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Dendritic cells as therapeutic agents against cancer

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1. ABSTRACT

Dendritic cells (DC) are antigen-presenting cells whose immunobiology has been proven to be central to the function of the immune system. Further understanding of these cells is leading the way to the manipulation of the immune system as a tool to cure and prevent a vast array of diseases including cancers. These cells have been used in trials as vaccine adjuvants in therapies that aim to break the body's tolerance to the tumor. From the first 1000 DC vaccinees in 2003 there has been a breadth of information on safety that is paving the way to the study of the efficiency of these therapies. This review aims to explore recent updates to the current literature on DC vaccine therapies in clinical trials and analyze their future. At this crossroads is where intricacies of the technique are being revised to explore the most efficient and effective parameters for the enhancement of DC adjuvant therapies.

2. INTRODUCTION

Research on the immune system continuously reveals roads to novel therapies for many diseases including infections, allergies, graft rejection, and cancer. Among the many cells involved in this intricate system are dendritic cells (DC) whose immunobiology have already proven to be central to the function of the immune system. Further understanding of these cells is leading the way to the manipulation of the immune system as a tool to cure and prevent a vast array of diseases including cancers. For instance, the discovery that tumor-infiltrating DC can naturally be found in renal cell carcinoma (RCC, 1). Our understanding of these cells has boomed in the recent years and led to DC-based cancer immunotherapy protocols, also referred to as DC vaccines. Some of these protocols include pulsing of autologous DC (with synthetic tumor antigens, idiotype antibodies, tumor lysates, mRNA or genetically

altered cancer cells), genetic altering of DC (by transfection with tumor DNA or viral transduction with specific genes) and creating tumor cell/DC fusions to stimulate DC (2). DC in cancer immunotherapy are used as vaccine adjuvants to induce specific immunity against tumor epitopes. In general, these therapies aim to break the body's tolerance of the tumor in order to fight it, control it and even perhaps eradicate it. Clinical trials and therapies in this area have advanced tremendously in just a couple of years. It was only in 2003 when a report on the first 1000 DC vaccines became available and now six years later some of these trials have proven safe and effective to a certain extent, which has been the main goal until now (Table 1, 3). This field is starting now to enter into trials where clinical efficacy is starting to become the main focus, rather than safety and feasibility. At this crossroads is where intricacies of the technique are being analyzed to explore the most efficient route for the goal of targeting various tumors. This review will explore recent updates to the current literature on DC vaccine therapies in clinical trials and analyze their future.

3. DC IMMUNOBIOLOGY

A critical aspect of the immune defense is mediated by helper function of newly activated T helper (T_H) cells and by professional antigen-presenting cells (APC). Dendritic cells (DC) are the most potent APC known, triggering T cells into cell cycle-progression (4, 5). In healthy individuals, these cells are a sparsely distributed, migratory group of bone-marrow-derived leukocytes that are specialized for the uptake, transport, processing and presentation of antigens to T cells (6). These cells have a distinct morphology characterized by the presence of numerous membrane processes that can extend for up to hundreds of micrometers resembling neuronal dendrites (ergo their name). Additional morphologic features include high concentrations of intracellular structures related to antigen processing such as endosomes and lysosomes. At an immature stage of development, DC act as sentinels in peripheral tissues, continuously sampling the antigenic environment by phagocytosis and micropinocytosis. Any encounter with microbial products or tissue damage initiates the migration of the DC to lymph nodes where they use their high stimulatory capacity to activate naïve T cells. They are 10-100 times more potent at activating naïve and memory T cells than other professional antigen presenting cells (6). This makes them important initiators of primary specific immune responses. They regulate both immunity and tolerance while other cells (i.e.: B cells, T cells and NK cells) are the effectors. Research groups regularly rely on this ability to induce proliferation in an allogeneic mixed leukocyte reaction (MLR). Using this assay system, they can stimulate T cells by presenting either self or exogenous antigens.

All of these characteristics make these cells unique candidates as cancer vaccine adjuvants since they have the essential features for the initiation of immunity. Sensitizing the immune system to specific antigens is certainly the most pertinent function for DC. Immature DC, located at sites of antigen entry such as the gut mucosa, are specialized for antigen capture but lack the ability to activate T cells (7). Current understanding of the maturation events shows that progenitors from the bone marrow become highly phagocytic DC precursors (immature DC) in peripheral tissues. As they mature, DC migrate to peripheral lymphoid organs where they lose the ability to capture antigen but now acquire MHC at the surface and thus acquire the capacity to activate naïve T cells carrying receptors for that antigen (5, 8, 9). Many products can lead to this maturation but in general, products that can be recognized by the body as a foreign entity (i.e.: CpG motifs in bacterial DNA, double-stranded viral RNA, lipopolisacharide (LPS), necrotic cell products) to activate DC. In order for this activation to occur, antigen capture should lead to signals associated with inflammation or infection, such as engagement of toll-like receptors (TLRs), production of interferons (IFNs), or up-regulation of members of tumor necrosis factor-receptor (TNF-R) family. *In vivo* this process is paralleled by migration of DC to T-cell rich areas of lymphoid organs, where they present antigen-derived peptides to antigen-specific T cells and direct their differentiation into T effector or T memory cells by clonal expansion (9-11). These mature DC can also induce NK cell activation, B cell differentiation into antibody forming cells and induce tolerance (12).

The importance of the DC/T cell interaction is highlighted by the fact that antigen capture in the absence of co-stimulation can lead to tolerance (12). All steps of the formation, maturation and presentation of antigens for the initiation of the adaptive immune response are critical at determining the response that ensues. Throughout the process of maturation DC undergo changes in markers and receptors that are critical as part of their differentiation (Figure 1). Freshly isolated DC are active at pinocytosis and possess nonspecific antigen uptake receptors though at lower levels. Some express FcγR (CD16, CD32) and complement receptors (CD11b, CD11c, CD35). CD11c may also act as a receptor for LPS as DC lack the classical LPS receptor, CD14, and yet respond to this stimulus. In addition, DC can also distinguish between tissue cells that die by the normal process of apoptosis and those that die by externally generated necrosis. The receptors that recognize these diverse stimuli vary from lectin-domain scavenger receptors that are similar to those on phagocytes to the TLRs (13). Most antigens used in cancer immunotherapy are targeted to stimulate $CD8^+$ T cells with the aim of inducing cytotoxic responses against tumor cells. It seems that the objective clinical responses observed with these types of cells tend to be sporadic albeit with a strong immune reactivity against tumors (14). This effect may be linked to the trafficking of these cells to tumor sites. In order to circumvent such a problem the recruitment of $CD4^+$ T_H cells by DC instead would induce a generalized antitumor immunity which should include the mobilization of CTLs (15, 16). Furthermore these cells could overcome the tolerance induced by the tumor microenvironment (17). Some of the reasons why these cells are not the target is because MHC class II restricted peptides are difficult to isolate, there are few methods for their identification and tumors express class I but not class II molecules (18). The development of improved methods of identification of

Table 1. Tumor-specific targetted approaches with DC in Phase I-III clinical trials

Patients and Cancer	C generation	Antigen Delivery Method	Dosage	Outcome	Trial Phase	Ref
512: IMPACT (IMmunotherapy for Prostate Adeno-Carcinoma Treatment)	Auto DC	Loaded with recombinant fusion of PAP antigen and GM-CSF (Provenge)	leukapheresis in weeks 0, 4, 8, and 24	All had immune response to fusion protein. 38% had immune response to PAP, 6 patients had PR. Phase III was looking for a minimum 22 percent increase in survival.	III; I/II	Results to be announced AUA in Chicago 2009; (146)
55: DTIC 53:DC and ITT Stage IV melanoma	Auto DC	loading with MHC class I and class II restricted peptides	s.c. biweekly, total of 5, then monthly;	Only 3.8% OR, trials closed early by recommendation	III	(139)
26: Progressive advanced breast cancer		loading with 3 wt and 3 P2 anchor HLA-A2 p53 peptides	$5x10^6$ cells s.c.in 1-2 week intervals, 10 total, + 6 doses IL-2	8/19 SD or Regression, 11/19 PD	II	(101)
15: Multiple Myeloma	Auto MoDC from CD14+ isolated cells. Cryopreserved	Loaded with autologous Id as whole protein or Id derived class I peptides + KLH	3 s.c. and 2 i.v. administrations of increasing doses. biweekly	8/15 T cell responses; 8/15 $IFN\gamma^+$ responses; 4/15 DTH $^+$. Peptides were as effective as whole protein. 7/15 SD, 1 PR, 7 PD	I/II	(147)
13: Metastatic CRC	GM-CSF/IL-13 MoDC; matured with Klebsiella-derived cell wall fraction+ IFNγ	loading with 6 CEA and MAGE-derived peptides, HLA-$A2^+$ restricted	$35x10^6$ cells, i.d. every 3 weeks	PD in all	I/II multi-center	(148)
24: Stage IV RCC	Monocytes, GM-CSF/IL-4	DC/tumor fusion	$4\text{-}10x10^7$ cells, 3 times at 6 week intervals	2 PR, 8 SD	I/II	(149)
46: stage IV melanoma	Cryopreserved DC	DC/tumor fusion		3 CR, 3 PR	I/II	(127)
10: CML Bcr/abl^+ in the chronic phase	auto MoDC; shared leukemia antigen	"leukemic" DC non irradiated	post IFNα and imitumab , 4 s.c. injection of increasing dose from1 to $50x10^6$ cells on days 1,2,8 and 21	4/10 patients improved their cytogenetic/molecular response	I/II	(97)
14: metastatic melanoma	Matured with: Ribomunyl+IFNγ	loaded with Melan-A/MART-1 and/or NA11-A +KLH	1i.l. then 2 i.n. 1 month apart each	DTH to NA17-A 6/10, DTH to KLH 4/11, DTH to Melan-A 3/9, T cell responses: 4/12 Melan A, 2/12 NA17-A, All SD, long term 3 alive	I/II	(130)
12: hormone and chemo refractory prostate cancer	auto DC	loaded with PSCA and PSA derived HLA-A2 binding peptides	$\sim 2.7x10^7$ cells s.c. biweekly, 4 total.	4 SD, 1PD	I/II	(135)
2 Melanoma	Auto	transfection with autologous tumor mRNA	long-term booster (already vaccinated)	Enhancement of already present presentation immune responses	I/II	(110)
20 metastatic RCC	Mature MoDC	pulsed with HLA-A2 binding MUC1 peptides + PADRE	s.c. biweekly total of 4 with scalating doses of IL-2; then monthly DC until progression	6 regressed; 1 CR, 1 PR, 1 MR, 1SD	I/II	(106)
22 advanced malignant melanoma	auto MoDC	treansfection with autologous tumor mRNA by electroporation	4 weekly vaccines i.n. or i.d.	8/18 positive DTH; 9/19 positive ELISPOT; 7/10 i.d. versus 3/12 i.n. OR	I/II	(150)
6 Hormone-refracto prostate carcinoma	auto DC	loaded with peptides from PSCA, PAP, PSMA, PSA; HLA-$A2^+$ restricted	i.d. injection six times at biweekly intervals	Significant CTL responses against cell antigens. Memory T cell responses also boosted	I/II	(134)
22 AML	DC derived from leukemic cells (DLLC)		s.c.weekly. Total of 4 scalating doses	5 CR, 2/5 endured	I/II	(115)
19; 15 Disseminated carcinoma	MoDC GM/IL-4; Matured with supernatants of OK432 stimulated PBMC	loaded with autologous necrotic whole tumor	s.c. injection biweekly i.v. of activated lymphocytes every 4 weeks	6 responders, 13 non responders; DTH in 13/15	I; I/II	(151)
9 CEA+ CRC or lung cancer	CD14+ MACS isolated	pulsed with CEA-derived altered peptide CEAalt + KLH	i.v. 1 or $5x10^7$ cells every 2nd week; total of 4	Increased $IFN\gamma^+$ peptide specific $CD8^+$ T cells 5/9; 3/9 CEA native peptide recognition	I/II	(152)
15 Stage III or IV melanoma	Auto MoDC GM/IL-13, serum free media	loading with allo tumor cell lysate + HBAg or TT	4 s.c., i.d., i.n. injections	4/9 had >20 months survival, 1 CR, 1 SD	I/II	(136)
24 grade 3,4 recurrent glioma	GM-CSF/IL-4MoDC: matured with supernatant of OK432	pulsed with autologous tumor lysate + KLH	i.d. or i.d./i.t. every 3 weeks	1PR, 3 minor R, 10SD, 10PD	I/II	(153)

22 Metastatic RCC	Allo MACS $CD14^+$ MoDC; Matured selected for $CD83^+$	loaded with tumor lysate + KLH	12 only DC, 10 DC + cyclophosphamide, days 4 and 3 pre-vaccination; monthly total of 3	2 MR, 3SD, 13 PD, 4 no-followup; KLH responses weak or absent; Cyclophosphamide: 2MR, 1 SD	I/II	(104)
20 Metastatic RCC	Auto DC	pulsing with TAA-MUC1 9 mer peptides HLA-A2 binding		6 regression; 1 CR, 2 PR, antigen spreading in all responders	I/II	(116)
20 Metastatic sarcoma	Auto GM-CSF/IL-4MoDC	Tumor cell lines + IFNγ, irradiated, frozen	$20x10^6$ s.c. + GM-CSF weekly, total of 3 doses then monthly total of 5 doses	16 PD, 8/20 positive DTH, 95% survival at 3 years	I/II	(92)
10 MM	MACS $CD14^+$ MoDC; matured with: TNFα or IL-1β, IL-6, TNFα, PGE2	Pulsed with Id whole protein +KLH	5, 10, $50x10^6$ cells 3 s.c. and 2 i.v.	Cryopreservation did not affect phenotype of functionality →continuation to scaled-up phase II recommended	I/II	(57)
11Prostate cancer, 5 RCC	Allo DC	Allogeneic tumor lysate pulsed, cryopreserved	$1\text{-}3x10^6$ cells + KLH i.d. biweekly 6 times then monthly	DTH^+ almost all; Increased T_H1, $IFN\gamma^+$ T cells; Reduction of PSA ; 2/5 SD	I/II	(8)
11 Malignant melanoma	Matured allo DC	Fusion with autologous tumor	i.c. or s.c. combined with s.c. IL-2	10 PD, 1 SD, none had DTH+ responses	I/II	(154)
14 NSCLC stage IA through IIIB	auto DC; matured with: original trial used DCTCMF, new trial uses immature DC	pulsed with apoptotic bodies from allo NSCLC cell line $Her2/neu^+$, CEA^+, $WT1^+$, $Mage2^+$, $survivin^+$	$8.1x10^7$ cells twice i.d., 1 month apart in patients w/ or w/o resection	Immune: 4/7 stage III, 6/7 stage I/II; Clinical: PD 4/7 stage III, RD 1/7stage I/II	continuation	(113)
18 Relapsed or refractory cancer	Monocytes, GM-CSF/IL-4; matured with: IL-1β, PGE2, TNFα	tumor lysate pulsed 100 ug/ml	2 vaccinations i.d., every 2 weeks then monthly	4 OR	I	(155)
Progressive cytokine-refractory RCC	Mature DC	loading with CA-9 peptide +KLH	5 vaccines	All developed DTH and antiKLH antibodies. No clinical responses observed	I	(156)
20 Stage IV melanoma	auto MoDC GM/IL4; matured with TNFα+CD40L	loaded with killed Colo829 allo cell line tumor cells	8 vaccines total, monthly	4 alive at 12 month, 9 at 24 months, 8 at end of study, 1 CR, 1partial regression, 3 T cell immunity	I	(150)
6 Advanced CRC	auto MoDC GM/IL4	pulsed with lysate from DDM-1.13 cell line	5 i.d. injections, 2 week intervals, total 5	Safe	I	(157)
8 Hormone-refractory prostate carcinoma	auto DC	loaded with peptide cocktail from PSA, PMSA, survivin, prostein, trp-p8 antigens, $HLA\text{-}A2^+$ restricted	biweekly vaccines total of 4	1 PR, 3SD	I	(141)
10 MUC^+ Adenocarcinoma	auto DC GM/IL-4	pulsing with mannan-MUC-1 fussion protein (MFP)	3 leukapheresis monthly then continue if there was a response	Especific $IFN\gamma^+$ T cell and DTH responses in 9/10 after one year. 2SD	I	(158)
18 Stage IV melanoma	CD34+ DC	loaded with Mart-1/Melan-A, trosinase, MAGE-3, gp100, FluMP, KLH; $HLA\text{-}A2^+$ restricted	total of 8 vaccines	4 patients alive for >20 months. Correlates with at least 2 anti-peptide responses	I	(159)
20 Stage III or IV melanoma	auto MoDC	pulsed with melanoma cell lysate	s.c. injections with or without low dose IL-2	10/20 increased IFNγ; 4/5 increased T cell responses; 6/13 DTH^+	I	(56)
28 Metastatic Prostate Cancer	auto DC	pulsed with PSA	i.d. injections of PSA +GM-CSF or i.v. DC at weeks 1,4 and 10	50% DTH^+; 6/7 TNFα/IFNγ increased response	I	(96)
9 Stage III or IV metastatic melanoma	auto DC	Transduced with fowlpox vector encoding CEA		4/9 NK activity increased, 2/9 stable, 3/9 decreased; 5 SD or NE, 4PD	I	(122)
22 Stage IV melanoma	$CD34^+$ - derived DC; matured with: IFNα	loaded with peptides derived from MART-1, tyrosinase, MAGE3, gp100 + fluMP+ KLH; HLA-A2+ restricted	8 vaccinations or more	No expansion of memory T cells; 6/7 Specific $IFN\gamma^+$ T cells; 4 > 26 months survival	I	(160)
12 Glioblastoma multiforme	Auto DC	pulsed with acid-eluted tumor-peptides	1, 5, $10x10^6$ DC in 3 i.d. weekly injections	6 CTL responses; 4/8 increased tumor infiltration; 1 OR	I; multi-cohort	(161)
1411CRC 5NSCLC		modified with a rFCEA and rF-CEA(6D)-TRICOM	1 or 2 cycles of triweekly s.c. or i.d. injections		I	(123)

		+ TT loaded DC as control + pp65				
14 advanced hepatoma	Immature auto DC		i.t. injection in 4 dose cohorts: 0.5, 1.5, 3 and 5x10^7 cells 2 days after radiotherapy. 2nd dose 3 weeks later	2 PR, 4 MR; 3/14 had decreased AFP, 8-6/14 had anti tumor immune responses	I	(162)
11 Stage IV neuro-blastoma	MoDC	pulsed with tumor RNA	2 courses carboplatin, chemotherapy, surgery, radiation, high dose therapy, stem cell regime and DCRNA	Carboplatin maintained lymphocytes, $CD4^+$ cells decreased, $CD8^+$ cells increased, $CD19^+$ decreased, humoral responses to recall antigen decreased, no OR	I	(163)
6 Stage IV melanoma	Auto CD34-DC	Transduction with MVA-hTyr	$1x10^8$ cells i.v. then 3 s.c. injections every 14 days	1 PR; significant and long lasting T cell responses	I	(125)
10 Stage II-IV melanoma	Auto DC	Pulsed: HLA-$A2^+$ immuno-domminant MART-1 (27-35)	10^7 cells i.d. biweekly; 3 doses	1 SD, 1 CR; determinant spreading observed; CTLA4 blockade may enhance immunogenicity	I open-label	(164)
9 Brain tumors	GM-CSF/IL4 MoDC	Pulsed with autologous tumor RNA	i.v. and i.d. biweekly	2/7 SD; 1/7 PR; 2/7 tumor-specific immune responses	I	(165)
32 Metastatic breast and RCC	Auto GM-CSF/IL4 and autologous plasma MoDC	Fusion with autologous tumor cells with polyethylene glycol	$1x10^5$ to $4x10^6$ in 3 week intervals	IFNγ^+ CD4 and CD8 cells increased; 2 PR, 2 SD in breast cancer; 5 SD in RCC	I	(166)
14 Glio-blastoma multiforme; anaplastic astro-cytoma	Auto DC	Pulsed with tumor lysate	3 total doses biweekly intervals	6/10 increased IFNγ, 4/9 increased CTL, 3/6 increased intratumor infiltrate; media survival 133 weeks	I	(167)
6 Advanced breast cancer	Auto DC	Loaded with 3 WT and 3 modified p53 peptides	HLA-$A2^+$	2 SD, 1 PR, 1 MR, 3 of those showed specific T cell responses	I	(168)

MHC-class II restricted peptide, such as the genetic targeting expression system or the definition of such peptides from candidate antigens should lead the way for this alternative to class I restricted peptides. Alternatively, the improvement of the currently used tumor antigens to make sure that they include $CD4^+$ T cell peptides could also benefit the improvement of this area of DC vaccine development.

After antigen uptake and reception of maturation signals, they are endowed with receptors to recognize antigens: lectins, Fcγ receptors, TLRs. They also change their chemokine receptor CCR6 to MIP3a which makes it home to lymph node after antigen encounter. Between days 8 and 11, the expression levels of the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) are up-regulated with activation, particularly with CD40 ligation (19). CD86 tends to appear earlier in maturation, while CD80, which is almost unmeasurable in blood precursors, appears later. Human DC precursors circulating in the blood initially can express CD2, CD4, CD13, CD16, CD32, and CD33. In contrast, up-regulation of the expression of MHC class II and the absence of lineage markers (markers that are specific for other cell types) including CD14 (monocyte), CD3 (T cell), CD19, CD20, CD24 (B cell), CD56 (natural killer cell), and CD66b (granulocyte) becomes a hallmark of fully mature DC. Because of their antigen presenting functions, DC also express various adhesion and co-stimulatory molecules like CD11a (LFA-1), CD11c, CD50 (ICAM-2), CD54 (ICAM-1), CD58 (LFA-3), and CD102 (ICAM-3, 20). CD83, the function of which is unknown, remains the best available maturation marker for mature DC although it is also unique to the DC differentiation pathway among other myeloid cells. Although some activated lymphocytes can express low levels of CD83, macrophages never do (20). The stimulatory milieu produced by activated DC, combined with the presentation of epitopes in MHC class I and class II and the expression of co-stimulatory molecules, contributes to the generation of potent antigen-specific immune responses.

4. DENDRITIC CELL SUBPOPULATIONS

Although there is general agreement that DC are derived from hematopoietic stem cells, studies indicate that they can arise from at least two distinct lineages. The several and often opposing roles now ascribed to them cannot all be carried out at once by the same cell at the same time, so it is theorized that there should be different sets of DC that perform different functions. Such specialized subtypes might represent different activation states of a single lineage, the functional differences depending entirely on local environmental signals (the functional plasticity model). Alternatively, the specialized DC subtypes could be the products of entirely separate developmental lineages. The signals that determine lineage segregation would then act earlier and the immediate precursors of the DC would already be separate and functionally committed (the specialized lineage model). The reality is a confusing mixture of these two models, and a large degree of functional plasticity seems to be a general feature of both DC and progenitor DC (13). The lack of markers makes understanding the relationship between DC subsets more complex. What is known about the human blood DC is that there is heterogeneity in their marker expression, but many of these reflect differences in the maturation or activation states of DC rather than separate sub-lineages, such as those described above. In a few cases,

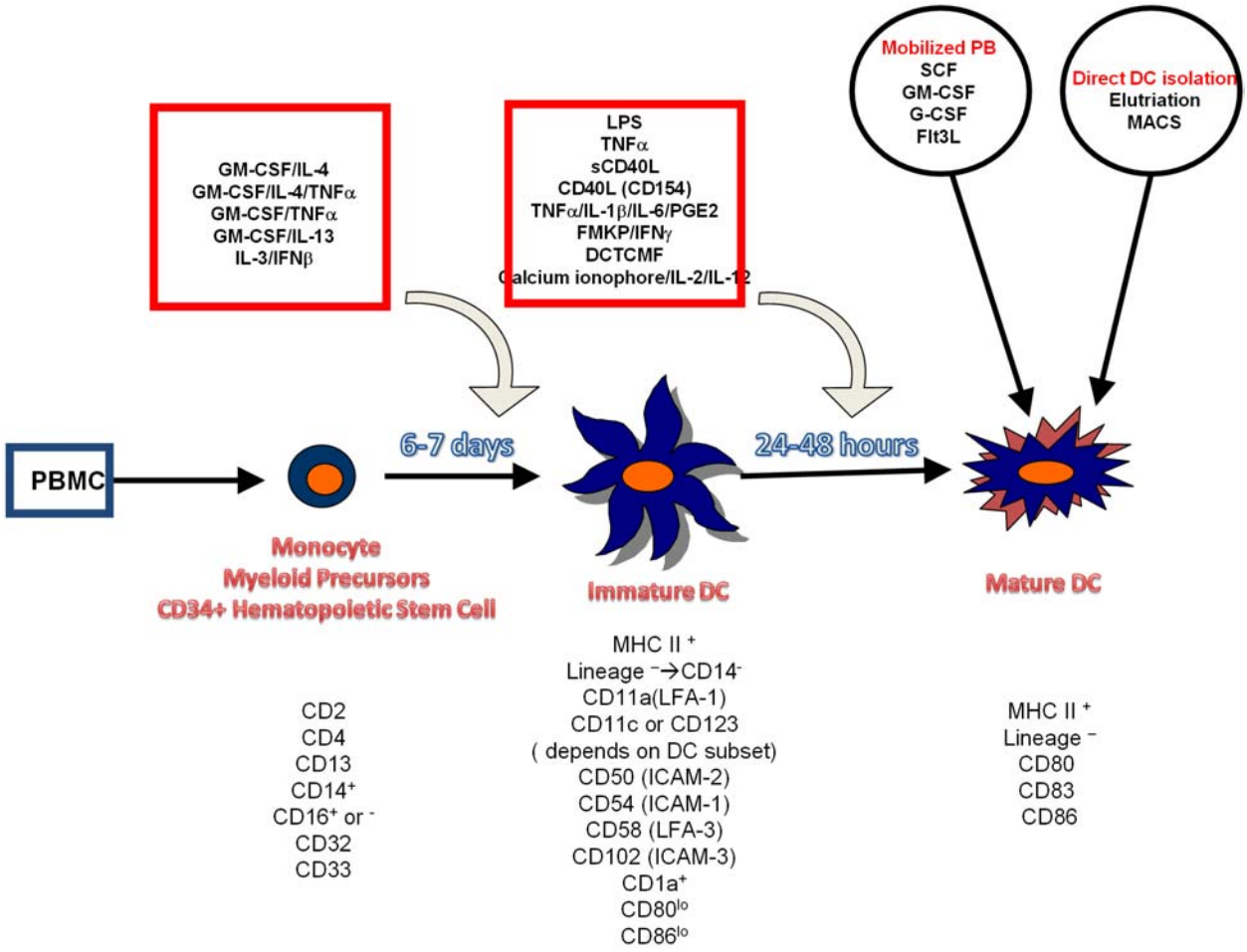

Figure 1. Diagramatic representation of DC generation methods used in therapeutic protocols. DC in therapeutic applications can come from different sources. It can be either isolated directly from PBMC or precursors from PBMC can be used to derive them. The first red box contains some of the growth factors used in the derivation of such cells wich usually takes from 6 to 8 days generating cells that have dendritic morphology and an immature phenotype as described in the text. Many methods can also be used to induce the maturation of these DC if the protocol calls for these cells. Conversely DC at both immature and mature stages can be generated by mobilization in vivo. Markers used to identified the different stages of these cells are denoted at the bottom of each representation.

human DC have been isolated from lymphoid tissues without any incubation steps to promote differentiation, and the mature DC analyzed. In general, human thymic DC are $CD11c^{+}CD11b^{-}CD45RO^{lo}$ and don't have myeloid markers while a small subgroup is $CD11c^{hi}$ $CD11b^{+}$ $CD45RO^{hi}$ and expresses many myeloid markers (21, 22).

DC in blood are generated from either myeloid or lymphoid bone marrow progenitors through intermediate DC precursors (iDC) that home to sites of potential antigen entry where they differentiate locally into mature DC. Two subsets of DC have been phenotypically described, a myeloid derived DC that captures antigen in the periphery and migrated to the draining lymph node and a lymphoid DC that resides in the lymph node. The $CD11c^{+}$ resident cells *in vivo* (intra-thymic, $CD11c^{+}CD11b^{-}CD45RO^{lo}$ and lack myeloid markers) in the human postnatal thymus are truly myeloid DC and have up-regulated GM-CSF receptor expression. These cells comprise the majority of the precursors while minorities of those DC are $CD11c^{hi}$ $CD11b^{+}$ $CD45RO^{hi}$ and express many myeloid markers (7, 13). Also $CD16^{+}$ cells were more effective than $CD16^{-}$ monocytes in reverse transmigration and differentiation into DC. $CD16^{+}$ also have higher expression of co-stimulatory molecules than other monocytes. Therefore it is suggested that $CD16^{+}$ human monocytes readily develop into DC via CCR-8 mediated signals (6). In humans, the more classical myeloid DC are derived either from a committed DC precursor or from a granulocyte/monocyte precursor. Conversely, data derived mostly from *in vivo* DC reconstitution assays in the mouse shows that the same DC subpopulations (including conventional DC and progenitor DC) can be generated from either myeloid or lymphoid progenitors. Recently, Liu *et al* have discovered in mice the point of diveregence between monocytes, cDCs and pDCs from their common shared progenitor macrophage-DC

precursor (MDP) in the bone marrow (23). More importantly, they show that T_{regs} control DC development in peripheral lymphoid organs and that DC division required an increased FLt3-L production. Further studies should reveal if a similar interplay is observed in humans as well.

Myeloid DC can also be derived *in vitro* from several cell types previously thought to be terminally committed. This will be further expanded in the following section. A lymphoid DC lineage has also been implicated from knock-out mouse models. In humans there are reports of a distinct human DC subpopulation which would be the lymphoid DC described in mice. This lymphoid precursor in humans expresses high levels of CD123 (IL-3 receptor) and CD4 and lack the CD11c myeloid DC marker. Identified in blood and tonsils, these $CD123^+$ DC precursors require IL-3 for survival and an activation signal, such as CD40L, for maturation (7, 24). They appear to bias $CD4^+$ T cell priming to a T_H2 response, in contrast to myeloid $CD11c^+$ DC, which preferentially induce a T_H1 response. This $CD123^{hi}$ DC also appears to be a major source of type I interferon and may, therefore, possess effector immune function as well. All of the experiments in this area do not support the existence of independent myeloid and lymphoid DC subpopulations as previously proposed but instead point to a DC differentiation model relying on contributions from both myeloid and lymphoid differentiation pathways. There is a possibility that all DC subsets might derive from a single DC common precursor (12, 25). A lympho-myeloid precursor population is thought to be seeding the postnatal thymus in humans (17). It is of note that most of the insights into human DC subsets and their development origins have come not from direct isolation of the mature DC from tissues but from indirect studies of their development in culture from iDC or pDC. These studies have led to the concept of distinct pathways of human DC development, although the correspondence between the DC generated in culture and naturally occurring DC subtypes in human or mouse is not clear (13).

5. THE TYPE1/TYPE2 DUALISM AND CANCER

A critical aspect of the immune defense is mediated by the function of newly activated $CD4^+$ T helper cells. Activation and proliferation of immune cells might lead to different patterns of cytokine secretion and the responses elicited can be polarized into several patterns, including type 1 and type 2 responses (26, 27). One theory to explain the selectivity of T cell responses postulates that cytokines secreted by neighboring cells drive resting naïve T cells down a particular differentiation pathway. However, Rissoan and colleagues challenged aspects of this model by suggesting that DC not only provide a common set of signals to initiate clonal expansion of T cells but also provide T cells with selective signals leading to either T_H1 or T_H2 immunity (26, 27). It is now clear that DC can influence, and likely dictate, this polarization in the proliferating T cells with whom they interact. DC are rich in class II HLA antigens and can be subdivided into two subgroups based on their polarizing function. Type 1 DC (DC1) expresses CD11c and polarizes naive T cells toward a T-helper 1 (T_H1) phenotype (6). Type 2 (DC2) does not express CD11c but does express CD123 and polarizes T cells toward a T_H2 phenotype (6). T_H1 and T_H2 cells differ in the cytokines they secrete and the type of response they elicit in target cells expressing cytokine-specific receptors.T_H1 induces inflammatory cytokine production such as IL-12, IFN-gamma and TNF-alpha. In contrast, T_H2 down regulates innate and acquired anti-tumor immunity and secretes cytokines such as IL-4, IL-5, IL-10 and IL-13. T_H2 cytokines can contribute to tumor rejection by boosting eosinophil function and increasing antibody concentrations (28).

The polarized subsets T_H1 and T_H2 both develop from the same T_H0 precursor. It's the dose of antigen, strength of signal through the T-cell receptor (TCR) and co-stimulation all influence the initiation of T_H differentiation. An important insight was obtained from the observations that the antigen-activated naïve T_H0 cell can be induced to differentiate into the $T_H1$or T_H2 lineage *in vitro* by the addition of exogenous cytokines. Furthermore, it is currently recognized that myeloid DC can generate either type of cytokine production in naïve T cells (29). The determining factor in skewing the T_H response by the DC is the secretion of IL-12 and the amount of IL-12 secreted by the DC varies with the conditions of DC generation and stimulation. The environment during antigen capture includes several factors as well as the antigen itself including microbial products, the CD40 ligand (CD154), stimulation from activated T cells and the appropriate cytokine milieu. Overall, the mutually antagonistic effects of IL-4 and IFN-gamma regulate T_H1/T_H2 balance and subsequent polarization (30). DC induce the development of naïve T_H0 cells populations into T_H1 cell populations, producing both IFN-gamma and IL-4, because interaction between DC and naïve T_H cells does not facilitate the induction of IL-12 production. These helper subsets can also cross-regulate each other. Therefore, the balance between T_H1 and T_H2 cytokines can determine whether the immune response is appropriate or will terminate in detrimental immunopathologies. In cancer it can mean the difference between clearing the tumor and allowing its expansion. Thus, dysregulation of these cytokines can lead to allergenic and inflammatory conditions. DC vaccines take advantage of this fact by trying to induce changes in the T_H1/T_H2 balance in a way that is favorable for tumor clearance. Despite this DC plasticity, each human DC subtype does seem to have a different functional bias which contributes to the overall immune response. Furthermore, it is important to notice that the evidence for this comes mainly from studies on the DC1 and DC2 populations generated from precursors in culture (13). It is clear that we must move from considering DC subtypes as being static elements in healthy individuals to considering the dynamic behavior of the entire DC system in response to infections or pathological states.

Another aspect of the DC-T cell interaction involves the induction of peripheral tolerance against antigens. Two general mechanisms have been proposed by which DC might maintain peripheral tolerance. The first is that a subtype of specialized regulatory DC is involved.

While there is some evidence for such a cell there is no consensus on whether these cells are the same as the previously stated or a different subtype on its own. The second is that all DC have a capacity for initiating tolerance or immunity depending on the maturation or activation state of the DC (13). The ability of DC to induce immunity or tolerance is dependent on the microenvironment during antigen capture and the antigen itself. Antigens that fail to induce an inflammatory stimulus are considered safe and induce tolerance, while antigens that are accompanied by an inflammatory signal elicit an immune response directed at antigen elimination (10). This is in opposition to type1/2 responses where high antigen doses induce T_H1 response while low doses induce a T_H2 profile in the case where both types of antigens induce an inflammatory reaction (7, 25). Therefore, knowledge of the type of response elicited by the antigen-DC-T cell combination should be considered carefully when designing DC vaccines to induce the appropriate type of reaction against the tumor.

6. GENERATION OF DC FOR THERAPY

There are relatively few studies on freshly isolated mature human DC. Blood is the only readily available source where they are scarce (around 1% of peripheral blood mononuclear cells (PBMC)) since it is mostly a source of progenitor and immature DC (11). Another challenge in obtaining these cells has been due to the lack of specific markers (20). Methods for obtaining high numbers of these cells have motivated the use of culture techniques to induce their differentiation as well as the use of mobilizing agents (19). Human DC can be enriched as circulating precursors from the blood (either monocytes or PBSC) by density-based purification techniques from leukapheresis products. Others perform counterblow centrifugal elutriation for monocyte enrichment before the addition of cytokines. The number of DC generated from one leukapheresis is sufficient for multiple vaccinations (on average 5 x 10^6 DC) and obviates the need for repetitive blood drawings. Mobilization of PBSC with agents such as G-CSF also helps by increasing the proportion of DC progenitors in blood (namely $CD34^+$ hematopoietic stem cells) All methods result in a relatively high proportion of contaminating lymphocytes and moderate DC purity. Apart from contaminating cell fractions, G-CSF mobilization attenuates T cell proliferation activity in response to PHA stimulation. At the same time this does not change the cytokine profile of G-CSF derived DC who maintain the high levels of IL-12 and low level of IL-10 characteristic of conventional DC (31). It also does not interfere with the ability of DC to induce antigen-specific T cell responses. Furthermore, some of these cells have been directly isolated from lymphoma or breast cancer patients where they maintained the characteristics just described. Conversely, the use of G-CSF should be taken with caution since it has been shown that it can down-regulate genes of the adaptive immune response which probably explains some of the immunosuppressive effects observed on T cells from G-CSF mobilized PBMCs (32). Alternatively, $CD34^+$ cells can be used directly to culture DC *in vitro* in large scale (33). These cells can be an attractive candidate when considering allogeneic DC preparations because they can not only be obtained from mobilized blood but also from bone marrow and cord blood as well (34-36). Furthermore, these cells have an amazing proliferation potential, increasing the amount of usable cells to produce DC from each procedure used to obtain them, as well as the ability to induce stronger CTL responses than monocyte-derived DC. Lastly, although not yet tried with human cells, some groups have started to develop and test DC derived directly from embryonic stem cells (37-39). While this would be a controversial progenitor to use, advances on the area of stem cell technologies may make this an attractive possibility in the future once all the ethical and technical questions get further resolved.

To increase DC purity, unselected PBMC can be cultivated together with GM-CSF and IL-13 first and then enriched by elutriation. While increasing purity these methods do not increase the quantities of DC to generate enough DC for repeated vaccinations. Therefore, the use of precursors in order to induce the development of DC *in vitro* has become one of the most important tools for DC vaccine design. Three different precursor-cell starting points have been used to generate human DC *in vitro*. One approach utilizes $CD34^+$ cells, the earliest hematopoietic precursors. Sources for these cells include bone marrow, cord blood, and G-CSF-mobilized peripheral blood. These cells can then be cultured in the presence of exogenous GM-CSF in combination with IL-4 and/or TNF-alpha and selected by the presence of CD1a or absence of CD14 and re-stimulated with GM-CSF (13, 24). As well as containing haematopoietic stem cells and other progenitor cells, this fraction contains some committed DC precursors capable of forming pure DC colonies in semisolid media. Also, stem cell factor (SCF) and/or Flt3-ligand (FL) are often added to increase DC yields by inducing the proliferation of DC progenitors as well as their mobilization into peripheral blood (13, 24).

An alternative approach is to expand DC *in vivo* with the use of agents that induce DC differentiation in the blood. GM-CSF is an agent that has been used, in combination with other cytokines, for differentiation of progenitor cells into DC and it is being studied as an adjuvant to cancer vaccines (40-42). In this setting, GM-CSF has been shown to increase protective immunity to melanomas when cancer cells are transduced with this gene (43-45). GM-CSF is also an approved growth factor that promotes granulocyte recovery (41, 46). In combination with either IL-4 or TNF-alpha, GM-CSF is known to elicit DC which have higher levels of co-stimulatory molecules, an indication of greater functional maturation (47-50). In these systems, GM-CSF stimulates an intense local immune response consisting of recruitment of DC, macrophages and granulocytes, as well as a marked expansion of DC locally and systemically that stimulates high levels of protective immunity. Our group has also recently demonstrated that this agent is capable of inducing type 1 pro-inflammatory responses *in vitro* and an expansion of the two DC subsets (myeloid and plasmacytoid, 51). FL is also used for mobilization of both $CD11c^+$ and $CD123^+$ DC into peripheral blood. The importance of such an expansion is

demonstrated by the fact that DC derived from peripheral blood have more favorable characteristics than monocyte-derived DC (MoDC) with respect to antigen processing and presentation, acquisition of a phenotype conducive to migration to lymph nodes, and subsequent presentation of antigen to T cells (52). For example, PBDC mature in response to minimal stimulation, rapidly acquire migratory function in the absence of PGE-2 containing stimuli and produce low levels of IL-12p70, IL-18 and IL-23 (52).

The last approach, and one of the most commonly used, is the use of blood monocyte precursors for the culture of DC *in vitro.* These cells are an immune reservoir of cells with dual potential that can be recruited to the tissues and differentiate into macrophages or DC depending on the tissue microenvironment. Monocytes are $CD14^+$ and $CD11c^+$ and can be either $CD16^+$ or $CD16^-$; their precursor is about 20% of the total blood cells. In the presence of M-CSF they will generate macrophages but, in the presence of GM-CSF and IL-4 enriched peripheral blood monocytes produce large numbers of immature DC *in vitro* after six to seven days with little or no proliferative expansion involved (53). These immature MoDC are morphologically and phenotypically similar to the ''classical'' density purified DC: they express MHC class II as well as low levels of co-stimulatory molecules CD80 and CD86, CD1a, lack CD14 (a monocyte/macrophage surface receptor) and are highly efficient in antigen capture but are poor stimulators of T cells. These cytokine-generated DC require additional maturation *in vitro* in order to fully stimulate in an allogeneic mixed lymphocyte reaction (MLR) or prime antigen-specific T cell responses *in vitro* and *in vivo*. After the addition of a maturation stimulus (a proinflammatory stimulus such as TNF-alpha, lipopolisacharide (LPS) or soluble trimeric CD40L (sCD40L)) MoDC up-regulate MHC II, CD80, CD86, and induce expression of CD83 (a DC specific cell surface marker). $CD4^+$ T_H cells, signaling by means of CD154 (CD40L), can also mature and activate these DC (13, 24). They also lose the expression of the monocyte/macrophage marker CD14, decrease mechanisms of antigen capture, are capable of potently inducing CTL responses and become highly immune-stimulatory just like an *in vivo* matured DC (54). Without this additional maturation step the DC phenotype can revert to that of a monocyte (11, 55). While they are all lead to the development of DC, it is important to consider the type of monocyte used as a precursor for vaccine design. For instance, $CD16^+$ cells are more effective than $CD16^-$ monocytes in reverse transmigration and differentiation into DC. $CD16^+$ also have higher expression of co-stimulatory molecules than other monocytes. It is suggested that $CD16^+$ human monocytes readily develop into DC via CCR-8 mediated signals (12).

In humans, the more classical myeloid DC are derived either from a committed DC precursor or from a granulocyte/monocyte precursor. Conversely, data derived mostly from *in vivo* DC reconstitution assays in the mouse shows that the same DC subpopulations (including conventional DC and progenitor DC) can be generated from either myeloid or lymphoid progenitors. Myeloid DC can also be derived from several cell types previously thought to be terminally committed. For example, as discussed, monocytes and granulocyte precursors can differentiate into DC when exposed *in vitro* to appropriate combinations of cytokines including GM-CSF or TNF-alpha with or without IL-4. Conversely, Shortman and colleagues also described a population of DC derived from lymphoid progenitors in mice. This other cell type appears to arise from $CD4^+CD8^+$ lymphoid precursors and can be induced to differentiate in culture without GM-CSF (13). Knockout mouse models have also implicated a lymphoid DC lineage. In humans there is a distinct DC subpopulation, which would be these lymphoid DC described in mice which express high levels of CD123 (IL-3 receptor) and CD4 and lack the CD11c myeloid DC marker. Identified in blood and tonsils, these $CD123^+$ DC precursors require IL-3 for survival and an activation signal, such as CD40L, for maturation (7, 24). These cells are the IFN alpha/beta producing plasmacytoid DC and they appear to bias $CD4^+$ T cell priming to a T_H2 response, in contrast to myeloid $CD11c^+$ DC, which preferentially induce a T_H1 response (13, 24). This $CD123^{hi}$ DC also appear to be a major source of type I interferon; and may therefore, possess effector function as well. So far, all of the experiments do not support the existence of independent myeloid and lymphoid DC subpopulations as previously proposed but instead point to a DC differentiation model relying on contributions from both myeloid and lymphoid differentiation pathways (12, 25).

There is yet no consensus on the optimal vaccine preparation or on its mode of injection. Therefore, when developing cancer vaccines using cultured DC careful attention needs to be paid to the steps involved in their production. For instance, culture and storage conditions can significantly alter the qualities of the vaccine product leading to neutral or undesirable results. Gradient solutions lacking potentially immunogenic proteins such as BSA and other fluids used in separation or culture are osmotically active to varying degrees and have additional stimulatory properties as well (13, 19). Further in the process, precursors can be either selected by negative/positive selection columns or by adherence in the case of monocytes. Enrichment by negative selection of PBMC using labeled CD2 and CD19 antibodies produces high numbers of DC and can even be performed in a closed system but so far the used antibodies do not meet clinical standards for *in vivo* use (56). Certain markers for positive selection, such as CD1a can vary their expression depending on culture conditions which may affect yields. Both methods may therefore affect maturation or change antigen presentation capabilities and cytokine release. Alternatively, by simple adherence of PBMC, the $CD14^+$ monocytes can be enriched in the adherent fraction to $\geq$ 60% with B and NK cells as contaminants (19). This seems to be a preferred method due to its low cost since it requires less reagents and fewer purification steps although it produces a less pure product. After culture, immature DC can be frozen in aliquots and matured after thawing for later use. Proliferation assays have demonstrated that the ability of fresh and cryopreserved DC to stimulate allogeneic T cells *in vitro* as well as the phenotype of DC was not be altered by cryopreservation (57, 58). In

particular, they can be effectively loaded with the tumor as a whole protein or peptide before storage apparently without loss of potency. The main concern with cryopreservation remains the low DC yields recovered after thawing.

Either before or after cryopreservation the association of purified antigens with mature rather than immature DC or the use of IL-12p70 rather than of IL-10 secreting DC should also be considered, as well as the choice of procedures to allow DC migration towards lymph nodes. In the latter, expression of the CCR7 receptor plays an important regulatory role. This receptor allows DC to recognize the lymph node-directing chemokines CCL19 and CCL21 and therefore must be present on DC used for vaccination when they are injected intradermally or subcutaneously. An alternative approach to overcome this constraint would be to inject DC intranodaly since it skips the need for DC migration. The study by Quillien *et al.* studied the path taken by 111In-oxine labeled DC on either of those three injection routes (59). They found no correlation with CCR7 expression on DC and migration was clearly detected as soon as the first hour onwards with kinetics close to that observed after intralymphatic injection suggesting that the movement from one node to another was passive and probably followed the efferent vessel route. DC injected by the intranodal route seemed able to perform their function of presentation to lymphocytes. Furthermore they observed the migration of radioactivity from the injected node to neighboring ones. From their studies it seemed that the maturation status of the DC administered as well as the type of maturation played a crucial role in the trafficking of these cells (which will be discussed in detail in the next section). In general, the intra-nodal route provided the best results for T cell sensitation. They stated that various studies in animals show that increasing the number of DC injected subcutaneously leads to a saturation phenomenon, whereby only 3% of the injected DC are found in the nodes. They suggested that it appears possible to increase this percentage appreciably by conditioning the site of injection with pre-injection of DC, which increases the local secretion of inflammatory cytokines, and that of CCL21 by endothelial lymphatic cells but they also note that this approach might only be possible with immature DC. While the technique they used to study the routes of migration of DC after injection provided insightful information it has the disadvantage of requiring the service of a radiologist for several hours which can lead to high cost of its implementation. Therefore alternative approaches to the study of DC migration after injection must be developed in order to address those questions further. Continued research in this area, as well as those mentioned earlier, should help elucidate the optimal conditions for DC preparations to use in cancer vaccination.

7. CONSIDERATION OF DC MATURATION STAGES IN VACCINE DESIGN

Maturation is a terminal differentiation process that transforms DC from poorly immune-stimulatory cells specialized for antigen capture into cells specialized for T cell stimulation. Only mature DC are capable of inducing antigen-specific CTL responses. It has been demonstrated that the efficacy of DC vaccines is critically dependent on the maturation state of the cells (60). It is not only that maturation may be a better candidate for inducing strong anti-tumor responses but it has also been observed that T cells co-cultured with immature, antigen-loaded DC only exhibited a slight immune response (61). This effect might be caused by the fact that immature DC can induce immune-tolerance rather than immunity. Therefore, a better understanding of the different stages of maturation of isolated or cultured DC has become another critical component of DC vaccine research. The differences in maturation and its effects can be observed from the choice of isolation protocol to the maturation cocktail used. *Ex vivo* generated MoDC were shown to be significantly more efficient than freshly isolated circulating DC to induce an anti-Id T cell response (57). The use of fetal calf serum in their culture also seems to be a prerequisite for obtaining high quality mature DC populations (61). Even under similar GMP conditions varying DC culture cytokine milieus in sequential DC cultures have been reported, even when derived from the same subject. Under Ribas *et al.* differentiation protocol CD14 levels persisted at varying levels in the loosely adherent population of cells harvested as DC, had low CD83, low levels of CCR6 and CCR7 trafficking receptors and variable levels of CD40 receptor (62). Culture supernatant from DC preparations generated in this protocol had uniformly low levels of type 1 cytokines IL-12p70 and IL-15 and on occasional preparations slightly higher levels of TNF-alpha and/or IL-1beta. Surprisingly production of type 2 cytokines that have been previously correlated with an immature phenotype of DC (IL-6 and IL-10) was higher than the type 1 cytokines on a pg/ml basis. This effect if observed when DC are removed from the GM-CSF/IL-4 medium and maintained in cytokine-free medium. All of these observations illustrate the variable nature of this form of therapy and the importance of the early stages of differentiation in the final maturation state of DC. Indeed it has been suggested that DC cultures that have not undergone a final dedicated maturation step or DC cultures preferentially producing T_H2 biasing cytokines such as IL-10 may induce antigen-specific tolerance rather than activation (62). On the contrary, a terminal maturation of DC is associated with an exhaustion state with decreased ability to produce IL-12 leading to a T_H2 polarizing signal. Although the production of IL-10 by immature DC has been associated with tolerogenic effects and T lymphocyte anergy, it has also been described that IL-10 does not affect mature DC antigen presentation or functional properties. Moreover, despite the high levels of IL-10 and the barely detectable levels of IL-12 MoDC were induced by bacterial infection or adenoviral vector to undergo phenotypic maturation and acquired antigen-presenting cell functions, activating T_H1 and epitope-specific CTL. These IL-10 expressing DC were even able to stimulate IFN-gamma expression by autologous T cells and by melanoma specific CTL lines demonstrating their antigen presentation capability (56). This finding is particularly important since only fully mature $CD83^+$ DC remains unaffected by inhibitory factors

such as IL-10 and VEGF and induces T_H1 polarization and CTL response even after cryopreservation.

There are many approaches to maturing DC after they have been isolated or differentiated from monocytes. In general, TNF-alpha seems to be an important component in maturation. Analysis of several published melanoma vaccine trials indicated that TNF-alpha-induced maturation of DC correlated with favorable clinical outcomes (60). But, as demonstrated in a set of experiments in a multiple myeloma trial, DC matured with TNF-alpha did not produce as high a percentage of fully mature $CD83^+$ cells as those matured with a cocktail containing TNF-alpha, IL-1beta, IL-6 and PGE2 as well as a diminished capacity to uptake FITC-Dextran (57). This cocktail may be required in order to fully express the adhesion molecules and chemokine receptors necessary for proper migration and stimulation of T cells (1). These DC showed a higher migratory response to CCL21 than DC matured with RBM and IFN-gamma despite comparable levels of CCR7 (59). Migratory capacities were also higher when DC matured for 48h than with DC matured overnight indicating that apart from the cocktail the time allowed for the stimuli influences their future abilities as well. Conversely the allo-stimulatory ability of DC matured with RBM and IFN-gamma was higher than that of DC matured for 24 or 48 hours with TNF-alpha, IL-1beta, IL-6 and PGE2. This cocktail had also being used to induce effective maturation in preclinical studies. Furthermore, the expression of IL-12 by DC after TNF-alpha and tumor lysate stimulation was below the limit of detection. These mature DC showed a high expression of TNF-alpha, IL-10 but not IL-12 (56). However, when mature DC were mixed with allogeneic IL-2-activated irradiated peripheral blood lymphocytes they began to express significant amounts of IL-12 which indicates a requirement for additional stimuli. Therefore, maturation with TNF-alpha improves when combined with a second maturation stimulus although this in itself does not indicate the use of a specific cocktail as the only option.

There are other maturation cocktails that have been seen with strong immunologic effects that can be potentially used in protocols for DC vaccination. IFN-alpha in combination with GM-CSF is another of those maturation approaches. This cocktail produces MoDC that express high HLA, co-stimulatory molecules and CCR7 (a chemokine receptor favoring migration in secondary lymphoid organs, 63). These DC spontaneously produce IL-15 (which promotes T_H1 responses and survival of T lymphocytes) and are comparable to cells generated by IL-3 and IFN-beta (another cocktail) since both types produce high IFN-alpha. Some other cocktails can involve the use of bacterial lysates since these are very immunogenic agents as long as they meet the GMP (good manufacturing practice) conditions. Such are DC generated through culture with GM-CSF and IL-13 can be effectively matured *in vitro* with a combination of IFN-gamma and bacterial membrane fraction from *Klebsiella pneumonia (*FMKP), both of which are available as clinical-grade reagents (64). It also appears that appropriate maturation stimuli are required for IL-12 production by DC based on Ebner *et al.* demonstrating that CD40 ligation and bacterial products (such as LPS a TLR4 ligand) are sufficient while monocyte-conditioned media are not (65, 66).

One important feature of matured DC is their ability to up-regulate molecules heavily involved in migration and trafficking of these cells. One main reason is that these cells need to be located in specific places to induce their effects, such as the tumor microenvironment. Conversely, the microenvironment where these cells are added forms the framework for their migration and it can change or modulate their maturation status as well as their immunologic effects. This is the reason some of the focus of the current DC vaccine research involves the understanding of the route of injection of the vaccine product into patients. Jacobsen *et al.* observed that the semi-mature thawed DC used in their vaccines would track to the regional lymph nodes after intra-dermal injection and during the migration would acquire a more mature phenotype to allow T cell priming to tumor antigens within lymph nodes (67). They also observed, while working with primitive murine hematopoietic progenitors, that the combination of FL with IL-3 or stem cell factor produced mainly mature myeloid cells, whereas the combination of FL with G-CSF, IL-11, or IL-12 produced mainly immature cell types. This indicates that mobilizing agents such as FL are not able to stimulate maturation by themselves even if they are capable of inducing the trafficking of DC. IL-3/IFN-beta DC migration was observed after intra-dermal but not subcutaneous injection indicating that subcutaneous administration of DC seems to be ineffective in causing DC migration to regional lymphatics (63). Furthermore intravenous administration of these cells results in their migration to the spleen whereas intradermal administration leads to regional transit in some patients. In this other maturation approach cells are able to produce high levels of IL-12 p70 upon re-challenge with CD40L. IL-3/IFN-beta DC was also observed to produce higher levels of IL-6 which is able to circumvent suppressive signals elicited by regulatory T cells which jeopardize anti-tumor responses (63). They also observed that GM-CSF/IL-4 DC had a significant percentage of both immature and mature DC remain at the injection site, higher than IL-3/IFNβ DC. In general, maximum migration observed was at 24h post injection for both groups. Another trial compared intralymphatic, intradermal, and intranodal routes for the injection of DC that were matured in the presence of a calcium ionophore, IL-2 and IL-12 (59). All three routes allowed the induction of an immune response with the emergence of specifically antigen-reacting lymphocytes. Even so, the authors commented on the possible importance of intranodal injection of semi-mature DC which induced observable immune and clinical responses in their trial. One future caveat of this approach would be its cost-effectiveness as well as the ease of the procedure for the patient, especially if multiple vaccine doses are required. An alternative approach was adopted, wherein DC were activated *in vivo* by injecting immature cells into a cutaneous site that had been treated with the topical TLR7 ligand imiquimod. This approach enhanced DC migration indicating that *in vivo* maturation may offer a superior method for generating immune-stimulatory DC (68).

Although some DC appear to be phenotypically mature, many authors have shown that tumor-related factors such as TGF-beta and IL-10 prevent the normal recruitment and activation of DC (60). If they are not fully matured they are not committed to either a T_H1 or a T_H2 profile (69). Therefore, the phenotypical maturation of DC does not seem to be the only measure to obtain highly immunogenic DC since this approach can also be misleading. This can result in impaired antigen presentation and T cell stimulation which can lead tolerance or anergy against some tumorigenic epitopes (8). This lack of an antitumor response can be explained by different poorly understood phenomena such as incomplete maturation or by the effects of differential maturation based on the stimulus received. Furthermore, a departure from the standard vaccination protocol uses subcellular vesicles, known as exosomes directly derived from DC but the maturation status of those cells can be the tipping point between tolerance and immunity (70, 71). This further highlights the need to better understand how to mature these cells to induce the right kind of immunity. However, there is yet no consensus on the optimal vaccine preparation, on the route of injection or on how these influence the maturation stage at which the function of the preparation is optimal for induction of anti-tumor immunity. All of these factors can affect and change the effect of the DC being administered. At best, certain options may be considered preferable, such as association of purified antigens with mature rather than immature DC, or the use of IL-12p70 rather than of IL-10 secreting DC. The area is still open to many changes as further knowledge of the different properties of these stages becomes available.

8. METHODS TO IMPROVE DC POTENCY

DC in cancer immunotherapy are used to initiate a response against the tumors that are non-immunogenic. Conversely, these cells can also alter or modulate the current response against tumors that have a low level of immunogenicity in favor of the correct adaptive immune response. This is particularly important since most tumors tend to induce a tolerogenic environment to prevent their destruction by the immune system. It has been theorized that a T_H1 profile is protective against tumor relapse and therefore beneficial; while a T_H2 profile may be deleterious because it may promote relapse as well as tumor growth and dissemination (72). Two markers seem to be important in determining the progress of tumors. We have recently published that increased circulating DC levels are correlated with improved survival and reduced cancer relapse after hematopoietic stem cell transplantation (HSCT, 73). Furthermore, we have demonstrated that the pro-inflammatory cytokine IL-12, a T_H1 cytokine, has been associated with improved relapse-free survival without increasing graft-versus-host disease (GVHD, 74). This graft-versus-leukemia (GVL) effect is usually mediated by alloreactive donor T cells, and/or NK cells. Furthermore, patients with both high numbers of DC and high IL-12 levels had reduced cancer relapse and improved survival (74, 75). This indicates that cellular therapies should aim not only to inject DC but to increase their numbers *in vivo* as well as induce the production of IL-12 and type 1 cytokines, like IFN-gamma, by resident cells.

Certain agents currently in use are capable of inducing modulatory effects as well as increase the levels of DC *in vivo*. One such agent is G-CSF. This growth factor is generally used as a peripheral blood mobilizing agent in HSCT and in enhancing neutrophil recovery in cancer patients (76, 77). Generation of DC from isolated $CD34^+$ progenitors also requires that patients be treated with G-CSF to increase mobilization of these cells into the periphery followed by a prolonged *in vitro* culture with a complex panel of cytokines. The use of autologous DC ensures that the antigen will be presented in a correct human leukocyte antigen (HLA) context and the use of G-CSF can produce an increase in $CD123^+$ plasmacytoid DC (78, 79). pDC normally are thought to induce a T_H2 response but some studies suggest that they can also induce T_H1 responses (80). However, G-CSF is also potentially an immunosuppresive agent since it induces the production of T_H2, like IL-4 (78, 79). Furthermore, G-CSF decreases inflammatory cytokines, such as IL-12 and TNF-alpha, which leads to a reduction in graft-versus-host disease (GVHD) in murine models and increased infection after haploidentical transplantation (72, 81, 82). So agents such as G-CSF might be used with caution to increase DC number for preparation of the vaccine product but care should be taken first to prime it to induce the correct immune response since DC progenitors obtained this way are capable of producing conventional like DC with higher IL-12 secretion (31). One trial attempts to circumvent this immunosuppressive type 2 activity by giving a receptor agonist, SD-9427, which is also an agonist for the FLT3 receptor (80). This approach would mobilize both subsets of DC (mDC and pDC) to fight the tumor hopefully without tilting the balance in favor of a T_H2 response. Unfortunately, while well tolerated there it induced an initial leukocytosis in all patients as well prolonged neutropenia in one patient (probably due to autoantibodies directed to the G-CSF domain).

GM-CSF is a clinically approved growth factor that promotes granulocyte recovery as well as DC growth, differentiation and survival from all precursors in both animals and humans (20, 41, 46, 83). It has also been used, in combination with other cytokines, to mobilize hematopoietic stem and progenitor cells for autologous transplantation and for differentiation of progenitor cells into DC *in vitro (*40-42, 84). It is currently being explored as an adjuvant to cancer vaccines because of its capacity to increase protective immunity against the tumor (43-45, 85, 86). It is an agent normally considered to promote an expansion in immune stimulatory DC , locally and systemically, after autologous hematopoietic stem cell transplantation although, in this setting, it has not been fully established whether it can induce a decreased relapse rate compared to G-CSF alone (41, 80). This enhanced activity results in more efficient T-cell stimulation and a more immunogenic response in the form of a type 1 response (by shifting the T_H1/T_H2 ratio, 84, 87-89). This is indeed one of the qualities that make GM-CSF such a good candidate for cancer vaccination, including non-DC

therapies (90). The type I IFN bias is further supported by studies where the outcome of GM-CSF administration is similar to that of patients receiving IFN-gamma as an adjuvant, by increased responses to tumor-derived antigens (80, 91, 92). Furthermore, T cells proliferate respond when stimulated with autologous tumor cells that secrete cytokines like IL-2, IFN-gamma, GMCSF and TNF-alpha (93). Although it is of note that the effects of GM-CSF may be different on PBMC derived from cancer patients as compared to healthy donors used (65). *In vitro* studies often combine GM-CSF with other cytokines while generating DC in culture. For instance, its combination with either IL-4 or TNF-alpha is known to elicit DC from monocytic progenitors which have high levels of co-stimulatory molecules indicating greater functional maturation (47-50). These DC stimulate an intense local immune response consisting of recruitment of immune cells such as macrophages and granulocytes, as well as a marked expansion of DC locally and systemically that stimulates high levels of protective immunity. We have also demonstrated the same effect *in vitro,* observed a general differentiation of monocytes into DC (where DC1 predominated over DC2), saw the enhancement in the proliferation of allogeneic T cells and an abrogation of any type 2 responses elicited by G-CSF (51). In addition, cells treated with GM-CSF had an enhanced production of IL-12 by T_H1 cells which may activate NK cells, further augmenting anti-tumor activity. Another group showed that pre-treatment with GM-CSF induced production of both type 1 and type 2 responses in rat respiratory tract DC that were ovalbumin-pulsed and used in adoptive transfer (24, 94). More recent studies targeted at GM-CSF focus on GVAX which is an autologous tumor vaccine expressing GM-CSF in malignancies such as non-small cell lung carcinoma (NSCLC) where durable responses have been observed (69). Other approaches include: the direct injection of GM-CSF into the tumor site, GM-CSF-peptide fusions transduced into DC, vaccines with multiple or single tumorigenic peptides plus GM-CSF as adjuvant as well as peptide-pulsed DC with GM-CSF as an adjuvant (either transfected into DC or administered concurrently, 83, 95-97).

A third growth factor of importance as an adjuvant in cancer immunotherapy is Flt3 ligand or FL. Flt3 is a tyrosine kinase growth-factor receptor that is expressed on progenitors of multiple hematopoietic lineages, including DC (68). One of FLs main functions is to support the expansion of DC in peripheral blood. It is also the only one to do that by itself *in vivo* by augmenting DC 40 fold when administered systemically (20, 98). It has been shown that FL expanded DC loaded with a single peptide could induce detectable immune and clinical responses (64). In animal models, FL caused tumor regressions that were associated with the development of antigen-specific, cell-mediated antitumor immunity (68). Altogether, the response to FL was a cyclical increase in the DC subsets (with overall higher DC1) that declined 7 days after the last administration of FL (68). But FL may have its downsides since it may not be effective at stimulating T cell proliferative responses while inducing IFN-gamma (98). This might have been linked to the development of autoimmunity in some of the patients and while in some animal models it has been shown to be effective at circumventing tolerance in other studies it might has been shown to actually enhance tumor tolerance (64, 98). A new interesting approach that could be combined with FL therapy combines DC vaccination with lymphodepletion or removal of Treg cells to enhance immune response (99).

Since one of the main goals of cancer immunotherapy is the induction of an inflammatory response against the tumor the direct use of some of the cytokines that are associated with such a state is another area of intense research in current trials. A variety of cytokines have been tested in small trials of melanoma peptides vaccination in an attempt to augment tumor antigen presentation and overcome immune suppressive influences in tumor-bearing patients (80). One such cytokine is IL-2, glycoprotein of 15kDa synthesized and secreted mainly by T helper cells and known clinically as PROLEUKIN (aldesleukin, 93). This cytokine can induce the activation and expansion of antigen specific $CD4^+$ and $CD8^+$ T lymphocytes cells (1, 99). A high dose IL-2 therapy has an overall tumor response rate of 10% to 20% with 5% being durable complete responses (100). This cytokine is capable of mobilizing T cells from the blood towards the tumor site (101). Unfortunately, IL-2 therapy can elicit a generalized immune response which is not targeted to the virus that ends in significant systemic toxicity. These high dose side effects include hypotension, lung edema, vascular leak syndrome, and renal and hepatic side effects (99). An alternative currently used is a lower dose of IL-2 in combination with other therapies since it is better tolerated (100). This low dose can increase the number of T cells and memory T cells while at the same time having a lower rate for the induction of vitiligo (56, 100). Low dose still has certain toxicities associated with it such as the development of thyroiditis and glucose leves should also be monitored with both doses. Care should also be taken since IL-2 can also be secreted by Tregs, so depletion with ONTAK (an anti-IL-2 toxin) seems to help improve immunotherapy (99). Cytokines that are involved in type 1 immuneresponses seem also appropriate as an adjuvant for cancer immunotherapy since they may be the key to breaking tumor tolerance. IL-12 has already been shown to be effective at this task and IFN-gamma did not cause any toxicity in patients although its efficacy as an adjuvant in humans was not clearly observed (64, 80, 102). Animal studies with IFN-gamma did demonstrate a considerable boost in antigen-specific CTL responses when combining it with LPS or CpG. Type I IFNs are not as well supported as type II since it produces a significant amount of toxicity (92, 99).

Other cellular components or its agonists can also be used to enhance the antitumor effects. Some of these include CTLA4, p53, VEGF/VEGFR, TLR agonists, and PADRE/KLH. Cytotoxic T lymphocyte- associated protein-4 (CTLA-4) is an activation-induced negative regulatory receptor which is involved in the prevention of overt reaction to self-antigens. In animal models blockage of this receptor by administration of CTLA4 blocking

antibodies induces rejection of established tumors and immunologic memory and it has also been used as a cancer adjuvant decreasing relapses (99). This type of therapy was shown to increase and maintain the frequency of peptide-specific, IFN-gamma-producing, $CD8^+$ T cells in peripheral blood. This type of therapy also enhanced the immune reactivity to recall antigens (62). P53 has been a widely recognized tumor antigen and has been the object of intense study by itself. More than 95% of p53 mutations result in single amino acid substitutions that lead to the synthesis of a stable, inactive p53 protein, which accumulate in tumor cells (101). It seems logical that a response to the tumor would be enhanced by adding therapies against this protein. In that light, it has been observed that induction of anti-p53 cellular immunity synergizes with subsequent chemotherapy to provide potent systemic antitumor activity (103). Perhaps this effect is due to the downregulation of suppressor cell activity as in the case of cyclophosphamide (104). Furtheremore, p53-peptide can also be modified in its MHC anchors to induce stronger responses by autologous DC which correlates with the observation that patients with sustained responses had higher expression of p53 than patients with partial responses in both Phase I and Phase II studies (101). Therefore while p53 can be a marker of a poor prognosis it can also mean a good target for immunotherapies. In the same sense another target that can be used to enhance immunotherapies are VEGF and its receptor. These antiangiogenic therapies have shown promising results in clinical trials and perhaps in combination with DC-based vaccination it can lead to improved results (99). Even more, a combination of both the study of the role of targets like VEGF and P53 and the cancer vaccine can improve the outcome of severe malignancies (105). In the case of TLRs agonists may act as DC activators enhancing the activity of DC in vaccine preparations expecially when overexpressing nonmutated proteins as antigens to break tolerance (105). Two of these agonists are the TLR7 agonist imiquimod and the TLR4 agonists LPS (68, 105). In the first case, imiquimod was able to increase the frequency of induced cutaneous reaction responses to test and tumor peptides and an increase in $CD8^+$ responses while LPS polarizes the DC towards a type 1 pro inflammatory response which ensured a robust IL-12 secretion after vaccination and preconditioned the cells for a subsequent IL-12 burst with CD40L (105). Lastly, another route of stimulation is by the MHC class II binding epitopes. One such molecule is PADRE which is a pan HLA-DR peptide that stimulates $CD4^+$ T cells and results in enhanced cytotoxicity against the tumor peptides in the vaccine preparation and also to be used as a control antigen (64, 101, 106). PADRE is most of the time found in combination with keyhole limpet haemocyanin (KLH) which is used because of its ability to shift immune responses towards pro-inflammatory responses and is as well used as a control antigen (64, 97). Both of these adjuvants can increase the frequencies of the specific proliferative responses and the magnitude of the responses to them is thought to correlate with an increasing time to progression (64). The mechanism of their action is probably due in part to the induction of maturation of DC which lead to all the effects just described (101). Lack of use of such adjuvants may hinder the maturation and hence the presentation of tumor antigens to T cells although this type of molecules can also generate non-targeted immune reactions as realized by DTH reactions triggered by them (1, 97).

9. CANCER-SPECIFIC TARGETED THERAPY WITH DC

While DC are an appropriate vaccine or vaccine adjuvant its specificity against the tumor relies on the recognition of its peptides as foreign. In this area, there have been varied attempts to induce specific anti-tumor responses to make the vaccine targeted and effective. As we discussed earlier, DC first need to be able to present tumor peptides in the context of the class MHC and second, it needs to be primed to induce a T_H1 pro-inflammatory response. This is the second step in the production of *ex vivo* DC for vaccination. It is known that the antigen presenting system of an APC works more effectively and efficiently when the protein/antigen is synthesized inside the cell rather than outside the cell, especially when cellular immune responses are considered. Therefore, a number of laboratories have attempted to introduce specific tumor antigens into DC using varied methods such as peptide pulsing, gene transfer, and tumor RNA transfection to name a few (107).

The most obvious and simple of these seems to be the direct injection of immature DC into the tumor site or its periphery putting these cells in close proximity with a wide range of very specific tumor antigens (108). Once the antigens have been captured, the antigen-bearing DC can mature and home to the lymph nodes to stimulate antigen-specific T cells. This technique has already demonstrated some efficiency in the clinic and it would be an affordable way to produce individualized vaccines since the procedure would be the same regardless of the patient. One caveat is that tumors are known to mediate the suppression of the host's cellular immunity and the amount of such suppression correlates with the amount of tumor and with metastatic disease (109). Furthermore, it has been shown that both the efficacy of DC vaccines and the host immunity are enhanced when tumor is resected before the vaccine is administered. This hindrance would mean that the vaccine would be less effective on those who patients who would benefit from it the most unless surgery is taken as part of the procedure. Therefore, it is not surprising to find that most of the current vaccine trials target a more involved approach where DC are loaded with tumor antigens before the administration of the vaccine product. One such source for autologous vaccine preparations can be the resected tumor when surgery has been necessary. Tumor can also be used to obtain allogenic sources of antigens such as those that share common proteins. When such tumors have identified, isolatable antigens, direct incubation with DC allow for passive exposure that leads to the acquisition by the cells for antigen presentation, a strategy that is widely used (3). To define those antigens, several characteristics must be met for them to be a good candidate to use in vaccination protocols: lack of pre-existing tolerance, differential expression on tumor versus normal tissue as well as in a significant proportion of

patients with that particular cancer, and a role in tumorigenesis or in survival of the tumor (99, 103). Tumor antigens are derived from actively transcribed genes in tumors, but not in normal cells, and are usually related to early steps in embryonic processes. Other groups of antigens are constituted by modified antigens also known as tissue-specific associated antigens (TSAA, 93). In general, the wider the tumor pool the better the chances to avoid tumor escape (110). A different, more recent approach has been the development of TAA that are specific to a wide array of tumor or all tumors for loading DC. One such TAA is the human telomerase reverse transcriptase (hTERT) which is expressed in ~85% of tumors but not on healthy tissues (111). This antigen for instance was has been proven to induce very strong $CD8^+$ T cell responses both *in vitro* as well as on clinical trials (112).

When using the whole tumor as a multivalent antigen source two main approaches have been used: the use of whole tumor or the fusion of DC with tumor cells tumor fusions. For the first approach, autologous or allogeneic tumor cells obtained from surgically resected solid tumors are co-cultured with DC and then analyzed prior to administration. These vaccines were well tolerated and capable of inducing immunologic responses in the majority of patients (108, 113). Resected tumors can be a great source of apoptotic bodies which can mature DC and allows them to effectively cross-prime CTL responses (99, 108). Because it is difficult to obtain sufficient quantities of tumor cells for loading DC *ex vivo* and because apoptotic tumor cells may not be well suited from antigen presentation, the use of allogeneic sources of tumor becomes of particular interest in these cases. A particular study showed the importance of tumor resection in conjunction with DC immunotherapy since the best responses were from patients with the higher tumor resection while those with unresectable tumor showed poor responses (~20% for 5-year survival). These immature DC preparations had a weak upregulation of a maturation marker (CD40) while other markers remained unchanged (CD80/86 or CD8) as compared to non cultured cells. This upregulation might be due to the acquisition of apoptotic bodies from the tumor and might suggest a role for CD40 as a determinant of vaccine potency *in vivo*. The second approach has seen the fusion of both autologous and allogeneic cells to produce a targeted vaccine product (3). In this approach, the entire repertoire of tumor antigens can be co-expressed in the context of the immune-stimulating machinery of the DC by exposing co-cultured cells to polyethylene glycol or by administration of electrical pulses (109). This format can also induce better anti-tumor immunity than DC prepared by tumor co-culture which time will tell if it can carry to the clinic since DC isolated or generated from cancer patients have limited efficacy. This immunity consists of the induction of $CD4^+$ and $CD8^+$, IFN-gamma producing, T cells which may be stimulated by the presentation of tumor-specific antigens by the HLA at a complex of the autologous tumor cell in the context of co-stimulatory, adhesion, and cytokine support provided by the allogeneic DC partner. Some of the drawbacks are the low class I MHC expression in tumor cells and the low efficiency of fusion with these methods (~20%) which would make this procedure not only costly monetarily but it would increment the burden on the patient. In general, this procedure was well tolerated, with no significant evidence of autoimmunity, no significant differences were appreciated in vaccine doses administered, patient age, sex, or prior therapy and clinical response correlated well with the immunological response to the tumor. Two twists in this approach are the tumor-DC hybrid cells (dendritomas) and the dendritic-like leukemia cells (DLLC, 114, 115). Dendritomas are cells purified from a tumor-DC fusion mixture that retain the characteristics of the tumor cell as well as the ability of the DC to act as an effective APC (114). These cells seem to be better at activating tumor-specific responses than when mixed with the rest of the fusion cells as demonstrated by an increase in IFN-gamma expressing $CD4^+$ and $CD8^+$ T cells. Whether the results observed are also enhanced by the injection of IL-2 immediately post-vaccination should be further evaluated. DLLC, on the other hand, are leukemia cells differentiated *in vitro* to obtain DC-like characteristics. These cells have enhanced autostimulatory capacity, including the *in vitro* development of anti-tumor CTLs, which is one of the main reasons why they might be useful as cellular cancer vaccines (115). In general these approaches are good for when tumor antigens are not well characterized as well as when there is enough source of autologous or allogenic tumor to produce such vaccine and can yield both MHC class I and II restricted immune responses.

A more costly but also more targeted alternative to the use of whole tumor cells as the antigen source is to load DC with tumor-specific peptides and to either add these immature DC to the tumor or, more likely, further mature them. This seems to be the most common choice for DC vaccination protocol and perhaps the one that has shown the best efficacy among vaccine preparations. Many of the original pilot studies in this area include mostly melanoma antigens, such as the MART peptide, because of the fact that this cancer posses the best immunologically defined antigens (62). The first one, by Nestle *et al*, used DC loaded with peptides or tumor lysates and Keyhole limpet hemocyanin (KLH) as an adjuvant (93). When the tumor antigens are not well defined, apart from tumor-DC fusions as described above, a lysate of the tumor can be use to load a myriad of antigens to DC which has the advantage of providing a broad spectrum of tumour-derived epitopes to generate a broader T-cell immune response although clinically, this cells may fail to fully activate T cells and be more on the tolerogenic side (99, 116). Conversely, some of the defined peptides can also be tolerogenic, as seen with MUC1. This protein can induce apoptosis of activated T cells *in vitro* and has also been shown to inhibit T cell proliferation (116). Interestingly this inhibition can be reverted by IL-2, anti-CD28 antibodies or by a 16 amino acid MUC1 peptide just as was also seen before in MUC 1 tolerant transgenic mice. This shows the importance of a combination of therapies to further boost the effects of DC vaccinations in order to break the tumor tolerance in patients and induce a strong response against it. These responses are not constrained to CTL response since these type of DC vaccines can induce high titers of anti-tumor

IgG (117). While some of the antigens are unique to the tumors, some are not such as the case of p53, a tumor suppressor gene that plays an important role regulating cell growth and differentiation in normal cells. In this case specific mutational differences are the target of the vaccine since when is present in tumor it has a prolongued half-life and it is present at much higher quantities inside the cell (103). Furthermore, this target is well characterized and provides a unique opportunity for synthesizing the peptides instead of using specific tumor antigens. The spectrum of antigens may be further extended by the use of peptide cocktails or allogeneic cancer cell lines although how these peptide pools correlate with the individual patient's tumor remain to be further investigated (110). Regardless of the peptide or its source peptide-pulsed DC products seem to be safe and feasible in addition to demonstrating immunological and clinical responses for several tumor types and have become increasingly popular as the choice for preparation of these cellular vaccines (107).

Tumor antigens can also be derived directly from the genes of tumors and in order to do that DC can be exposed *ex vivo* to tumor mRNA or by RNA or cDNA encoding for TAA (3, 116). One group for instance developed a melanoma vaccine based on autologous DC transfected with autologous tumor mRNA since transfection with all of the tumor's mRNA pool allows for a wide spectrum of tumor antigens that can be expressed by the vaccine DC (110). This approach also would bypass the requirement for defined HLA alleles matching peptides and lead as normal protein processing inside the cell allowing for a more targeted antigen. In this trial they were able to observe an increase in IFN-gamma secretion but only after 4 vaccine boosts while IL-10 was never changed but in general T cells produced equally T_H1 and T_H2 cytokines. They demonstrate that this type of vaccine design can recruit multiple T cell clones but their cytokine profiles do not follow the classical T_H1/T_H2 delineation and seemed to have observed a wide range of cytokine profiles. Based on their observations a vaccination schedule with different boosts of vaccine may probably lead to a stronger immune response.

An alternative delivery method of tumor antigen genes is by the use of viral vectors such as adeno, vaccinia, retro and fowlpox viral vectors with the goal of targeting tumor cells with high efficiency (118). These viruses provide an effective means of activating DC since not only do they deliver the genetic material but they can also by themselves induce the maturation of these cells (103). Several initial experiments with retroviruses, for instance, demonstrate of targeting the production of inflammatory cytokines like IL-2 and IFN-gamma into explanted tumor cells (119). Even the mere addition of a virus (regardless of its payload) can lead to benefitial (and controvertial) anti-tumor effects. For instance, adenoviral transduction results in up-regulation of cell membrane MHC class II and co-stimulatory molecules on DC, and an increased production of pro-inflammatory cytokines such as IL-12 due to their high immunogenicity (120, 121). A safer alternative is the use of vaccinia virus or fowlpoxvirus but these vectors have a reduced collateral damage probably due to a dimished anti-tumor response (122-125). This method of transduction normally results in a high level of transgenic protein production samples. However it is also fraught with many disadvantages including: 1) the limited ability to identify all of the important specific tumor antigens, 2) the limited ability to map the genes of the specific tumor antigens, 3) only one or a small number of the known tumor antigens can be introduced into the DC and 4) the possibility that some of these peptides can modulate tolerance towards the tumor (84).

10. POST-THERAPY MONITORING OF DC EFFICACY

The most common problem still faced in the development of an effective cellular vaccine remains the lack of correlation between the detection of a strong antitumor immune response and the observed clinical benefit (126). Two criteria form part of this correlation: one is the clinical measurements used to identify the quantity or quality of the response of the vaccine against the tumor while the other is the immunological response as measured by *in vitro* assays of patient blood or other tissue. For both of these types of measurements there has not been a particular agreement as to which of these provides the best information on the effectiveness of the vaccine. For the clinical data gathered in the majority of the trials guidelines, like RECIST (response evaluation criteria in solid tumors), were used to avoid ambiguity as to the status of the tumor. However, in diseases such as melanoma whose natural course is characterized by a highly variable rate of response among different patients, 'stable' disease or 'mixed' response were not categorized under objective clinical response (127). These criteria, as well as similar others normally used in the clinic, can be ambiguous and empirical which can lead to wrong correlations, especially in large trials.

Providing tools to measure clinical tumor remission in a more accurate way should and is a main goal in tumor vaccine strategies. One such study reports on the PET and CT responses of six patients treated with a DC vaccine (128, 129). One of three patients with measurable, macroscopic disease responded to vaccine therapy. This response was accurately measured with an increase in 18F fluorodeoxyglucose (FDG) uptake by tumor cells (128). FDG-PET is based on the increased glucose metabolic activity of most malignant cells and it is demonstrated to be a useful tool for evaluating treatment efficacy. This technique seems highly accurate in the early prediction of the response to chemotherapy as it is proposed to detect tumor in persistent masses. For instance, in the same study, progression to CR in a patient with smallest macroscopic tumor burden was successfully monitored this way even though there was no indication of such a response by measuring cellular or humoral anti-lymphoma responses. This same technique can probably also be used as a predictor of future relapses. Conversely, instead of staining the tumor cells, a tracker dye or radiolabelling the vaccine or immune cells can also indicate if there is an active response against a tumor even if that tumor is not detectable any other way allowing the observation of the

vaccine product early in the protocol which gives a snapshot of the early events of the vaccine against the tumor and their migration patterns (128, 129). This way of tracking cells has been used to assess the most efficient route of administration by injecting 111 Indium or Techtenium-labeled macrophages or DC showing that the route of administration should be correlated with the migration patterns as well as with the maturation status of the immune cells to avoid tolerogenic effects, by observing the migration patterns of these cells after injection (130, 131).

Further in the vaccination protocol clinical responses are commonly and accurately measured by delayed-type hypersensitivity (DTH) test (132). This measurement of cell-mediated immunological memory while extremely useful suffers from the same consequences as the RECIST criteria for it measures the immune response against the tumor after vaccination in a empirical way (56, 133). Furthermore, these responses depend on the ability of the immune system to respond against the tumor and would not take into account the stage of the tumor or the tumor burden which might account for different results depending on the advancement of the tumor. For instance, stage II melanoma patients significantly surpass DTH responses of stage IV patients in strength and their reactivity is long lived. This may have been due to the fact that stage IV patients had higher frequencies of tumor antigen-specific T cells before immunization that were only moderately expanded by vaccination so in the end both stage II and stage IV patients had the same amount of antitumor CTLs. Furthermore, this type of test can produce false negatives as it has been shown to induce responses to unpulsed DC as well (134). On the plus side, this method conveys the information that the vaccine has generated recognition by the immune system of the peptides that were used in the vaccine formulation and is the reason behind some of the control peptides and neoantigens added to the preparations, such as KLH, the HIV-gag peptide tat or HBV antigens, since they would also generate a response that can provide a measurement for the efficacy of the vaccine (126, 135). For the most part control antigens seem to correlate with the response to anti tumor epitopes used in vaccine formulations even though they seem higher at the beginning of the therapy and that is not known if loading these antigens induces a competion for antigen by T cells which diminishes the overall response to the tumor (134, 136). Most of the trials reviewed that used DTH testing showed a strong correlation with prevention of disease progression although this test did not seem to differentiate the strength of the response between individuals or the optimal number of DC in the preparation.

Other immune tests seem to provide further insight into the antitumor response provided by these vaccines and that is why they are all also used routinely. T cell responses to tumor antigen, for instance, have been correlated with the release of type 1 cytokines such as IFN-gamma which has been associated with tumor regression (8). Interestingly, these immune tests do not seem to correlate with the clinical data obtained (136). This may indicate that that these methodologies may be capable of measuring independent immunological events and therefore a further understanding of the meaning of the results of these tests would be pertinent to the study of cellular vaccines (56, 132-134). If anything they demonstrate that the DC vaccines currently under study are biologically active and show the potential to succeed someday. It is now recognized that these vaccinations can result in objective clinical responses and regression of metastases in a selected populations of patients, although only a few studies have really established a correlation between immune and clinical responses (61, 80, 136). It is possible that the reason is that no single parameter of the immune monitoring is going to be a predictive marker for the clinical course of individual patients by itself. Therefore, the parallel use of different assays would be necessary for an appropriate immune evaluation of vaccinated patients.

11. FUTURE OF DC IMMUNOTHERAPY: DEFINING SUCCESS VERSUS FAILURE

Many hurdles need to be overcome in order to obtain the goal of a complete cellular vaccine against cancer. The lack of complete response in many of the trials studied in this review serve as a witness to the fact that consensus needs to be reached in many areas before results can be seen (69, 127, 132). For instance, the patients studied in many of these trials have large tumor burdens, rapidly growing tumors or are at the late stages of disease and have already tried chemotherapy and resection or do not reach the evaluation time due to disease deterioration (105, 132, 134). Previous therapies, as well as an advanced tumor, may account for suppressive immune responses post vaccination. Conversely, the advanced stage of a rapidly spreading disease does not allow enough time for a fully functional immune system to produce an anti-tumor response (133). It stands to reason that the therapy would be of most benefit to those at the end stages where other, cheaper and more feasible, protocols have been exhausted. Comparative studies at different stages of disease as well as to how to circumvent the shortcomings of the advanced tumors should shed some light on this area. Tumor induced immune suppression can also lead to immune evasion at any stage. Some of the tumor strategies include secretion of cytokines such as IL-10 and TGF-beta which can inhibit antigen presentation, induction of $CD4^+CD25^+$ regulatory T (Treg) cells, mutation or downregulation of immunodomminant antigens or immune molecules (HLA, costimulatory, etc), expression of FasL to induce apoptosis of active T and NK cells (93, 99, 131, 137). The importance of these mechanisms in the reduction of the potential of any immunotherapy should be fully established so that tumor suppression and perhaps even eradication can be achieved (93, 131, 137). Therefore, a more careful comparison of the patients, their tumor and their immune systems should be one of the basic goals for future studies.

Agreement on the many areas of the vaccine preparation as well as the administration protocols need to be reached since there is a lot of methodological uncertainty not limited to the number of patients and the variability between them (138). At the top of the list is the DC themselves. Should they be autologous or allogeneic?

In theory autologous DC are better than allogeneic DC as tumors can be heterogeneous in terms of phenotype and growth kinetics. However, the generations of autologous cells has been perceived to be expensive, complicated and time consuming compared to allogeneic cells since it would require for the patient to undergo additional procedures while donor cells that are readily available would be used otherwise which would make them impractical for routine use (98, 139, 140). On the reverse side, allogeneic DC may actually increase anti-tumor immunogenicity and further add to the effectiveness of the treatment (1). Should DC be isolated or cultured? If cultured, what would be the individual components in the media used to prepare them? Can they conserve their effectivity after cryopreservation? Every single step can induce drastic changes in the quality and safety of these preparations and therefore careful attention to protocols should lead to an optimized, more immunogenic, product (3, 61, 63, 69, 98, 105, 127). After these cells are prepared the maturation stimulus (if any) can lead to very different types of responses and can have a significant impact on the induced response although there seems to be a shift towards the use of matured DC since most of them can induce potent T_H1 responses (99, 131, 132). Furthermore, immature DC might act in an immunosuppressive way protecting the tumor instead which might excerbate the damage (103). This seems based on observation of the number of immature cells in the periphery rather than the ones being given with the vaccines which indicates that research in the role of immature DC *in vivo* would be important to understanding their real role in immunosuppression of anti-tumor responses (20, 52, 101, 104, 130, 133). Perhaps just understanding the effective concentration of DC to be delivered in the vaccine should help break through tolerance and help induce a strong anti-tumor immunity. Thus, the development of ways to obtain larger numbers of monocytes that can differentiate into clinically competent DC seems worth pursuing (54). DC by themselves cannot bring about the immunity against the tumor and need to be prepared with a target antigen but the type of antigen to use or the delivery of it is also riddled with variability. Which type of antigen induces the most effective immune response to cancer *in vivo* has not been well studied and leads to empiricism when preparing DC vaccine trials. Whole tumor antigens from lysates, apoptotic or necrotic cells, tumor/DC chimeras, or RNA/DNA transfections are the most economical and less cumbersome option but their effectivity is still questionable although it is still the most used (1, 56, 99, 107, 134). Their goal of inducing broad responses to minimize tumor escape seems to backfire and induce immunesuppression in some instances although this in itself is uncertain since such a broad antigen base is difficult to study immunologically. Individual antigens are more costly and can lead to an ineffective tumor vaccine because of escape mechanisms but this can be changed not only by choosing appropriate antigens but by antigen modification to make them more immunogenic (99, 105, 106, 116, 131). While specific antigens might be present in all tumors their distribution varies considerably and lose of peptide reminds that they need to be used in a timely manner. In general these types of vaccine preparations can induce broader responses than anticipated because of epitope spreading mechanisms (56, 106, 130). Perhaps a compromise such as the use of several well-defined antigens, as well as enhancing antigens like PADRE, would lead to the most immunogenic DC and a higher quality vaccine (90, 106, 141).

Most of the attention in this field has been focused on the *ex vivo* preparation of DC but the specific details of the protocols used also need to be fine-tuned. While freshly cultured DC may provide the best responses to the tumor a need for frequent administration would create practical difficulties for manufacture and coordination, so optimization of cryopreservation protocols would enhance the feasibility of this type of therapy (127). There is also considerable variation in the routes of administration used and in few studies available demonstrate that different routes and sites of injection can lead to differences in the effectiveness of the vaccine (102, 131, 136). For instance, migration of DC was observed with intranodal but not with subcutaneous administration while intradermal injection is more localized as compared to intravenous administration which leads to a more systemic spread of the cell (8, 63). Overall, for effectiveness, intranodal injection seems to be the best delivery method as long as care be taken not to disrupt the microarchitecture of the lymph node and the inconvenience of the patient in trying to reach the closest node to the tumor are taken into account (96, 99, 130-132). Apart from these, the immune responses to intranodal injection should also be addressed since it has been shown that the method of delivery can have an impact in the pattern of cytokine secretion (96). Co-administration of factors to stimulate anti-tumor reactivity, such as IL-2, IFNs, GM-CSF or cyclophosphamide, would not only circumvent such a problem but has also been used effectively to enhance the normal protocol with apparently low toxicities to the patients of those trials (99, 104, 110, 132). Lastly, the dosing and schedule of vaccination can not only shift the response to the tumor but be the most restrictive constraint of the procedure in economical as well as methodological terms (62, 127, 137). There is a tendency for "more is better" because the degree of administered DC that accumulates in lymphoid tissues is a factor that can enhance or restrict the therapeutic effects of this type of immunotherapy. Mature DC are not very mobile so apart from the induction of a specific anti-tumor response the upregulation of specific chemoquines and their receptors might aid in moving these cells from the periphery and into the lymphoid tissues where they can prime the anti-tumor response (142-144). Therefore careful consideration should be taken when designing subsequent trials and studies on the particulars of the vaccination protocol would be a much welcomed addition to the current literature.

12. CONCLUSIONS

DC vaccination, as it stands today, has the capacity to be a successful therapy against cancer. Success or failure will depend on characterization and optimization of DC *in vitro* and *in vivo*. There is still a long way to understand the normal physiology of DC *in vivo* and how these cells compare to their culture counterparts. The host-

tumor relationship should therefore be addressed to overcome tolerance which seems to be the main reason behind the lack of strong responses in the current trials. "Proof-of-concept" studies such as those on melanoma and prostate cancer will provide clues that can be replicated in other systems to avoid limitations in further trials (83, 135, 145). Safety does not seem to be an issue yet as no strong toxicities (as measured by the Radiation Therapy Oncology Group (RTOG) scale or the National Cancer Institute Common Toxicity Criteria (NCI-CTC)) or development of autoimmunity (as evidenced by symptoms of diabetes, vitiligo, eczema or other type of self reactions) has been observed (131). However, since changes in the immunogenicity of the vaccine preparations are in order due to the lack of strong anti-tumor responses and only few complete remissions, those results should be taken with caution as the enhancement of immunogenicity may increase the danger of developing such secondary reactions.

In order to fully establish the feasibility of these vaccines in late stage patients, perhaps trials on a more immunogenic vaccine preparation should be carried out. Afterall, the fight against cancer at that stage seems to be a balance between a cure and the secondary effects of the therapy on the patient. This also brings the necessity for further bench research to better understand why the current trials where not able to clear the tumor even with clear increases in immunogenicity. The development of better immunological markers to routinely test in such trials should help in the determination of immunogenicity and perhaps in preventing or ameliorating such side effects (98, 99, 101, 110, 115, 127, 130). At this crossroads, and armed with varied clinical trial results, a consensus guideline should be stablished that let's researchers know what has been already tried with success in these patients. This would mean a new base for starting trials to build upon and gather fresh information. Better questions would be asked this way because there would not be a need to ask the same questions repeatedly. Due to the low toxicity profile of this therapy it would also be appropriate to conduct well designed clinical trials in the adjuvant setting to recruit locally effective killer cells in low tumor burden patients.

Despite methodological uncertainty of DC production and lack of complete tumor remissions in many of the trials it should not be forgotten that a significant amount of improvement in the survival of terminal patients has been observed and may give an edge against other therapies being developed. Case in point, at the time of writing of this manuscript, results from a Phase III trial for Provenge (sipuleucel-T) with 512 Prostate Cancer patients with an increased median survival of 4.1 month and increased 3 year survival by 38% was presented, as a live webcast, on April 28, 2009 at the meeting of the American Urological Association in Chicago (146). If confirmed, it would indicate the importance of harnessing our own body to fight cancer using DC. The dream for a cancer cure has always been to have a magic pill that will kill only cancer with minimum side effects. To get help from some of the systems already available in our body might not be the single cure expected for a disease, but then again, cancer is not just one disease.

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