response and the use of objective clinical endpoints. Furthermore, DC-based immunotherapy could benefit from combination with additional therapies

been well documented that $CD4^+$ T-cell help is required for efficient priming of memory $CD8^+$ T-cell responses (reviewed in [20]). For this to occur, DC need to be licensed by activated $CD4^+$ T cells through reciprocal interactions between CD40 on the DC and CD40L on the activated $CD4^+$ T cells, leading to IL-12 production by the DC (signal 3) [12, 73, 163]. The IL-12 produced by DC polarizes the T-cell response towards a Th1 profile, which is believed to be preferential for cancer immunotherapy.

The DC population residing in the human body is roughly divided into two groups: $CD11c^+$ $CD123^{lo}$ myeloid DC and $CD11c^-$ $CD123^{hi}$ plasmacytoid DC (pDC). Plasmacytoid DC are important mediators of innate antiviral immune responses and are the main producers of IFNα in the body (hence they are also called natural interferon producing cells, NIPC). pDC are located in blood and lymphoid organs. Myeloid DC reside in the blood and lymphoid organs as well as in the dermis and the interstitial tissue of most organs. A special subtype of myeloid DC, the Langerhans cells, reside in the epidermis and mucosal tissues [43, 148]. Since most research in the field of DC vaccination is focused on monocyte-derived myeloid DC, this review will refer to this cell type, unless specifically mentioned.

Generation of clinical grade dendritic cells

Critical parameters for DC vaccines

When chosing a generation protocol for DC intended for vaccination purposes, several critical parameters must be considered.

A first parameter which is important for the generation of DC vaccines is the number of DC that need to be isolated/generated. Since it is believed that repeated immunizations are beneficial and most protocols use around 10^7 DC per injection, it is necessary to obtain large numbers of DC ($\sim 10^8$ DC per preparation) which can be frozen in aliquots for repeated vaccinations. DC viability should be >75%.

Contaminating cells in the DC preparation could affect the efficacy of DC vaccination. Since the type of contaminating cells present in the DC preparation can vary between protocols and in order to minimize the effect of the contaminants, it is necessary to aim for the highest possible DC purity. Based on data from the literature, we propose to aim for DC purities of >75% [60].

As discussed later in this review, the maturation state of DC is critical for their effectiveness. It is therefore recommended to use mDC for vaccination or to combine the use of iDC with the in vivo administration of maturation stimuli. For each DC isolation/generation protocol, it should be assessed whether the resulting DC are immature or mature and, if immature, investigated whether they can be induced to mature with activation stimuli.

DC isolation and generation

DC can be isolated directly ex vivo from the blood of patients, either through positive selection using DC-specific markers or by depletion of contaminating cells or by a combination of both. Myeloid-derived DC and pDC can be distinguished through the differential expression of CD11c/BDCA-1 and CD123/BDCA-2/BDCA-4 on these DC types [62, 78, 103, 144, 179]. A first approach to enrich blood DC entails the use of sequential density centrifugation of apheresis PBMC followed by a culture period of 24 h. Administration of Flt3-L significantly increases the number of DC obtained and the isolated DC show a mature phenotype. However, reagents for density gradient centrifugation are not GMP-grade and it is difficult to carry out this approach in a closed system [62]. Another method for the isolation of blood DC involves depletion of lineage marker positive cells, which can be followed by positive selection using DC-specific markers. The isolated DC are immature and comprise both myeloid DC and pDC. Maturation is achieved by in vitro culture [144, 179]. Recently, Fearnley et al. described a method for the isolation of high-potency blood DC by using the CMRF-44 antigen. Using this method, only mature DC are isolated. The DC preparation contains both myeloid DC and pDC. However, yield and purity of the isolated DC are highly variable [4, 56, 97]. DC can also be enriched by exploiting membrane expression of DC markers (e.g. CD1c, BDCA-4,...) for magnetic or flow cytometric enrichment. It has to be mentioned that only the pDC-specific antibodies are truly DC-specific, whereas other DC markers are also expressed in low amounts on other cell types, which need to be depleted. Myeloid DC and pDC can be isolated separately or together and the DC show an immature phenotype but can be matured with appropriate stimuli [78]. Nevertheless, despite the fact that it has now been shown that Flt3-L can be used to mobilize blood DC, yields are generally too low to obtain sufficient numbers of DC for vaccination [62, 103]. On the other hand, Lopez et al. recently described the isolation of blood DC from apheresis products in sufficient numbers for immunotherapy [4, 97] and Campbell et al. [31] described the isolation of large numbers of blood DC using the CliniMACS system. It remains a matter of debate, though, if it will become feasible to use DC isolated from the blood of cancer patients for vaccine preparations because some reports have been published showing that significantly reduced DC numbers were found in peripheral blood of cancer patients compared to healthy donors [156].

A second source for DC generation is the proliferating $CD34^+$ progenitor cell. These $CD34^+$ cells can be isolated from blood or bone marrow by positive selection through magnetic separation. In order to obtain higher yields of $CD34^+$ cells, these cells can be mobilized into the blood via G-CSF administration alone or by a combination of stem cell-mobilizing chemotherapy (e.g. cyclophosphamide), G-CSF and/or IL-3 [120, 150]. However, caution has to be taken regarding the use of G-CSF for stem cell mobilization, since recent reports indicate that G-CSF can skew immune responses towards the Th2 phenotype and can induce/recruit Treg, which may both be undesirable for cancer immunotherapy [63, 86]. After isolation, $CD34^+$ precursors can be differentiated into DC by the addition of different cytokine mixtures. Most frequently, $CD34^+$ cells are cultured with GM-CSF and TNFα. After a 2 week culture period, iDC are obtained which mature asynchronously during the next 2 weeks, resulting in a mixture of iDC and mDC. A small proportion of the cells display the characteristics of Langerhans cells. IL-4, Flt3-L or c-*kit* ligand can be added to enhance DC yield. Purities are variable and rather low, with the contaminating cells being mainly granulocytes [98, 99, 162, 174]. Another protocol consists of a 2 week culture in the presence of GM-CSF and IL-4, resulting in the generation of iDC, followed by a 7 day period of maturation using CD40L or TNFα [38]. $CD34^+$ cells can also be differentiated into iDC using GM-CSF and IL-13 during 2 weeks [98].

The most commonly used cell type for DC generation is the peripheral blood monocyte. Monocytes can be easily collected via buffy coat preparations or leukapheresis and can be enriched in various ways. The easiest and most cost-effective way for DC generation is through adherence of monocytes to plastic which has been developed for use in closed systems. However, the variability in DC purity of this approach remains an important shortcoming [17, 57, 108, 135, 167, 172, 183, 189]. Highly purified monocytes can also be obtained by positive immunomagnetic selection of $CD14^+$ cells, but the reagents required are expensive, which limits their clinical use [10, 52, 57, 66, 108]. Furthermore, positive selection of monocytes raises concerns about the use of xenogeneic antibodies and possible activation/alteration of the monocytes [25, 54]. Although it has never been investigated whether monocyte activation has a negative effect on DC generation, this can be circumvented by a negative magnetic enrichment, but this method yields highly variable monocyte and DC purities [57, 108, 135, 172, 197]. Another technique to enrich monocytes involves the use of density gradient centrifugation, but this technique is difficult to integrate in a closed system and the available reagents are not GMP-grade [34, 93]. Recently, elutriation has been described as a means to isolate highly pure monocytes. This technique is based on a counter-flow centrifugation to physically separate cells depending on their size and density. The development of the Elutra™ device by Gambro.BCT facilitated the use of this approach in a closed system. Elutriation has been described to be fast and very cost-effective. Important parameters to consider when using the Elutra™ system are: (1) the dimensions of the elutriation chamber and the resulting requirement for high numbers of input cells (minimum 1×10^9 monocytes and minimum 5×10^9 PBMC); (2) due to the design of the system where cells are separated on the basis of cell size, there is no discrimination between monocytes and granulocytes leading to granulocytes being the major contaminants of the purified monocyte preparation; and (3) red blood cell (RBC) levels in the apheresis product should be kept low because RBC interfere with the purification process [2, 18, 66, 149, 167, 178, 197] (Dr. H. Vrielink, Sanquin Blood Bank, Amsterdam, The Netherlands: personal communication). Although significant differences exist in culture characteristics and study endpoints (iDC or mDC, evaluation of purity/yield) used by different groups, we attempted to compare the characteristics of the different available systems for monocyte enrichment and DC culture. An overview of the methods used for monocyte isolation, along with the resulting purities and DC yields is given in Table 1. In view of possible differences in phenotype and function of DC, some groups have compared DC generated from monocytes that were obtained using different isolation techniques. Again, results from different groups cannot easily be compared, due to differences in medium, cytokines and culture vessels used for DC generation. Table 2 gives an overview of the studies that have compared DC from differentially isolated monocytes at phenotypical and functional level. Taken together, these data indicate that the method of monocyte isolation has no major implications on DC phenotype and function.

Classically, immature, "myeloid-type" DC are generated from enriched monocytes by a 5–7 day culture in the presence of GM-CSF and IL-4, which can afterwards be matured using different stimuli. IL-4 can be replaced by IL-13, which induces the same type of DC. Recently, several groups have published optimized protocols to reduce this culture period to 48 h without affecting the phenotypical or functional properties of the resulting DC [46, 121, 133, 198]. This shorter culture period has several advantages such as reduced labor, cost and time. Since it has been proposed that DC generated in the presence of IL-4 display several functional alterations, a search for other differentiation cocktails has been carried out [180]. DC develop quickly in a model of trans-endothelial trafficking, which has been suggested to be operating in vivo [138]. In the presence of GM-CSF and type I IFN monocytes also develop quickly into DC, which might therefore be more physiological. However, although several authors have

Table 1 Characteristics of the different available closed systems for monocyte enrichment and subsequent DC generation

	Adherence	Positive selection	Negative selection	Elutriation
Cost	Low	High	High	Low
Monocyte purity[a]	±60%	91–99%	8–75%	55–90%
Main monocyte contaminants[b]	B, NK cells	/	NK cells Granulocytes	Granulocytes (especially neutrophils)
Monocyte recovery	ND	27–100%	43–97%	53.3–88.2%
Closed culture recipient	Cell Factories™ Gas-permeable bags	Gas-permeable bags	Gas-permeable bags	Gas-permeable bags
Monocyte activation	Possible	Possible	No	No
DC purity[c]	34–98%	59.5–98%	31–86%	62–98%
DC yield (PBMC)	2.7–20%	1–2%	4.8–13%	5–12%
DC yield (monocytes)	12–68%	4–41%	16–95.1%	20–100%
References	[17, 57, 72, 108, 135, 167, 172, 183, 189]	[10, 52, 57, 66, 108]	[57, 108, 135, 172, 197]	[2, 18, 66, 149, 167, 178, 197] Dr. H. Vrielink (personal communication)

[a] Monocyte purity was assessed by flow cytometry using the monocytic marker CD14

[b] Contaminants of monocyte preparations were assessed by flow cytometry using lineage-specific antibodies

[c] DC purity was evaluated by FSC/SSC characteristics or by CD83 positivity

reported the efficient generation of highly stimulatory DC using GM-CSF and type I IFN in a short culture period, some contradictory results have been published [45, 113, 153, 154, 182]. Recently it was discovered that DC can also be differentiated from monocytes in the presence of type I IFN and IL-3, where substitution of GM-CSF by IL-3 rescues the cells from apoptosis [24, 29, 142]. Another pathway of monocyte differentiation into DC has been described where incubation of monocytes in the presence of GM-CSF and IL-15 results in differentiation of Langerhans-like cells [111].

Comparison of different DC types

Freshly isolated DC or DC generated from different precursors are not equivalent, which raises the question of which DC type is optimal for the induction of an anti-tumor response. Many comparisons between these different DC subtypes have been published, which we briefly summarize hereafter. Again, these studies are difficult to compare due to differences in media and cytokines used.

Several groups have compared *freshly isolated blood DC and monocyte-derived DC*. These DC types are phenotypically and functionally distinct. At the phenotypical level, blood DC seem to be more activated, because blood DC require a short period of culture to mature, whereas the maturation of monocyte-derived DC is variable and largely dependent on the stimulus. However, compared to monocyte-derived DC, blood DC are relatively poor cytokine producers, are reported to be less active in endocytosis and lack DC-SIGN expression. Both DC types are equal stimulators in allo-MLR, but blood DC seem to be better initiators of Ag-specific T-cell responses and have a higher Th1 polarization capacity [79, 84, 126].

Many groups have reported comparisons between *CD34- and monocyte-derived DC*. In general, yields of CD34-derived DC, as calculated from the starting number of PBMC, appeared to be somewhat higher. Depending on the cytokine combinations used to generate DC, iDC or mDC were obtained, but both CD34- and monocyte-derived DC showed a comparable morphology and phenotype. However, when CD34- or monocyte-derived DC displaying comparable phenotypes were used for the stimulation of T cells, the induction of Ag-specific T-cell responses was enhanced when using CD34-derived DC [11, 37, 174].

The group of Dauer et al. performed an extensive comparison between the *classical monocyte-derived DC* generated in the presence of GM-CSF and IL-4 during 5–7 days and *DC generated from monocytes in a reduced period of 48 h*. They show that both DC populations have a comparable phenotype, IL-12 secretion and Ag uptake, processing and presenting capacity. Both DC types migrate equally well towards the CCR7 ligand 6CKine/CCL21. However, yield and purity of the fast DC population are higher compared to the classical monocyte-derived DC. Furthermore, fast DC induced similar numbers of Ag-specific T cells, but with higher lytic capacity compared to T cells induced by classical monocyte-derived DC [46, 121, 198].

Many reports have been published comparing the *classical monocyte-derived DC (GM-CSF + IL-4) with DC generated in the presence of GM-CSF/IL-3 and type I interferon*. In general, DC differentiation seems to occur more rapidly in the presence of type I IFN and these DC

Table 2 Effect of monocyte purification on DC phenotype and function

Reference	Monocyte selection	DC phenotype	DC function
Suen et al. [172]	Negative selection vs. adherence	Similar CD1a, CD80, CD83, CD86	Similar uptake FITC-dextran DC from negative selection slightly better in allo-MLR
Pullarkat et al. [135]	Negative selection vs. adherence	Similar CD11c, CD40, CD44, CD58, CD80, CD83, CD86, HLA-DR DC from negative selection higher CD1a expression Similar phenotype stability	Comparable allogeneic and peptide-specific (IMP-1, gp100) proliferative T-cell responses and gp100 specific cytotoxic T-cell responses
Berger et al. [18]	Elutriation vs. adherence	Comparable CD1a, CD14, CD25, CD83, CD86, HLA-DR Equal phenotype stability	DC from elutriated monocytes were better in allo-MLR
Felzmann et al. [57]	Adherence vs. positive selection vs. negative selection	No differences in CD1a, CD14, CD45, CD80, CD83, CD86, HLA-ABC, HLA-DR	No difference in IL-12 secretion or allo-stimulatory capacity
Wong et al. [197]	Elutriation vs. negative selection	Equal CD1a, CD14, CD80, CD83, CD86, HLA-DR DC from elutriation higher contamination with $CD3^+$ cells	No difference in allo-MLR or TT/influenza recall response
Garlie et al. [66]	Elutriation vs. positive selection	Similar CD11c, CD83, CD86, HLA-ABC HLA-DR higher on DC from elutriated monocytes	Similar FITC-dextran uptake and induction of allogeneic MLR responses
Meyer-Wentrup et al. [108]	Adherence vs. positive selection vs. negative selection	DC from positive or negative selection slightly more mature than adherence-DC (CD83, CD86, HLA-DR, CD14)	No difference in allo-MLR

acquire a semi-mature phenotype and a high migratory capacity at day 3 of culture without any other maturation stimulus, in contrast to IL-4 DC which need exogenous maturation stimuli to mature. The cytokine secretion pattern is largely different between type I IFN DC and IL-4 DC, with the major difference being a decreased IL-12p70 secretion and an increased IFNα secretion by type I IFN DC as compared to IL-4 DC. However, type I IFN DC are short-lived because a considerable percentage of the cells undergo apoptosis by day 5 of culture, which can be counteracted by adding IL-3 [24, 29, 49, 113, 142, 154, 182]. The comparison of T-cell stimulation by these two DC types has been troubled by the fact that immature type I IFN DC already display a semi-mature phenotype. Consequently, when immature type I IFN DC and immature IL-4 DC were compared, type I IFN DC were more potent at inducing Ag-specific T-cell responses than DC differentiated with GM-CSF and IL-4 [131, 154]. However, the capacity to induce Ag-specific T-cell responses was comparable between immature type I IFN DC and mature IL-4 DC [182]. When both DC types were matured, conflicting results have been published, showing either an equal capacity of both DC types to induce Ag-specific T-cell responses [142] or an enhanced capacity of type I IFN DC [24, 113]. Type I IFN DC have been shown to polarize $CD4^+$ T-cell responses either only towards Th1 [154] or to both Th1 and Th2 [29, 49]. In contrast, Dauer et al. [45] reported that DC fail to develop in the presence of type I IFN and that type I IFN even disables DC precursors. It should be mentioned however that these authors compared both DC derived in the presence of IL-4 or type I IFN at day 6 of culture, a time point at which type I IFN DC already undergo apoptosis. McRae et al. [106] described that when type I IFN is added to cultures of monocytes together with IL-4 and GM-CSF, TNFα-mediated maturation is impaired, resulting in a decreased T-cell stimulatory capacity and reduced IL-12 secretion. This contrasts with findings described by Radvanyi et al. [136], who observed that addition of type I IFN to GM-CSF/IL-4 cultures of monocytes accelerated DC generation and maturation. Furthermore, Tamir et al. reported that the environment in which type I IFN is present plays a central role in determining its effects on DC function and Lehner et al. showed that adding both type I IFN and bacterial stimuli to GM-CSF and IL-4 containing DC cultures induces apoptosis in monocyte-derived DC [92, 176].

Differentiation of monocytes with GM-CSF and IL-15 was decribed by Mohamadzadeh et al. and compared to the *classical DC generation* in the presence of GM-CSF and IL-4. The authors reported that both DC types share: (1) the basic DC phenotype; (2) the FITC-dextran uptake capacity; (3) maturation capacity upon incubation with various stimuli; and (4) the capacity to stimulate allogeneic and Ag-specific T cells. As opposed to GM-CSF/IL-4 DC however, DC generated in the presence of IL-15 express several Langerhans cell markers: E-Cadherin, Langerin and CCR6. As a consequence, IL-15 DC migrate towards the CCR6 ligand MIP-3α/CCL20 [111].

Importance of maturation state

The induction of a successful anti-tumor immune response requires the use of immuno-stimulatory mDC, because of their enhanced capacity to induce Ag-specific T-cell responses and because iDC tend to induce tolerance. Furthermore, iDC have been shown to expand regulatory T cells (Treg), although it has been described that DC matured with certain stimuli can also induce Treg expansion and even provoke de novo Treg generation from $CD4^+CD25^-$ effector T cells [14, 48, 105]. In addition, mDC have been shown to be resistant to immunosuppressive factors produced by tumors and are phenotypically and functionally stable in the absence of cytokines [145, 168]. Maturation can be achieved by a wide array of different stimuli: monocyte-conditioned medium (MCM), different pro-inflammatory cytokine cocktails, TLR ligands,... [1, 32, 39, 90, 109, 122, 152, 166]. These stimuli are schematically represented in Fig 2. Currently, the most widely used stimulus is a cocktail containing IL-1β, IL-6, TNFα and PGE_2 but extensive controversy exists about which stimulus might be optimal. The different stimuli used to induce maturation give rise to subtle differences in the DC maturation stage. These differences can be observed at the phenotypical level (expression of maturation markers CD25, CD40, CD83, CCR7,...), the functional level (allo-MLR, Ag-specific T-cell induction...) and at the level of cytokine/chemokine secretion (IL-6, IL-10, IL-12p70, IFNα...). Furthermore, the DC phenotype, functional characteristics and cytokine/chemokine secretion pattern can also be influenced by the culture medium used to generate the DC [54, 181]. The concept arising now is that DC do not need to have a completely mature phenotype, but need to secrete cytokines/chemokines which polarize T-cell responses and express chemokine receptors for efficient migration to lymphoid organs.

Recent insights indicate that phenotypic DC maturation is not a distinguishing feature of immunogenic versus tolerogenic DC, since tolerance can be induced by phenotypically immature, semi-mature and fully mature DC. In this regard, semi-mature DC, which express high amounts of MHC and co-stimulatory molecules and produce high amounts of IL-10 but only trace amounts of IL-12, have been implicated in the conversion of naïve T cells into Treg [151]. Recently, IDO expressing mDC were shown to be tolerogenic and could be further identified by CD123 and CCR6 expression. These authors suggest that IDO expression

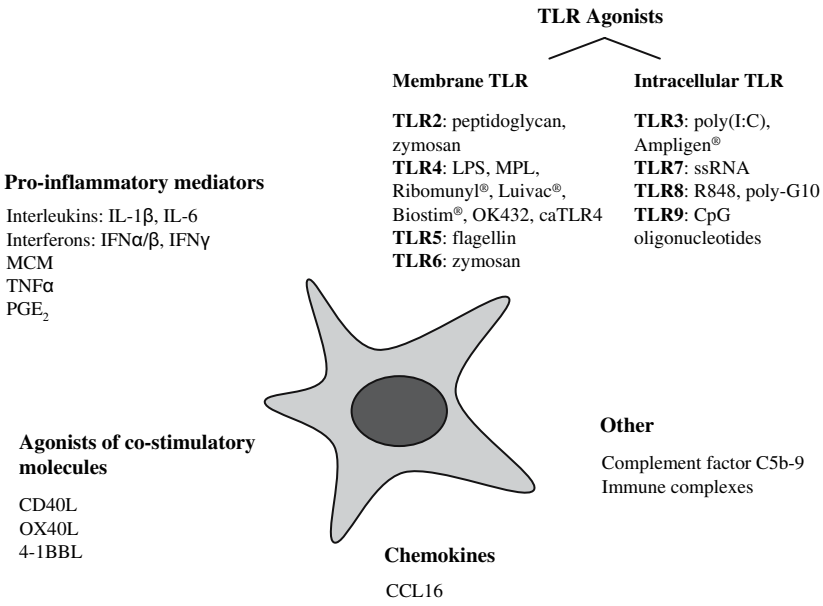

Fig. 2 Schematic representation of the currently available maturation stimuli. To date, several stimuli have been identified which promote DC maturation. The extent to which DC maturation is affected varies considerably between individual stimuli. In addition, it has been shown that distinct combinations of maturation stimuli can act synergistically to promote DC maturation and Th1 polarizing capacity of DC. Ongoing research will possibly identify optimal combinations of stimuli for DC maturation. Although clinical-grade bacterial immunomodulators (Ribomunyl®, Luivac®, Biostim®, OK432) are classified in this figure as TLR4 agonists, the nature of these stimuli suggests that they possibly trigger a combination of distinct TLR. Agonists of co-stimulatory molecules can be delivered by agonistic Ab, soluble ligands (if available) and transfection with ligands. *MCM* monocyte-conditioned medium, *MPL* monophosphoryl lipid A, *caTLR4* constitutively active TLR4

by mDC might be determined by the prevailing regulatory influences during maturation [116]. This has led to the concept of defining DC not only by their maturation status but also based on effector function [141]. However, the precise DC characteristics leading to Th1, Th2, Th17, Treg or cytotoxic T-cell development are not yet completely identified. It is conceivable that small differences in environmental stimuli drive DC to acquire different effector functions, which could be characterized by subtle differences in the expression of various known molecules or DC longevity. In this view, distinct DC subsets can each acquire characteristics of immunogenic and tolerogenic DC depending on the stimuli they receive.

Although it is difficult to define precise characteristics for DC preparations, we can point out some parameters that can be considered important. First of all, mDC can be characterized by a high expression of MHC and co-stimulatory molecules and the expression of CD83, which has recently been shown not only to be a marker of phenotypical maturation, but also a modulator of the immune response [3]. Next, expression of CCR7 on DC displays their capacity to migrate to the lymphoid organs. In order to get licensed by $CD4^+$ T cells for the induction of a memory $CD8^+$ T-cell response, DC should express the CD40 molecule for signalling through CD40L, which, in turn, leads to IL-12p70 production. Cytokine secretion is another means by which DC can modulate T-cell responses. IL-12p70 and IFNα can induce a Th1 response, whereas IL-6 can rescue effector T cells from the suppressive effect of Treg cells. In contrast, expression of IDO and secretion of IL-10 can lead to the induction of Treg and is therefore undesirable.

Clinical-grade DC

DC for use in cancer immunotherapy should be produced according to good manufacturing practise (GMP) guidelines. This implies that the procedure of DC generation is validated and that protocols are available for each step in the DC generation process. Finally, a quality control system must be developed to examine the quality of every final DC preparation. In order to avoid infections, DC cultures should be performed in closed recipients or gas-permeable culture bags with sterile connections, using GMP-grade reagents and culture media (AIM-V, X-VIVO-15, X-VIVO-20) either serum-free or supplemented with autologous heat-inactivated plasma to avoid exposure of DC to xenoantigens [65, 145]. Recently, several groups have developed closed culture systems for DC generation. In view of repeated immunizations, it is desirable to generate a large amount of DC from one single leukapheresis, which can then be frozen and thawed before each injection [59, 94]. The generated DC vaccines should be subjected to an

extensive quality control procedure before use. Since our understanding of DC biology and T-cell activation is continuously evolving based on ongoing research, a standardized set of specific parameters for DC quality which have to be fulfilled can not be defined. Instead, a minimal set of release criteria have to be fulfilled and additional parameters, which are thought to be important, can be analyzed to gain information for later comparison. Microbiological tests have to be performed in order to demonstrate the lack of bacterial, fungal or mycoplasma contamination and absence of endotoxins in the DC preparation. The viability of the final DC preparation before injection should be at least 75%. The DC preparation should display the typical morphologic features of DC with maximally 25% of contaminating cells. Minimal phenotyping should be performed to ensure that the DC are MHC class I and class II positive and express the co-stimulatory molecules CD80 and CD86 to some extent. In addition to these minimal criteria, we recommend the examination of additional parameters which give more information about the phenotypical and functional characteristics of the DC vaccine. Complete phenotyping provides additional information about the activation status of the DC (CCR7, CD1a, CD11c, CD40, CD80, CD83, CD86, CD123, HLA-ABC, HLA-DR) and the type of contaminating cells (CD3, CD14, CD16, CD19, CD56). However, it should be mentioned that different culture media can give rise to phenotypic differences. DC with a fully mature phenotype maintain their morphological and phenotypical characteristics after cytokine withdrawal, which can be examined using the washout test. The T-cell stimulatory capacity of the DC can be tested using an allo-MLR (non Ag-specific) or by an Ag presentation assay (Ag specific). Another characteristic of DC that can influence the induced T-cell response is their cytokine secretion pattern (IL-6, IL-10, IL-12p70, IFNα,...). Functionally, fully mature DC can also be characterized by their capacity to migrate towards the CCR7 ligand CCL19 [60].

Dendritic cells in cancer patients

Some authors have reported alterations in number and phenotype of peripheral blood DC as well as functional defects in freshly isolated DC from the blood of cancer patients compared to healthy donors. These defects appear to be more severe in more advanced stages of disease and are induced by factors secreted by tumor cells (IL-6, IL-10, TGF-β, VEGF,...) [200]. This has been described for patients with AML [112], CLL [124], multiple myeloma [139], colorectal cancer [50] and breast, head and neck and lung cancer [5]. This raises the question whether DC generated from precursors obtained from cancer patients will also display differences compared to DC from normal donors. Although several authors have reported no phenotypical and functional differences between DC derived from precursors from cancer patients compared to healthy donors [10, 88, 201, 203], recently some concerns have been raised regarding the generation of DC from cancer patients. Orsini et al. [125] describe that monocyte-derived DC from CLL patients with active disease display an abnormal phenotype and functional defects, whereas monocyte-derived DC from CLL patients in remission show no differences compared to DC from normal donors, indicating that patients with minimal residual disease (MRD) after conventional treatment are the most suitable candidates for DC immunotherapy. Schütt et al. [159] report the efficient generation of monocyte-derived DC in multiple myeloma patients, but found that their phenotype can be altered depending on the treatment the patients received previously. Pedersen et al. [132] show that monocyte-derived DC from breast cancer patients are more activated but less sensitive to maturation signals compared to healthy donor-derived DC. However, these DC can mount Ag-specific T-cell responses in vitro. Makino et al. show intrinsic abnormalities of monocytes and a defect of DC differentiation in adult T-cell leukaemia (ATL) patients. The authors provide evidence that these alterations arise because of infection with the human T lymphotropic virus type I (HTLV-1), which is responsible for the induction of ATL [102]. Furthermore, factors in the patient's autologous serum can influence DC generation from precursors in cancer patients, but this could be easily circumvented by using serum-free culture conditions [139]. Clearly, DC generation in cancer patients can be altered, but a lot of reports have been published where DC from cancer patients are able to induce Ag-specific immune responses. It is therefore recommended to carefully test DC generated from all cancer patients before using them in immunotherapy trials.

Antigen loading

The ideal target for cancer immunotherapy would be a TAA which is exclusively expressed in tumor cells and not in normal tissues, to avoid potential induction of autoimmunity. A prerequisite for a broad therapeutic potential in several cancers is the wide expression of the TAA on different tumor types. In addition, the TAA should be important for tumor growth and survival, so down-regulation is impossible [71, 202]. Most TAA are self-derived proteins and thus poorly immunogenic. Nevertheless, DC loaded with these Ags can be used to initiate Ag-specific T-cell responses. In recent years a large number of strategies have been developed to deliver Ags to DC, using defined epitopes, specific TAA or whole tumor cell material and employing both viral and non-viral techniques.

Peptide/protein approaches

The most commonly used protocol for loading Ags onto DC is pulsing with synthetic peptides. Advantages of this technique are the ease of manufacturing GMP-grade peptides, obviation of the need for tumor tissue and simplification of immunomonitoring. Important drawbacks of this technique are MHC restriction, the need for identification of TAA epitopes, low affinity binding of self-derived peptides and lack of $CD4^+$ T-cell help (since only a limited number of CD4 epitopes are known) [194]. The need for MHC typing and peptide identification can be circumvented by using acid-eluted peptides from autologous tumor cells, but in general the large amounts of tumor material needed for this procedure are not available. Moreover, tumor cells also present shared self Ags, which could give rise to unwanted autoimmune responses [51]. In order to increase the binding affinity of self-derived peptides, peptide analogues (so-called heteroclitic peptides) can be generated by modifying the anchor residues which mediate binding to MHC molecules [130]. However, it was recently described that vaccination with these heteroclitic peptides results in poor recognition of endogenous peptides and less efficient tumor cell killing [170]. $CD4^+$ T-cell help can be obtained by addition of a xenoantigen such as Keyhole Limpet Hemocyanin (KLH). Along with the discovery of more CD4 epitopes, the use of long peptides comprising both CTL and CD4 epitopes has been developed to generate both Ag-specific $CD4^+$ and $CD8^+$ T cells for optimal anti-tumor immune responses [204, 206]. In order to avoid tumor escape by TAA down-regulation, a mixture of different TAA peptides could be loaded onto DC. However, this could lead to epitope competition which, in turn, can be easily avoided by loading the different peptides on different DC batches [127]. In addition, the opposite phenomenon termed "epitope spreading" where vaccination with a single TAA epitope results in the induction of T-cell responses directed against other, non-related TAA, has also been reported [100].

In order to address some of these issues, purified whole TAA proteins have been used for loading DC [193]. This method has the advantage of being independent of the knowledge of the MHC haplotype of each patient and of prior identification of defined TAA-derived peptide epitopes. Furthermore, multiple immunogenic epitopes can be processed by DC in the context of both MHC class I and class II, resulting in both $CD4^+$ and $CD8^+$ T-cell responses. However, proteins are only efficiently taken up by iDC; protein loading should therefore occur in an immature state, after which maturation has to be induced. In addition, because of the combined use of standard and immunoproteasomes by mDC, some epitopes could be less efficiently processed and presented to T cells. Proteins can also be delivered as immune complexes, enhancing the efficiency of MHC class I presentation. Antigens can be conjugated to IgG mAb for uptake by Fc receptors or to Ab targeting endocytic receptors (mannose receptor, DEC-205, DC-SIGN). The latter receptors are more DC restricted and can thus be used for in vivo DC targeting. Furthermore, loading DC with immune complexes has been shown to bypass the need for CD4 licensing of DC [137, 160, 175].

Genetic approaches

Problems related to whole protein loaded DC (such as the intensive process of protein purification) can be overcome by gene-based delivery of TAA into DC. Advantages are the ease of cloning genetic constructs and the possibility to include sequences for improving Ag presentation in both MHC class I and class II. Furthermore, different TAA can be simultaneously delivered to DC, thereby broadening the T-cell repertoire that can be activated. DNA and mRNA can be delivered as naked strands, but transfection efficiencies are enhanced by lipid-mediated transfection or electroporation. Transfection of DC with DNA has not met with great success and important concerns can be raised regarding safety, because DNA can integrate into the host genome [190, 191]. mRNA delivery to DC proved to be more effective and safe and is surrounded by significantly less safety issues, because mRNA is only transiently expressed in the cells and does not integrate into the genome. Recently, several groups have developed mRNA electroporation strategies, resulting in very high transfection efficiencies of DC [69, 188, 190]. This technique has also been applied to amplified whole tumor mRNA. Using microscopic amounts of tumor tissue, total tumor RNA is amplified by a PCR-based protocol. Thus, this approach can even be applied when TAA are not defined and only limited amounts of tumor material are available, giving the opportunity to induce a broad patient specific immune response against both known and unknown TAA [16, 118].

Viral gene delivery systems are very efficient strategies to introduce genetic material into various cell types, including DC. We will only give a brief description of currently used viral vector systems for DC transduction, a more comprehensive overview has been given by Breckpot et al. [26]. As with non-viral gene delivery methods, several TAA can be combined in one viral vector and target sequences to obtain Ag presentation in both MHC class I and class II can be incorporated. A large variety of viral vectors have been developed and optimized for high-efficiency transduction of DC: adenovirus, adeno-associated virus, herpes simplex virus, vaccinia/pox virus, retrovirus and lentivirus. Depending on the viral system used, great variability exists in the size of genetic material that can be incorporated, the stability of transgene expression, the capability to transduce both

dividing and non-dividing cells and the viral titres that can be obtained. Furthermore, large scale production of viral vectors needs further research. In addition, major concerns are raised regarding possible effects of viral transduction on DC phenotype/function, immunogenicity of the virus, insertional mutagenesis and biosafety; issues which have to be resolved. Nevertheless, some viral vectors are already being used in cancer immunotherapy.

Whole tumor approaches

Methods that take advantage of the complete protein content of the tumor cell, thereby broadening the induced immune response and avoiding tumor escape, have been developed, including loading DC with tumor lysate, engulfment of necrotic/apoptotic cells by DC and fusion of DC with tumor cells. Tumor lysates contain the whole protein content of lysed tumor cells, which can be loaded on iDC in the same way as purified proteins. Using this approach, induction of Ag-specific $CD4^+$ and $CD8^+$ T cells can be achieved, but a relatively high amount of tumor cells is required [21]. Induction of necrosis and apoptosis in tumor cells can be achieved by mechanical/thermal lysis and UV irradiation, respectively. However, it is not easy to induce pure necrotic or apoptotic cell populations and it is still a matter of debate which type is needed for induction of immunity. The general concept arising now is that necrotic cells induce immunity, whereas apoptotic cells induce tolerance, because of the lack of DC activation signals. However, although this is an important issue in vivo, it can be circumvented by exposing ex vivo generated DC loaded with apoptotic cells to additional maturation stimuli to ensure full maturation of the DC [58, 77, 155]. Furthermore, using these techniques, it is essential that every tumor cell is rendered necrotic/apoptotic, because residual viable tumor cells in the DC preparation could theoretically lead to metastatic spread in patients. The requirement for relatively large amounts of tumor material is another important drawback. Another attractive approach consists of fusion of DC with tumor cells, which generates hybrids expressing the DC characteristics of Ag processing and presentation together with the unaltered antigenic spectrum of the tumor cell [87, 147, 185]. Fusion can be obtained by using either chemical fusogens or electrofusion. However, until now, simple and reliable protocols to generate a highly efficient DC-tumor cell fusion are not available and caution has to be taken regarding the safety of this technique. As for loading DC with tumor lysates and necrotic/apoptotic cells, this technique also requires the availability of a large number of viable tumor cells. An important issue to take into account when using whole tumor cell-derived material is the risk of inducing autoimmunity. Furthermore, evaluation of the immune response becomes more complex. Another concern relevant to the use of whole tumor cell-derived material (tumor mRNA, tumor-DC fusions and possibly also tumor lysates and necrotic/apoptotic tumor cells) is the risk of transferring immunosuppressive factors from the tumor cells to the DC, which would generate deficient DC.

A recently developed approach consists of the use of DC derived exosomes. Exosomes are 50–90 nm vesicles originating from multivesicular endosomes and contain Ag presenting molecules, adhesion and co-stimulatory molecules, i.e., the necessary machinery required to generate potent immune responses. Exosomes need to be transferred to mDC to promote T-cell activation leading to tumor eradication. Exosomes pulsed with tumor peptides can successfully prime Ag-specific CTL responses [7, 36, 55].

Monitoring the immune response and clinical outcome

Reproducible monitoring of the immunologic outcome of DC vaccination could facilitate the interpretation of study results. The establishment of reliable, reproducible and quantitative assays to evaluate vaccine-induced immune responses should be regarded as of critical importance [41, 85]. Immunomonitoring methods depend on the vaccine design: strategies employing defined epitopes, defined TAA, or undefined Ags need different approaches. Another issue complicating immunomonitoring of the vaccine-induced response is the phenomenon of epitope spreading: even when immunizing with a single peptide, the immune response can be broadened to other epitopes [30, 143, 192]. Thus, when measuring the immune response after vaccination, one has to discriminate between anti-vaccine and anti-tumor T cells. During the course of tumor progression, a spontaneous anti-tumor T-cell response develops, but these anti-tumor T cells become inactivated due to tumor-induced immunosuppression. Upon vaccination, anti-vaccine T cells are induced and migrate to the tumor. In some cases, these anti-vaccine T cells are able to overcome immunosuppression, thereby destroying local tumor cells and activating both pre-existing and new anti-tumor T cells which can eliminate the bulk of the tumor cells. This concept, introduced by Boon et al., implies that successful vaccination does not depend on the number of the induced anti-vaccine T cells, but rather on the production of an anti-vaccine T-cell clone which is able to migrate to the tumor and resist local immunosuppressive mechanisms [22, 100]. Thus, besides quantification of the number of anti-vaccine T cells, it is also important to determine their qualitative aspect. In principle, the elicited anti-vaccine T cells should have the ability to migrate to the tumor site, produce cytokines, proliferate after Ag re-exposure and mediate tumor cell lysis. Therefore, both T cells at the tumor site and in the

circulation should be analyzed if possible, as the immune response monitored in the blood does not always reflect the situation in the tumor [8, 91]. In addition, tumor-specific T cells should be analyzed by combining different methods in order to get a complete picture of the induced T-cell functional profile. Furthermore, if possible, the tumor site should also be screened for Treg, because intra-tumoral accumulation of Treg is associated with poor prognosis and it has been described that certain DC vaccination modalities can induce/expand Treg [14, 42]. Up to now, a big discrepancy has been observed between induced immune responses and clinical outcome of the patients, which is probably related to the breadth and quality of the induced T-cell response, resulting in the frequent observation of induction of anti-vaccine immune responses in the absence of an objective clinical response. Thus, either the induced anti-vaccine T cells in patients lacking a clinical response are not capable of destroying the tumor, or the tumor-induced immunosuppressive mechanisms between patients with and without a clinical response are of a different magnitude. The methods for immunomonitoring described hereafter are also schematically represented in Fig 3.

Target cells and time schedule for immunomonitoring

When immunizing with DC, it is important to evaluate immune responses with cellular targets other than DC, since this could result in significant background responses. When using peptide-pulsed DC, other target cells expressing the relevant HLA type pulsed with either an irrelevant peptide or the immunizing peptide can be used (e.g. T2 cells for HLA-A2 restricted peptides). However, with all other approaches for Ag loading of DC, this method is not applicable, since the immune response can be directed against various epitopes (both MHC class I and class II restricted). In this case, it is recommended to use autologous APC as targets (e.g. EBV transformed B cells or PHA blasts), since these cells express all the relevant MHC molecules. These autologous APC can then be loaded with the Ag(s) used for vaccination using one of the approaches described for loading DC with Ag. However, if possible, it would be optimal to use a distinct approach as the one used for Ag loading of DC for vaccination to avoid background. Perhaps the best target to use, at least for MHC class I responses, is the autologous tumor or cell lines derived thereoff, which is, however, not always available.

Regarding the time schedule at which samples should be taken for immunomonitoring, comparisons of pre- and post-vaccination samples are probably most informative, and should preferably take place concomitantly with the evaluation of clinical parameters. When the DC vaccine is repeatedly administered, several samples for monitoring can also be taken during the course of vaccination. If the patient is subjected to a follow-up period (either with or without any further treatment), it is advisable to take samples for monitoring at later time points as well to address the induction of a sustained/memory response.

T-cell receptor assessment

A strategy to enumerate the percentage of T cells recognizing a certain epitope in the context of a defined MHC molecule is by using tetramers. Tetramers are soluble complexes of recombinant MHC molecules folded in the presence of antigenic epitopes. MHC class I tetramers are relatively easy to produce, whereas MHC class II tetramer production is more challenging. With these reagents, T cells recognizing specific antigenic epitopes can be enumerated, but it does not give information about their functional capacity. However, tetramers can only be used when the patient's HLA haplotype is known and screening can only be done for known TAA epitopes. Tetramer staining can be combined with other techniques, described hereafter, in order to obtain information about the phenotypical and functional profile of the T cells [19, 196]. The group of Coulie et al. developed a tetramer-based mixed lymphocyte peptide culture (MLPC) approach to carefully estimate the frequency of peptide-specific CTL. In this assay, PBMC are re-stimulated

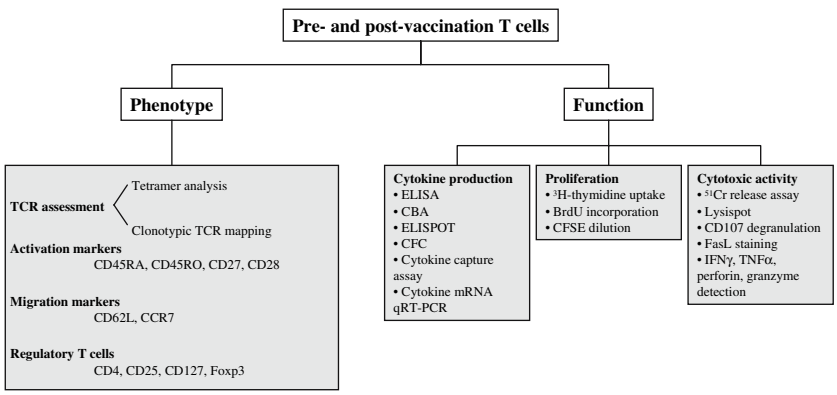

Fig. 3 Schematic overview of immunomonitoring methods to characterize the induced T-cell response. Several assays have been developed to determine phenotypical and functional characteristics of T cells. The combined use of these assays provides information about the breadth and the quality of the induced immune response

twice with peptide and cytokines in limiting dilution conditions. After this culture period the separate cultures are stained with tetramers and the CTL precursor frequency is deduced from the proportion of positive wells. Cells from the positive cultures are subcloned and the growing subclones are then tested for specificity with tetramers and analyzed further with a variety of techniques to determine their functional characteristics [70, 83]. Recently, a qRT-PCR-based method has been developed for clonotypic TCR mapping by which vaccine-reactive T-cell clones can be identified and enumerated [82, 157].

Analysis of T-cell phenotype

Analyzing the T-cell phenotype might be valuable to gather information about the activation status of Ag-specific T cells. Discrimination of naïve and activated/memory CTL solely on the basis of differential CD45RA/CD45RO expression has proven to be unreliable, since CD45RA is also expressed at high levels on stable resting memory $CD8^+$ T cells which did not encounter their cognate Ag for a long period of time. Further characterization can be achieved using markers such as CD27 and CD28 and the lymphocyte migration markers CD62L and CCR7. Table 3 shows the expression profile of these molecules on $CD8^+$ T cells in the naïve stage and during the course of activation. By combining tetramer staining with these cellular markers, the activation status and homing potential of the antigen-specific $CD8^+$ T cells can be assessed [6, 33, 134, 165]. Treg can be detected through the combined staining of CD4, CD25, CD127 and Foxp3, together with several other non-distinctive markers like CTLA-4 and GITR [95, 161].

Measurement of cytokine production

An important parameter of CTL effector function is cytokine secretion. Depending on the stimulus, CTL can produce a variety of cytokines including IL-2, IL-4, IL-10, IFNγ and TNFα. Over the years, several assays have been developed to measure cytokine production after Ag-specific stimulation. Bulk assays such as ELISA and cytometric bead array (CBA) measure the total amount of cytokines secreted by a whole cell population and do not provide information about the percentage of cells producing these cytokines [177, 195]. Furthermore, the detection limit of ELISA is rather high. Newer methods were then developed at the single-cell level: ELISPOT, cytokine flow cytometry (CFC) and cytokine capture assays. Using these assays, the percentage of $CD8^+$ T cells secreting a specific cytokine can be enumerated. In ELISPOT, cytokine secreting cells are visible as single spots on a nitrocellulose membrane and the frequency can be calculated from the number of cells plated [110, 196]. CFC measures the intracellular cytokine content of individual cells and offers the subsequent advantage of combined evaluation of T-cell phenotype [173, 196]. The above-mentioned assays do not provide the opportunity to specifically isolate cytokine secreting cells. Therefore, cytokine capture assays were developed: this assay uses bispecific antibodies that bind the cell surface and capture specific cytokines directly after their release by the cell. These cytokine secreting cells can then be isolated and either used in other assays or cloned [28]. Recently, a sensitive functional assay to directly measure CTL anti-tumor activity by qRT-PCR of cytokine mRNA was developed [81].

Proliferative capacity

Another characteristic feature of effector T cells is their capacity to proliferate upon Ag recognition in order to expand to sufficient numbers. The standard assay used to measure proliferation consists of the uptake of ^{3}H-labelled thymidine by proliferating cells and subsequent measurement of radioactive signals. This assay, however, does not provide information on the percentage of proliferating cells, nor on their phenotype. In order to obviate the need for using radioactivity, another assay was developed where the incorporation of 5-bromo-2′-deoxyuridine (BrdU) is measured by ELISA. BrdU is a pyrimidine analogue and is incorporated instead of thymidine into DNA of proliferating cells [107]. In addition, BrdU can be coupled to fluorochromes for FACS analysis. Dilution of carboxyfluorescein diacetate succimidyl ester (CFSE) is another FACS-based technique to measure cell division. CFSE fluorescence is halved upon every division and can be measured by FACS. Advantages of this technique are the possibility to enumerate

Table 3 $CD8^+$ T-cell characterization according to expression of activation markers and chemokine receptors

	CCR7	CD62L	CD45RO	CD45RA	CD27	CD28
Naïve T cells	+	+	−	+	+	+
T_{CM}, central memory T cells	+	+	+	−	+	+
T_{EM}, effector memory T cells	−	−	+	−	−	−
T_{EMRA}, stable resting Ag-experienced T cells	−	−	−	+	−	−

Central memory T cells home to the lymph nodes, whereas effector memory T cells home to the tissues

proliferating cells at the single-cell level, which can be simultaneously evaluated for their expression of other activation markers. Furthermore, the ability to manipulate the fluorescence intensity of a stained population allows for the differential labelling of two or more cell populations and the technique can be used both in vitro and in vivo [101].

Cytotoxic activity

CTL-mediated cytotoxicity involves three distinct pathways: (1) indirect killing through release of cytokines IFNγ and TNFα; (2) induction of apoptosis through Fas–FasL interactions using FasL expressed by the CTL; (3) direct killing by secretion of perforin and granzymes into the intercellular space [6]. A variety of methods have been developed to measure either total cytotoxicity or one of the aforementioned aspects in particular. The golden standard method is the ^{51}Cr release assay, where total lysis of target cells by CTL is measured. An important drawback however is that no information is obtained about the actual percentage of lytic cells. Consequently, the Lysispot assay was introduced where lytic CTL are visualized as single spots from which the precise percentage of lytic cells can be calculated [164]. Recently, the CD107 assay was shown to be a very attractive method to calculate the percentage of lytic CTL. As a marker of degranulation, CD107a/b is transiently expressed on the plasma membrane, and lytic cells are stained by adding anti-CD107a/b mAbs during culture. An extra advantage of this technique is that it can be combined with tetramer staining and membrane or intracellular FACS staining for activation markers and cytokines, respectively [19]. Other techniques providing information about speciWc aspects of CTL-mediated cytotoxicity comprise membrane staining of FasL and intracellular staining of IFNc, TNFa, perforin and granzymes.

Delayed type hypersensitivity reaction (DTH test)

The DTH test is a method to assess the anti-tumor immune response initiated by a vaccine in vivo. The Ag(s) used for vaccination are injected intradermally into the patient, which attract immune cells to the sensitization site, leading to induration and erythema. The extent of induration/erythema is then a measure for the strength of the immune response, but this assay is not reliable unless the immune cells invading the sensitization site are phenotypically and functionally characterized, providing detailed information about the in vivo immune response against the immunogen(s) [47].

Clinical response

In cancer immunotherapy, as with any cancer therapy, the desired outcome of any treatment is tumor control (either by prolonging the tumor free interval following resection of all disease, tumor stabilisation or regression). The most important appraisal for the success of cancer immunotherapy therefore remains the evaluation of objective clinical responses. At present most clinical trials with DC vaccines have been conducted in pretreated advanced-stage patients, very often with a large tumor volume. In such patients objective clinical endpoints would be easy to assess but have been rarely observed. Moreover, DC vaccination might not be able to induce tumor regression, but might result in slowing the rate of progression. In addition, these patients often have a compromised immune system, which makes them not the ideal patient population to test DC-based therapy which depends on an effective immune system for activity. DC vaccines can also show a delayed onset of activity, based on the time required to initiate an immune response. Therefore, patients could show early tumor progression before eventual tumor regression, which is another factor complicating the evaluation of the clinical response. Furthermore, the tumor can also be controlled by the immune system without complete tumor eradication, in which case only prolonged survival or time-to-progression are good criteria for evaluating the clinical response. In less advanced patients with no or clinically not-evaluable disease or patients with minimal residual disease (MRD) after debulking by other approaches, clinical estimation of the effect of immunotherapy would only be possible by analysis of the time to progression/recurrence and demonstration of a prolonged overall survival of the patients [76]. In some specific types of cancer, tumor markers in the blood could serve as a surrogate (e.g. PSA, idiotype protein, CEA,...). However, it is important to determine objective criteria (e.g. the recently proposed Response Evaluation Criteria in Solid Tumors, RECIST criteria) to evaluate the clinical benefit, as the use of non-standard criteria can lead to over-optimistic interpretation of the results [146].

Overview of clinical trials

A substantial number of clinical trials using dendritic cells has been carried out over the last decade. A recently updated list of published trials has been made available on the Internet by the group of Dr. D. Hart (http://www.mmri.mater.org.au/). Overall, tumor-specific immune responses have been frequently observed in patients vaccinated with DC, but durable clinical responses were exceedingly rare. In general, results from clinical trials published by different groups are difficult to compare because of a variety of reasons: (1) the variability in the type and activation status of the DC used (blood, CD34- or monocyte-derived DC, iDC versus mDC, cytokines used for DC generation,...); (2) the variation in Ag loading methods; (3) the use of different

immunomonitoring methods; (4) the use of non-objective clinical criteria, resulting in an over-optimistic representation of clinical outcome; (5) overall study design (number of DC injected, route and number of vaccinations,...). Nevertheless, certain conclusions can be drawn from these trials. An important overall observation that can be made is that DC vaccination is safe, as no or only mild and self-limiting adverse effects have been reported in a small number of patients. We will now discuss in more depth some trials which highlight aspects that could be important for future design of clinical trials using DC.

Several trials have assessed the influence of the *maturation status of the DC* on clinical and immunological responses. Jonuleit et al. compared GM-CSF/IL-4 monocyte-derived iDC or mDC matured using a cocktail of IL-1β, TNFα, IL-6 and PGE_2 loaded with distinct peptides and recall Ags for vaccination of melanoma patients. Peptide-specific CTL and recall Ag-specific $CD4^+$ T-cell responses were enhanced when mDC were used for vaccination, indicating that mDC are superior to iDC for use in cancer vaccination [80]. A study by de Vries et al. reported vaccination of melanoma patients with either GM-CSF/IL-4 monocyte-derived iDC or mDC matured with MCM pulsed with peptides, followed by vaccination with peptides alone in combination with KLH. KLH-specific cellular and humoral responses were enhanced when using mDC. DTH responses were only observed in patients receiving mDC and T cells isolated from DTH sites showed peptide specificity. No clinical responses were observed in patients vaccinated with iDC ($n = 8$), whereas in patients receiving mDC ($n = 9$), four had disease progression, three had stable disease, one showed a mixed response and one showed a partial response [48]. A recent report by Yamanaka et al. used GM-CSF/IL-4 monocyte-derived iDC or mDC matured with OK-432 (a lyophilized mixture of Streptococcus pyogenes and benzylpenicillin) pulsed with tumor lysate and KLH for vaccination of glioma patients. A higher percentage of patients vaccinated with mDC developed a tumor-specific DTH reaction and tumor-specific $CD8^+$ T cells in blood, compared to patients receiving iDC. In the group of patients vaccinated with iDC alone ($n = 17$), six had stable disease, nine progressive disease and two showed a mixed response. In the cohort of patients treated with mDC or mDC combined with iDC ($n = 7$), there was one partial response, one mixed response, four patients had stable disease and one progressive disease. Altogether, results obtained in these trials clearly indicate that mDC are required for optimal induction of tumor-specific immune responses in cancer patients [48, 80, 199]. However, recent evidence indicates that iDC can still be used for vaccination, when combined with in vivo maturation approaches [117].

Another variable possibly affecting the effectiveness of DC vaccination is the *route of vaccine administration*. Fong et al. used protein-pulsed DC isolated from blood for vaccination of metastatic prostate cancer patients by intravenous (i.v.), intradermal (i.d.) or intralymphatic (i.l.) route. All patients developed Ag-specific T-cell proliferative responses, regardless of the immunization route. TNFα secretion was only observed after i.v. vaccination, whereas IFNγ was only detected after i.d. and i.l. vaccination. None of the patients developed an IL-4 response. Ag-specific Abs were predominantly detected in i.v. treated patients. In conclusion, i.d. and i.l. DC vaccination leads to induction of Th1 immunity, whereas i.v. vaccination leads predominantly to a humoral response [61]. The group of Bedrosian reported a study in melanoma patients where monocyte-derived mDC were administered i.v., intranodally (i.n.) or i.d. Tetramer-positive $CD8^+$ T cells were induced/enhanced in the majority of patients but IFNγ production by these T cells was only seen in 6/7 i.n. treated patients and 2/6 i.d. treated patients. In the i.v. treated group 4/8 patients had stable disease and 4/8 had progressive disease. In the i.n. group, 2/8 patients showed a minor response, 2/8 remained stable and 4/8 progressed. In the i.d. treated group, 1/10 patients had a minor response, 3/10 had stable disease and 6/10 progressed. These results point to the intranodal route as the preferred mode of DC injection [15]. Kyte et al. describe the treatment of melanoma patients with monocyte-derived mDC transfected with autologous tumor RNA using either i.d. and i.n. injections. Tumor-specific T-cell proliferation was observed in 6/10 i.d. immunized patients and 4/12 i.n. immunized patients, whereas tumor-specific DTH responses developed in 6/10 i.d. treated patients and 2/12 i.n. treated patients. Clinically, in the i.d. treated group ($n = 10$), two patients had no evidence of disease, one remained stable and seven progressed. In the i.n. treated group ($n = 12$) 1 patient was stable and 11 progressed, which indicates that, in this study, i.d. administration of DC was more efficient [89]. Recently, Trakatelli et al. reported immunization of patients with IFNβ/IL-3 monocyte-derived mDC pulsed with peptides via the combined subcutaneous (s.c.), i.d. and i.n. routes. DC migration was only observed after i.d. injection, not after s.c. injection. Peptide-specific CTL were detected in 3/8 patients and these patients also showed DC migration. Regarding clinical outcome, 3/8 patients had no evidence of disease, 1/8 remained stable and 4/8 progressed [184]. Butterfield et al. compared i.v. and i.d. administration of peptide-pulsed iDC in melanoma patients. Both routes of immunization resulted in development of peptide-specific T cells in the same percentage of patients, but higher levels of IFNγ were secreted by these T cells in the i.d. group. Determinant spreading occurred in one i.d. treated patient and not in the i.v. group and this was correlated with the induction of a durable complete response [30]. Altogether, these results indicate that i.v. injection primarily induces humoral immune responses,

whereas i.d. and i.n./i.l. injection mediates induction of Th1 immunity. Intradermal DC administration induces DC migration, as opposed to s.c. injection. Since i.n. administration is rather complicated and because i.v. injection is less effective at inducing Th1 responses, results so far indicate that i.d. administration of DC is probably preferable for inducing anti-tumor immunity and clinical responses [15, 30, 61, 89, 184]. However, these results are very preliminary, since only a small number of patients have been treated in these studies.

As already mentioned, *epitope spreading* occurring after vaccination might be an important factor to counteract tumor escape and elicit durable clinical responses. Brossart et al. vaccinated seven breast and three ovarian cancer patients with peptide-pulsed monocyte-derived mDC. Upon vaccination two patients developed disease stabilization whereas the others progressed. Ag-specific T-cell responses developed in five patients, with two of them showing evidence for epitope spreading. These same patients also showed a period of disease stabilization, suggesting that epitope spreading could be correlated with clinical response [27]. The group of Trefzer vaccinated melanoma patients with irradiated fusions of allogeneic mDC and autologous tumor cells. Upon vaccination 1/17 patients developed a complete response, 1/17 developed a mixed response, 6/17 patients remained stable and 9/17 patients progressed. Tumor-specific $CD8^+$ T-cell responses were mounted in 11 patients, 3 of which showed epitope spreading. However, in all patients analyzed ($n = 6$) immune evasion was detected, as tumor cells were found to lose either TAA expression or molecules of the Ag presenting machinery, or both [185, 186]. These data indicate that the phenomenon of epitope spreading is often correlated with a positive clinical outcome and could thus be a predictive factor of vaccination efficiency [27, 185, 186]. Although these data are promising, more patients have to be treated in order to be able to draw definitive conclusions.

The group of Su et al. investigated whether *targeting of the Ag for presentation in both MHC class I and II* could improve anti-tumor immune responses. DC were electroporated with either hTERT mRNA or LAMP-hTERT mRNA. After injection, 9/11 hTERT immunized patients and 9/9 LAMP-hTERT immunized patients developed pronounced inflammatory responses at the injection site. Immune reactions were more pronounced in the LAMP-hTERT group. hTERT-specific $CD8^+$ T cells were detected in the blood of 8/9 hTERT immunized patients and 9/9 LAMP-hTERT immunized patients. hTERT-specific $CD4^+$ T cells were detected in 6/9 hTERT vaccinated patients, compared to 9/9 LAMP-hTERT vaccinated patients. Furthermore, $CD4^+$ T-cell responses induced in LAMP-hTERT vaccinated patients were of higher magnitude compared to responses induced by hTERT vaccination. CTL from LAMP-hTERT immunized subjects showed higher lytic activity against hTERT-expressing targets than CTL from the hTERT group. In addition, whereas CTL from hTERT immunized patients developed mainly into effector memory T cells, CTL from LAMP-hTERT vaccinated patients developed into central memory T cells. hTERT-specific $CD4^+$ T-cell proliferation was only observed in LAMP-hTERT vaccinated patients. Four out of nine patients in the LAMP-hTERT group had circulating tumor cells, all of which were transiently reduced during and after vaccination. Six out of nine patients in the hTERT group had circulating tumor cells, which were transiently cleared in 5/6 subjects. Altogether, these data suggest that the use of LAMP targeting can induce more pronounced anti-tumor immune responses with an improved T-cell memory [171]. These results thus seem promising and are prompting us to investigate this issue in larger cohorts of patients to draw further conclusions.

A key obstacle hindering the induction of successful anti-tumor immune responses by DC vaccination is the presence of *suppressive mediators*. One of the factors that has clearly been implicated in suppression of tumor specific immune responses are Treg. These cells are able to suppress Ag-specific effector T cells and were found in elevated numbers in the peripheral blood of cancer patients compared to healthy volunteers. Furthermore, large numbers of Treg were found intratumorally. Dannull et al. investigated whether elimination of Treg using denileukin diftitox/ONTAK (recombinant IL-2 diphteria toxin conjugate) could enhance the efficacy of tumor RNA-transfected mDC vaccines. In this respect, we have to mention that although Dannull et al. observed a reduction of Treg numbers using this regimen, this result could not be obtained by Attia and coworkers [9]. $CD8^+$ T-cell responses were increased 2.7-fold in patients receiving only DC and 7.9-fold in patients receiving ONTAK and DC. $CD4^+$ T-cell responses were increased 2-fold in patients immunized with DC alone, compared to 7.2-fold in patients vaccinated with ONTAK and DC. The results of this study indicate that combination of Treg depletion by ONTAK and DC vaccination might lead to improved anti-tumor immune responses [44]. The group of Höltl et al. investigated whether co-treatment with cyclophosphamide could enhance the efficacy of vaccination with allogeneic monocyte-derived mDC pulsed with tumor lysate and KLH. Although the dose and administration schedule of cyclophosphamide used here do not mediate Treg depletion [68], cyclophosphamide could down-regulate Treg activity. KLH-specific proliferative responses were only observed when DC vaccination was combined with cyclophosphamide, whereas tumor-specific responses could not be detected in any group. In the group receiving DC alone ($n = 11$), two patients remained stable and the others progressed. In the group receiving combined cyclophosphamide and DC treatment ($n = 7$), two patients developed

a mixed response, one remained stable and four progressed. These results suggest that combining DC vaccination with cyclophosphamide administration could be an effective means to counteract the suppressive effect of Treg on anti-tumor responses [75]. Although the number of treated patients was rather low in these studies, results indicate that either Treg depletion or inactivation combined with DC vaccination might lead to improved anti-tumor immune responses [44, 75].

Very few data are available on *the role of tumor volume on the outcome of DC vaccination* therapy. It has been postulated that in patients with large tumor burden active suppressive mechanisms of the tumor prevent the induction of effective anti-tumor immune responses. A study reported by O'Rourke and colleagues used the measurement of the S-100B protein in the blood of stage IV melanoma patients as a means to estimate tumor burden. They show that patients with low S-100B concentration (<0.36 μg/ml plasma) before vaccination show a significantly better survival upon DC vaccination compared to patients with high S-100B levels before vaccination. Furthermore, patients with initially low S-100B levels and concomitantly low bulk disease were shown to have significantly better objective clinical response rates. Thus, S-100B levels in melanoma patients could function as a measure for the tumor burden and could be predictive for the outcome of therapy. The authors postulate that a large tumor burden prevents the induction of an anti-tumor response by DC vaccination and suggest that surgical debulking before DC vaccination could improve the outcome of DC vaccination [123]. Another study reported by Tuettenberg et al. compared results of a study in stage II melanoma patients with MRD at high risk of relapse with previous results of a study in stage IV melanoma patients with large tumors. They point to several important differences: (1) the strength of DTH responses obtained in stage II patients was significantly higher and longer-lived compared to stage IV patients; (2) although tumor-specific $CD8^+$ T-cell responses were observed in both stage II and IV patients, the expansion of these tumor-specific $CD8^+$ T cells was much higher in stage II patients; (3) vaccine-induced IFNγ producing effector $CD8^+$ T cells were observed in a larger proportion of stage II patients compared to stage IV patients. Altogether, vaccination-induced expansion and differentiation of Ag-specific $CD8^+$ T cells was more prominent in stage II patients [187]. Although preliminary, these results suggest that a large volume of tumor indeed has a negative effect on the outcome of DC vaccination. Therefore, surgical debulking before vaccination to obtain a state of MRD could be advantageous for subsequent DC vaccination.

Although many issues still need to be resolved, the following conclusions can be drawn from the clinical trials carried out with DC so far. First, the present view is that mDC need to be used for vaccination. Maturation stimuli can be provided either in vitro or in vivo. However, considerable controversy still exists about the selection of optimal maturation stimuli. Second, concerning the immunization route, the few studies that compared different routes point to i.d. administration as the preferred method. However, combining different routes of immunization might also be beneficial because depending on the location of the tumor a different injection route might be required [115]. Third, a whole variety of methods has been used to load DC with Ags. Immunization with a single antigenic epitope could lead to the emergence of Ag-loss variants, although it is now clear that T cells directed towards the immunizing epitope can lead to secondary activation of T cells recognizing other TAA. Furthermore, the emergence of anti-tumor T cells or epitope spreading could be predictive for a clinical response. However, the use of approaches employing different TAA could select for the most immunogenic responses and, when a genetic approach is used, the TAA can be targeted for presentation in MHC class II, thereby providing $CD4^+$ T-cell help. Little is known about the optimal number of vaccinations, but it is generally believed that repeated injections are beneficial. However, there is little or no information on the optimal interval between vaccinations [60]. Finally, a number of studies combine DC therapy with other agents. KLH has been frequently used to provide non-specific $CD4^+$ T-cell help, but more recently other stimuli have been used in conjunction with DC vaccination to either activate DC in vivo (e.g. TLR ligands), activate the immune system (e.g. cytokines) or attenuate tumor-induced immunosuppression (e.g. cyclophosphamide, ONTAK). However, more research is needed to identify possible advantageous combinatorial therapies.

Concerning the design of clinical studies using DC, minimal criteria have to be fulfilled in order to be able to compare different studies and eventually come to a standardized protocol [60]. The method of vaccine preparation must be carefully described and it must be indicated if DC vaccination is combined with other treatments. The studies should provide precise information on the maturation stimulus used and the quality of the injected DC. Next, there has to be a careful description of the route of DC administration, the number of DC injected, the vaccination schedule and the time points at which samples are taken for monitoring. Lastly, a careful description of clinical outcome of all treated patients together with the results of immunological monitoring before and after treatment needs to be provided.

Conclusions and future perspectives

Promising results have been obtained with DC vaccination in mouse models, with induction of both anti-tumor

immune responses and tumor regression. Initial clinical trials in humans highlight the potential of DC for the induction of tumor-specific immune responses. Nevertheless, durable clinical responses have been rarely achieved and often a correlation between the induction of tumor-specific immunity and clinical outcome could not be observed. Several factors might contribute to this discrepancy. First of all, patients treated up to now generally suffered from advanced-stage disease, were heavily pre-treated and had a compromised immune system, which has considerable impact on the efficacy of DC vaccination. Furthermore, most patients have excessive, highly vascularized tumor burden, which may be difficult to reject by T cells induced by DC vaccination. We should now move to vaccination of less-advanced patients or even patients with MRD, which might be the ideal treatment population for DC vaccination. Another important variable which complicates comparison of clinical trials is the use of different DC subsets for vaccination. New insights in DC biology are continuously generated which makes it difficult to standardize the DC population to be used in cancer trials. Although DC vaccination proved to be safe in the short term in these first trials, concerns are raised regarding the long-term effect since it has been described that some DC subsets can induce/expand Treg. Preferably, DC generated from distinct progenitors and activated using different stimuli should be compared in small clinical trials in order to define optimal DC preparations and standardize vaccination protocols. Maybe a DC vaccine consisting of several DC types could be developed in order to try to activate all components of the immune system. The same holds true for the Ag type to be used for DC loading. Although epitope spreading has been described when using only one defined epitope for vaccination, optimal loading strategies should use a broad spectrum of potential TAA, in order to avoid tumor escape. Next, immunomonitoring methods used should preferably be standardized in order to be able to compare immunological outcomes for different trials. A broad spectrum of methods should be used to not only enumerate tumor-specific effectors, but also to characterize their activation status and functional properties. Furthermore, in order to fully characterize the immune response, it is important to analyze both T cells in the circulation and at the tumor site, since important phenotypical and functional differences were found, which might in part explain the lack of clinical efficacy of DC vaccination. One important contributing factor is probably the existence of a suppressive tumor microenvironment. Several mechanisms cooperate to establish this tumor-induced immunosuppression. Tumor cells display an over-consumption of glucose, leading to glucose deprivation which could inhibit effector T-cell functions. B7-H1/PD-L1 expression by human tumors leads to inhibition of T-cell responses through interaction with PD-1 on activated T cells. Tumors show an elevated expression of COX-2, leading to secretion of PGE_2 which inhibits T-cell effector functions. Indoleamine 2,3-dioxygenase (IDO) is frequently expressed and activated in tumor cells, which leads to tryptophan deprivation and production of toxic metabolites, causing cell-cycle arrest and T-cell death. Myeloid suppressor cells (MSC) are present in various tumors and express Arginase I and the inducible nitric oxide synthetase (iNOS), which lead to arginine deprivation and the production of toxic O and NO radicals within the tumor. Soluble immunosuppressive mediators (TGF-β, VEGF, IL-10, IL-23) are present within the tumor microenvironment and interfere with effective T-cell function. Finally, Treg are recruited and/or induced in tumors and exert direct immunosuppressive effects on effector T cells. Therefore, it will probably be advantageous to combine DC vaccination with agents tackling these immunosuppressive mechanisms. Among these agents are blocking Abs (PD-1/B7-H1, TGF-β, VEGF, IL-10, IL-23), enzyme inhibitors (IDO, Arginase I, iNOS) and strategies to eliminate or inhibit Treg (low-dose chemotherapy, ONTAK, CTLA-4 blockade, TLR8 stimulation) [35, 40, 64, 74, 96, 114]. Thus, in a first stage it is necessary to gather more information about the variety of escape strategies used by tumors. In the next step, we can identify which mechanism(s) is used by the tumor of individual patients in order to select for each patient the most appropriate approach to counteract tumor escape.

It has become clear that, in order to eradicate large tumors, a combination of vaccination approaches targeting several aspects of the immune system is necessary: (1) adoptive transfer of tumor-specific T cells to directly attack the tumor; (2) TAA-expressing DC to induce tumor-specific effector and eventually memory T cells; (3) interference with the suppressive tumor microenvironment; and (4) administration of immune-activating cytokines (GM-CSF, IFNα, IL-2, IL-15, TNFα...).

In conclusion, DC vaccination has proved to be very safe and can induce tumor-specific immune responses. However, objective clinical responses so far have been scarce. Further optimization of several parameters is needed, including defining the preferred DC type and the optimal Ag loading technique, which will eventually all lead to a standardized DC vaccine. Efforts should also focus on the combination of DC vaccination with other therapies, to further enhance clinical efficacy.

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