Published in final edited form as:

Semin Immunol. 2010 June ; 22(3): 173–182. doi:10.1016/j.smim.2010.03.002.

Polarized Dendritic Cells as Cancer Vaccines: Directing Effector-type T Cells to Tumors

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Abstract

Ex-vivo-generation and antigen loading of dendritic cells (DCs) from cancer patients helps to bypass the dysfunction of endogenous DCs. It also allows to control the process of DC maturation and to imprint in maturing DCs several functions essential for induction of effective forms of cancer immunity. Recent reports from several groups including ours demonstrate that distinct conditions of DC generation and maturation can prime DCs for preferential interaction with different (effector versus regulatory) subsets of immune cells. Moreover, differentially-generated DCs have been shown to imprint different effector mechanisms in CD4⁺ and CD8⁺ T cells (delivery of “signal three”) and to induce their different homing properties (delivery of “signal four”). These developments allow for selective induction of tumor-specific T cells with desirable effector functions and tumor-relevant homing properties and to direct the desirable types of immune cells to tumors.

Keywords

dendritic cells; IL-12; immunotherapy; cancer; vaccines; cytokines; chemokines; chemokine receptors; Th1; CTL; NK; Treg

Introduction

Established therapies of cancer, such as surgery, chemo- and radio-therapy, are usually effective in reducing major tumor mass in patients with established tumors, but often fail to eliminate residual cancer cells and prevent disease recurrence. This particular deficit led to attempts at utilizing patients' own immune system, specialized in generating sterilizing immunity to invading microbes, in order to identify and destroy persisting cancer cells.

Therapeutic “cancer vaccines” share many features of traditional protective vaccines against microbial pathogens. Similar to protective vaccines they need to induce immune responses of defined specificity and high magnitude. Although tumor cells are less different from the hosts healthy cells than virally-infected cells (what renders them less immunogenic), extensive research of the last twenty years allowed to identify unique tumor antigens and the ways of effectively presenting them to the immune system in the context of strong adjuvant signals (delivery of “signal 1” and “signal 2”) (1–5).

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However, despite the increasingly-high immunologic effectiveness of new cancer vaccines and indications of their ability to delay cancer progression (2,6–9), the overall effectiveness of the currently-available therapeutic vaccines against cancer still trails the effectiveness of preventive vaccination against infective agents (1,2,10–16). In particular, while current cancer vaccines show early promise in inducing disease stabilization and prolonging patients' survival (14,17–19), they remain poorly effective in inducing regression of bulky tumors (13). In this review, we will focus on two aspects of T cell function essential for their ability to induce cancer regression: their cytolytic effector functions and their ability to migrate to tumors, and the corresponding requirements for DC-based vaccines to preferentially support the activity of the effector-type (T_{eff}), rather than regulatory type (T_{reg}) T cell responses, and to deliver to T cells two types of signals regulating the acquisition of effector functions (signal 3) and tumor-relevant homing properties (signal 4).

Therapeutic vaccines against established cancer: Special requirements

Several aspects of vaccination relevant to therapeutic vaccines and several types of resulting challenges are less relevant to preventive vaccines, making it less clear whether the paradigms and practices successfully established during the development of protective vaccines are indeed relevant to therapeutic vaccination, in patients with advanced cancer.

The goal of protective vaccines is to induce the expansion of pathogen-specific T cells and establish immune memory (Figure 1). Subsequent exposure of the host to pathogenic challenge induces both the secondary expansion of the vaccine-induced memory T cells, but also their acquisition of effector functions associated with a switch of chemokine receptors and acquisition of peripheral homing function (20–26). Moreover, since the pathogen itself is inducing local tissue damage and/or activation of peripheral DCs and other types of antigen-presenting cells (APCs) that act as sources of the effector cell-attracting chemokines, the arising effector cells are immediately directed to the site(s) of pathogen entry (4,24,27). In contrast to such recall responses to microorganisms, the vaccination-induced T cells of cancer patients are not exposed to pro-inflammatory alarm signals from infected tissues and innate immune cells, and may need to rely on the vaccines themselves to acquire effector functions and peripheral homing properties. Moreover, therapeutic vaccines need either need to be particularly effective in inducing T cells highly sensitive for the chemokines expressed in the tumors (that often themselves rely on chemokines for growth, metastatic spread, and survival (28–33)), or to be combined with additional signals able of modulating the expression of homing molecules on T cells or the expression of tumor-produced chemoattractants.

The second group of factors limiting the effectiveness of immunotherapies of advanced cancer is tumor-associated immune dysfunction, associated with the expansion and hyperactivation of regulatory T_{reg} cell system (34–37), and the dysfunction of endogenous APCs (5,38–40). DCs present in patients with advanced cancer are a target of tumor-associated suppressive factors, resulting in their suppressed ability to support effector functions in tumor-specific lymphocytes (41,42). Known mediators of DC dysfunction include IL-10, TGF- β , VEGF, IL-6, and COX2 products, particularly PGE₂ (43–47). DCs that develop in their presence show impaired maturation and reduced expression of costimulatory molecules needed to support T cell activation, and suppressed production of cytokines needed to support survival and anti-tumor effector functions of tumor-specific T cells (5,48–51). Such functional defects have been observed in endogenous DCs from melanoma-, ovarian-, breast-, renal, prostate-, lung-cancer patients, as well as in the patients with head and neck cancer (49,52–56).

In addition to the endogenous DCs dysfunction, patients with advanced cancer have been shown to have elevated levels of circulating and tumor-associated T_{reg} cells and hyperactivation of their suppressive functions (34–37). While pre-existing T_{regs} can limit the

effectiveness of cancer vaccines (36,57), their numbers can be even further expanded by some of the currently-applied cancer vaccines (37).

The above data suggest that in contrast to preventive vaccines, therapeutic vaccines against cancer not only need to be highly-effective in inducing the expansion of cancer-specific T cells (despite the dysfunction of endogenous DCs, but they also need to avoid interaction with T_{regs}. They also suggest the need to compensate for the absence of pro-inflammatory cytokines and chemokines in inducing tumoricidal effector functions and tumor homing properties of T cells.

Ex vivo-generated DC as “cancer vaccines”: Benefits and caveats of DC maturation

DCs, the APCs specialized in inducing primary immune responses (58–61), are also essential in supporting the survival and functions of previously-primed T cells and in mediating overall communication within the immune system (62,63). Since in contrast to the DCs that develop in the context of tumor-related suppressive factors, fully mature DCs acquire significant resistance to such inhibitory factors (64–66), the therapeutic use of *ex-vivo*-generated DCs became a logical option.

After the initial success of the therapeutic vaccines involving partially-mature “first generation” DCs in follicular lymphoma and melanoma in 1990-ies (67,68), such DCs have been used to treat patients with numerous other malignancies. Immature, or only partially-mature DCs, express suboptimal levels of costimulatory molecules, at least directly after their removal from generation cultures, and constitute a weaker immunogen than the subsequently-implemented mature DCs, constituting “second generation” of clinically-applied DCs (69, 70).

However, while the incidence of “objective” clinical responses (as measured by RECIST or WHO criteria) to such DC preparations, often containing significant amounts of other cell types, were comparable to other vaccine types and rarely exceed the 10–15% (3,13,17–19, 71), two recent phase III trials (8) of a “first generation” DC-based vaccine against prostate cancer (Sipuleucel-T/Provenge), demonstrated their ability to prolong overall survival of the vaccinated patients with advanced cancer (6–9,18,72,73).

These data demonstrate that even immature DCs can offer therapeutic advantage in the settings of advanced cancer and raise the question of whether the occurrence of “objective” clinical responses detectable by RECIST criteria, that were developed to monitor the direct cytotoxic effects of chemotherapeutic agents, can indeed predict long-term advantage of cancer vaccines (2,16). This may not be surprising in view of the key difference in the modes or function of cytostatic drugs or such “passive” immune therapies as antibodies or adoptively-transferred effector cells, and cancer vaccines. While these first two groups of treatments directly target tumor cells, cancer vaccines represent active therapies and target patients’ own immune system that needs to be re-programmed from tolerating tumor cells to mounting an effector-type immunity and to develop a set of features allowing it to eliminate cancer. Therefore, while cytostatic drugs or adoptively-transferred tumor-specific CTLs can mediate immediate anti-tumor effects themselves, cancer vaccines need to first re-program the complex set of interactions within the patient’s immune system and between the immune cells and tumor. In effect, the therapeutic effect may occur with delay and it may not necessarily involve an early “acute” phase, in analogy different forms of autoimmunity or transplant rejection that can be manifested by a rapid destruction of the target organ (in case of acute pancreatitis or acute transplant rejection), but can also proceed in a more stealth although similarly damaging form

as, insulinitis leading to diabetes or chronic kidney disease leading to renal failure, or to chronic rejection of transplanted organs.

In attempt to overcome the limitations of the first generation of DC-based vaccines, significant efforts were dedicated to the development of clinically-suitable protocols of generating fully-mature DCs in clinically-desirable serum-free culture conditions. Two modalities, both involving PGE₂, macrophage-conditioned medium (74,75) and a cytokine cocktail involving IL-1 β , TNF α , IL-6 and PGE₂ (76), were successfully used to induce fully mature DCs with high expression of co-stimulatory molecules. An additional desirable feature of such PGE₂-matured DCs was their elevated expression of CCR7 and high migratory responsiveness to lymph-node-associated chemokines CCL19 and CCL21 (77,78).

Such “second generation” DC vaccines induced by the IL-1 β /TNF α /IL-6/PGE₂-containing cytokine cocktail (76), are clearly superior to immature DCs with respect to their immunogenic capacity (69,70) and migratory responses to LN-associated chemokines (77–79), and have been widely tested in clinical trials. Unexpectedly, however, a recent phase III trial in advanced melanoma showed their very limited effectiveness of less than 10%, similar to dacarbazine (80). While it remains unclear how far these limited effects resulted from the selected type of the maturation cocktail, one obvious possibility is the undesirable negative impact of PGE₂ on the production of IL-12p70 (47,65,66,81), the factor with numerous activities central to the induction and survival of type-1 immune cells (82), and high activity of such cells in activating Treg cells (37,83) are possible culprits.

Type-1-polarized DCs: Mature DCs with elevated, rather than “exhausted” ability to produce type-1-inducing factors and to induce type-1 immunity

Induction of anti-tumor CTL responses by DC-based vaccines benefits from high expression of costimulatory molecules and CCR7 responsiveness, typical of mature DCs (69,70,79,84). At the same time, high IL-12p70 secretion has been shown to dramatically enhance the ability of DCs to induce tumor-specific Th1 cells and CTLs, and to promote tumor rejection in therapeutic mouse models (85–95). Unfortunately, obtaining DCs with all the above three features (combination of high immunostimulatory function, high migratory activity, and high capacity to produce IL-12p70) proved to be difficult: “First-generation” DC-based vaccines utilized relatively immature or only partially-mature DCs, that were immunogenic and able of mediating clinical activity (67,68), but suboptimal with regard to their lymphnode homing ability and T cell-stimulating activity (69,70). “Second-generation” DC-based vaccines involving mature DCs, particularly the DCs matured in the presence of an IL-1 β /TNF α /IL-6/PGE₂-containing cytokine cocktail (76), showed a desirable fully mature status, but a reduced ability to produce bioactive IL-12p70, also referred to as DC “exhaustion” (64,96,97).

In attempt to circumvent the above limitation, several groups, including us demonstrated the feasibility of inducing mature but “non-exhausted” DCs, by exposing immature DCs to type-1 and type 2 interferons combined with TNF α or TLR ligands, or alternatively, by exposing immature DCs to activated NK cells or memory CD8⁺ T cells (65,97–106). The resulting “type-1 polarized” DCs (DC1s; as DCs inducing Th1-polarized responses) show dramatically enhanced capacity to induce long-lived tumor-specific T cells with strongly pronounced anti-tumor effector functions in human *in vitro* and mouse *in vivo* models, and high activity in enhancing antitumor functions of NK cells.

Originally, we observed that while the DCs activated by inflammatory cytokines alone, by the individual bacterial-type stimuli, such as LPS or SAC, or by a T cell-related DC activating signal delivered by CD40L, in all cases mature and produce numerous other cytokines, such as TNF α , IL-12p40, or IL-8, exclusively the “two-signal-activated” DCs, activated by the

combinations of at least two of the above factors, undergo an inflammatory pathway of maturation, associated with the production, the bioactive IL-12p70 (107–109). These observations have been extended by the subsequent demonstration that the combination of defined TLR-ligands are indeed much more effective than the individual stimuli (or cytokines only) in inducing IL-12p70 production and inducing Th1 responses of CD4⁺ T cells (110–112).

The most interesting of these observations was that some of the above combinations (such as LPS plus IFN γ) were not only successful in inducing the maturation-associated IL-12p70 production in DC cultures, but could prime DCs for a non-exhausted, that is type-1-polarized status, manifested by strongly elevated, rather than suppressed ability to produce IL-12p70 at the mature stage, after DC removal from the maturation cultures and subsequent re-activation or the interaction with T cells (65). This led to strongly-enhanced induction of Th1-type CD4⁺ T cells specific for superantigens (65) and tumor-relevant antigenic epitopes (103). Even more interesting, the combination of TNF α /IL-1 β with IFN γ could induce the same polarized DC1 phenotype (priming the DCs for high IL-12 production at later stages), despite lack of immediate ability to induce IL-12 during the maturation (65).

Since it is the ability of DCs to produce IL-12 after their administration to cancer patients (DC polarization rather than IL-12 production during maturation) that is the most likely to translate in improved induction of type-1 responses, the above findings prompted us and others to develop non-exhausted (or polarized) DCs suitable for clinical application. While the combination of TNF α /IL-1 β with IFN γ that induced complete maturation and polarization of DC1s in fetal calf serum (FCS)-supplemented cultures, was suboptimal in inducing DC1 polarization and the optimal CCR7 expression on polarized DC1s, we observed that additional inclusion in the maturation cocktail of IFN α and polyinosinic:polycytidylic acid (poly-I:C) overcomes this problem, allowing us to obtain CCR7-expressing, DC1s, able of high IL-12 production in such serum free media as AIM-V or Cellgenix (97). When directly compared with standard (s)DCs matured by IL-1 β /TNF α /IL-6/PGE₂ (76), which are frequently used in “second generation” DC-based vaccines, such IFN α -supported DC1s (α DC1) loaded with different forms of tumor-relevant antigens (peptides whole tumor cells) induce an average of 20–70 fold higher numbers of functional tumor-specific CD8⁺ T cells than PGE₂-matured DCs (97,113). An additional bonus of the inclusion of IFN α in the DC1-inducing maturation cocktail is its strong impact on the production of chemokines by DCs ((47), *see below*).

So far, our data from melanoma (97), CLL (113), head and neck (114), and several other forms of cancer, uniformly demonstrate the feasibility of generating α DC1s from patients with advanced cancer, their loading with peptide antigens (97) or apoptotic tumor cells (113) and their high effectiveness in inducing tumor-specific CTLs.

While our recent work focused on IFN α -supported DC1s (97,103) and DC1s with similar properties induced by “two-signal-activated” autologous NK cells or memory-type CD8⁺ T cells (100,101,104), the data from the groups of Brian Czerniecki and Gary Koski (102), Marieke van Ham (115) and Walter Storkus (103), showed the feasibility of generating clinically-compatible DC1s induced by the combination of IFN- γ with LPS (including its clinical-grade “detoxified” form, MPLA) and their high effectiveness in producing high levels of IL-12p70 upon interaction T cells and inducing strong Th1-type and CTL responses (65, 102,103,115).

The above approaches utilizing IFN γ (or both IFN γ and IFN α / β) to prime DCs for high IL-12 production upon interaction of T cells (type-1 DC polarization) need to be compared with the approaches utilizing DC exposure to the combination multiple TLR ligands that synergistically induce high levels of bioactive IL-12p70 (107,109,111,116,117), and with DCs engineered to

express IL-12 under non-genomic promoters (85–90,92–95). Another recently identified possibility may be the induction of Th1- and CTL-inducing DCs that show elevated ability to induce type-1 responses in IL-12-independent fashion, using *t-bet* transduction of DCs (118). So far, the above forms of type-1 DC polarization have been applied to the monocyte-derived DCs generated in the presence of GM-CSF and IL-4. However, it is possible that additional benefits can be observed by combining the phenomenon of DC1 polarization with alternative means of generating highly-immunostimulatory DCs, such as the use of IL-15 (instead of IL-4) to promote early DC development (119), B7-DC-cross-linking (120), or inhibition of p38MAPK (121,122), in order to generate DCs with the optimal combination of the desirable features.

Preferential interaction of polarized DC1s with the desirable immune cells (CTLs, Th1- and NK cells)

In addition to improving their overall immunostimulatory function, another aspect that needs a thorough evaluation is the possibility to manipulate vaccines to selectively enhance the interaction of the antigen-carrying DCs with the desirable types of immune cells, such as Th1, NK and CTL (allowing to selectively expand these subsets and support their functions), and to avoid their interaction with suppressor/regulatory cells. The need for such manipulations has been highlighted by the observations of preferential expansion and activation of FoxP3⁺ regulatory T_{reg} cells in cancer patients receiving standard, PGE₂-matured vaccines (37,83).

As a possible remedy to this situation, we and others have recently observed that the conditions of DC maturation imprint the differential ability of mature DCs to secrete particular classes of chemokines and to preferentially attract and distinct types of immune cells (47,123). While the presence of PGE₂ during DC maturation primed the resulting DCs for subsequent production of CCL22 (MDC) and attraction of Tregs (47), it suppressed the ability of DCs to produce such Th1-CTL- and NK cells chemokines as CXCL9, CXCL10, CXCL11, and CCL5, and the ability of DCs to recruit CXCR3⁺ effector T cells and NK cells (47,123). In contrast, the inclusion of IFNs in the DC maturation cocktails, particularly when combined with elimination of PGE₂, suppressed the DC secretion of CCL22 (and the ability to attract T_{regs}) and enhanced the secretion of effector T cell-attracting chemokines: CCL5 and CXCL10 (and other CXCR3 ligands).

Importantly for the ability of the DCs matured in different environments to interact with different subsets of immune cells *in vivo*, the ability of (PGE₂-matured) “standard” DCs and αDC1s (matured in the presence of type I and type II IFNs), to produce different sets of chemokines was stable and could be also observed after harvesting of the DC and their additional culture in the absence of the above maturation factors. These data implicate that the application of polarized DC1s may help to avoid the preferential interaction with T_{regs} and to limit the undesirable expansion of T_{reg} cells promoted by other types of DCs (37,83,124–126), preferentially supporting the desirable types of immune cells, such as CTLs, Th1- and NK cells.

Selective induction of type-1 immune functions by polarized DC1s (Delivery of signal 3)

DCs provide T cells with antigen-specific “signal 1” and costimulatory “signal 2” (127–129), promoting the expansion of tumor-specific T cells. DCs are also known to provide T cells with an additional “signal 3” (polarization; (63)), selectively driving the development of type-1 or type-2 immunity, associated with differential involvement of particular effector mechanisms and different abilities to induce cancer rejection (63,82,127–135). In addition to their role as

initiators of Ag-specific responses of CD4⁺ and CD8⁺ T cells, DCs also activate and support the tumoricidal functions of NK cells (123,136).

In order to determine if the mechanism of superior induction of tumor-specific CTLs and Th1 cells by polarized DC1s involve mainly a preferential enhancement of the pre-existing type-1 responses, or they also involve a superior *de novo* induction of CTLs from naïve precursors, we have compared the results of priming of naïve T cells by different types of non-polarized DCs (including TNF α /IL-1 β /IL-6/PGE₂-matured sDCs) and polarized DC1s (including the TNF α /IL-1 β /Poly-I:C/IFN γ /IFN α -matured α DC1), using superantigen model (137). We observed that while both polarized and non-polarized DCs were similarly effective in inducing the expansion and CD45RA to CD45RO conversion of naïve CD8⁺ T cells, the induction of granzyme B and perforin, as well as acquisition of CTL activity, all required priming by polarized DC1s (137). Although the advantage of DC1s in inducing qualitatively superior CTLs was also observed in case of tumor-specific recall responses (to MART-1), the magnitude of the difference was relatively lower, suggesting the key role of the induction of the functional CTLs, rather than their selection in the overall high CTL-promoting activity of DC1s.

In accordance with the central role of IL-12 in the development of CTL activity in CD8⁺ T cells, the neutralization of that factor abrogated the DC1-mediated GrB induction. A similar key role of IL-12 in the induction of tumor-specific Th1 responses of CD4⁺ cells by polarized DC1s was also observed by Wesa and colleagues (103), while our (unpublished) data demonstrate that IL-12 is key to the effective DC1-mediated NK cell activation. These results indicate that while the preferential ability to promote type-1 immunity may involve additional mechanisms, such as elevated production of CTL-, Th1- and NK cell-attracting chemokines (47,123), their production of IL-12 is critical to their function.

In order to evaluate the immunologic and anti-tumor effectiveness of DC1-based vaccines in preclinical *in vivo* settings, we and others have developed several models of polarized DC1s, generated from mouse bone marrow (138–140). Immature DCs from C57BL/6 mouse-derived bone marrow cells (BMCs) were cultured for additional 24 hours with either IFN- γ , IFN- α , IL-4 and poly-ICLC; with LPS, IFN- γ and IL-4; or with the combination of IFN- γ , CpG and poly-IC; in all yielding mature DCs able of producing elevated levels of IL-12. When compared to tumor-loaded (or tumor-relevant antigen-loaded) nonpolarized DCs, DC1s showed strongly enhanced ability to induce tumor-specific CTL responses *in vitro* and *in vivo*, elevated ability to induce high level of tumor infiltration effector cells, and strongly enhanced antitumor effects in mouse preventive and therapeutic models of lymphoma, glioma and melanoma (138–140). Similar enhancement of immunostimulatory and antitumor functions against mouse models of colorectal cancer and lymphoma was also observed in case of DC1s induced *in vivo*, but promoting the interaction of tumor-loaded DCs with memory-type CD8⁺ T cells specific for tumor-unrelated antigens of LCMV or ovalbumin (138–140).

Induction tumor-relevant homing function in tumor-specific T cells (Delivery of Signal 4)

The observations of high immunologic activity of many cancer vaccines, combined with their limited effectiveness in inducing cancer regression (13,141), suggest that the currently used vaccines may be suboptimal in inducing relevant tumor-homing properties in the vaccination-induced T cells, and that the effectiveness of cancer immunotherapies may benefit from the means to enhance the expression of tumor-relevant homing receptors on cancer-specific T cells. The differences in homing properties between different T cell subsets are generally recognized (26,142–147), but only more recently it was demonstrated that T cell homing properties can be regulated by DCs (148–153). DCs developing in different organs have been shown to utilize vitamin A and vitamin D to, respectively, induce the CCR9 (154) or CCR10 (155), the T cell

chemokine receptors allowing to preferentially migrate to the gut or to the skin. DCs from Peyer's Patches or DCs treated with vitamin A derivatives are highly effective in inducing gut-homing properties in T cells (149–152).

In additional support of the role of DCs and DC-related factors in induction of different migratory properties of T cells (*delivery of "Signal 4"*), it was shown systemic infusion of IL-12 to melanoma patients induces enhanced T cell expression of functional CLA (cutaneous homing receptor; ligand for skin endothelium-expressed ELAM), resulting in enhanced migration of CTLs to melanoma lesions in the skin (156). Similarly, it was recently shown that vaccination with monocyte-derived DCs can induce melanoma-specific T cells able of homing to both the skin and visceral metastases of melanoma (157).

Although it is likely that different tumors may express different patterns of chemokine production, implicating the need for different expression of CKRs on the vaccination-induced T cells against different tumor types, the known importance of chemokines in cancer biology (as factors dictating the pattern of metastasis formation and supporting tumor survival) suggests that the paradigms of T cell entry may be conserved within a tumor type. For example, since melanomas in general are known to over-express CCL5/RANTES (29,158), on which they rely as an autocrine growth factor (29,30), CCL5-responsive T cells may be more likely to enter melanoma tissues, compared to their negative counterparts. In support of such role of T cell-expressed CCR5 (as opposed to tumor-expressed CCR5 that may support tumor survival), it was shown that while the overall populations of melanoma patients lacking functional CCR5 (CCR5Delta32⁺ individuals) and have similar course of disease as CCR5-competent melanoma patients, the expression of functional CCR5 is correlated with positive outcomes of several forms of immunotherapy (159). Similarly, in accordance with the elevated expression of CXCR3 ligands: CXCL9/MIG and CXCL10/IP10, observed in melanoma tissues (160) and the presence of CXCR3 on tumor-infiltrating lymphocytes in regressing melanoma lesions (161), high levels of CXCR3 on circulating CD8⁺ T cells has been recently implicated in effective control of advanced melanoma (162).

Guided by these observations, we analyzed whether the induction and persistence of CCR5 (receptor for CCL4, CCL5, and CCL8) and CXCR3 (receptor for CXCR9, CXCR10 and CXCR11) on developing CD8⁺ T cells can be regulated by DCs maturing in different inflammatory environments. In different sets of *in vitro* sensitization (IVS) of HLA-A2-restricted melanoma-specific CD8⁺ T cells, using MART-1_{27–35}-loaded polarized DC1 or nonpolarized DCs (involving the cells from melanoma patients and healthy controls), we observed that MART-1-specific CD8⁺ T cells sensitized by polarized α DC1s also showed elevated levels of both CCR5 and CXCR3 in MART-1-specific CD8⁺ T cells, compared to the cells sensitized by sDCs (137). Interestingly, similar results were obtained in case of healthy controls and melanoma patients, suggesting the ability of DCs to differentially regulate chemokine receptor expression both in naïve and tumor-primed T cells.

While the nervous system (CNS), an "immunologically privileged" site (163), is often believed to be able to exclude effector immune cells from entry (164), migratory APCs may actually be able to prime T cells for integrin-dependent entry into the brain (165). As demonstrated in cases of paraneoplastic cerebellar degeneration (166,167) and in mouse model of experimental allergic encephalomyelitis (EAE), which resembles the pathology of multiple sclerosis in human (168), exposure of CNS-derived T cell antigens to the systemic immune system can lead to induction of specific T cell responses that recognize and attack immune targets located in the CNS.

Our studies demonstrated that tumor-specific IFN γ -producing type-1 CD8⁺ T cells (Tcl), but not type-2 CD8⁺ T cells, are able to efficiently traffic into CNS tumor sites and mediate

effective therapeutic effects, (169), in a mechanism involving CXCL10 (138,169,170) and an integrin receptor VLA-4 (171–174). Consistently, mouse DC1s induced CXCR3-expressing CTLs more efficiently than non-polarized DCs (138). Subcutaneously administered DC1s effectively migrated into draining lymph nodes, induced Ag-specific CTLs, and suppressed Treg accumulation. In addition, the immunization with DC1s loaded with glioma-associated Ag (GAA)-derived CTL epitope peptides prolonged the survival of glioma-bearing mice, which was associated with efficient tumor-homing of glioma-specific CTLs. The therapeutic vaccines of GAA peptide-loaded DC1-based vaccines were further increased by their intratumoral injection, suggesting the role of such cells in the attraction and/or supporting local functions of the effector cells. The antitumor effects of DC1s generated from CXCL10^{-/-} mouse-derived DC1s were completely abrogated, indicating an important role of the chemokine system in DC1 function.

The possibility that improved tumor homing may translate into better outcomes of active immunotherapies is supported by the observations that the level of T cell infiltration is a strong independent prognostic marker of the survival of cancer patients with melanoma (175), colorectal cancer (176–178) and malignant glioma (179,180). Of interest, in case of colorectal cancer, the highest benefit appears to be associated with the infiltration of the effector and effector-memory T cells (178), suggesting the importance of the defined set of chemokines able of attracting such cells. Therefore, the ability of DC vaccines to induce the CCR5- and CXCR3-expressing CTLs may help them to act as therapeutic cancer vaccines.

Since several types of tumors, notably including ovarian cancer often associated with poor prognosis, can evade immune surveillance by preferentially attracting T_{regs} and suppressive types of DCs in a CCR4/CCL22- and CXCR4/CXCL12-mediated mechanism (34,35,55), it remains to be tested whether the effectiveness of cancer vaccines can be enhanced by the pharmacologic modulation of tumor environments, in order to suppress the local production of Treg-attracting chemokines and to increase the expression of effector cells such as CTLs, Th1 and NK cells. Since several tumors have been shown to over-produce PGE₂ (43–46) and PGE₂ has been shown to be a potent enhancer of CCL22 production and a suppressor of the production of CXCR3-binding chemokines in APCs (47), it remains to be tested whether PGE₂-inhibiting treatments (including salicylates, indomethacin, sulindac, celecoxib or rofecoxib), used alone or possibly as parts of combination therapies, may reduce CCL22 levels and Treg infiltration, and promote tumor entry of the CXCR3-expressing effector cells. The possibility that tumor-specific chemokine modulation in tumor-infiltrating DCs may indeed enhance overall effectiveness of cancer immunotherapies has been supported by the demonstration that DCs present in regressing tumors show particularly CXCL9 expression and effectively attract CXCR3⁺ T cells (160,181), and it will be directly tested in our upcoming clinical trials. Another area of our research is the possibility to uncouple the “signal 3” from “signal 4” in order to generate type-1 effector cells with Th2/T_{reg}-type homing patterns (and *vice versa*), in order to be able to target effector and regulatory T cells to desirable tissues.

Clinical evaluation of polarized DC1s: towards next generations of DC-based therapies

The clinical activity of type-1 polarized DCs generated in the presence of IFN α (α DC1s) are being currently evaluated in a variety of cancer types, including glioma, melanoma, colon and prostate cancers at the University of Pittsburgh Cancer Institute (*see ClinicalTrials.gov: NCT00390338, NCT00099593, NCT00766753, NCT00558051 and NCT00970203*), with the clinical trials of other types of such “third generation” DC-based vaccines ongoing in other centers. In our trials, peptide-, autologous- or allogeneic tumor cells-loaded DC-vaccines are administered via a variety of routes including, intra-lymphatic (melanoma, colorectal ca.),

intra-nodal (malignant glioma, colorectal ca.) or intradermal routes (colorectal- and prostate ca.)

In our phase I/II trial in malignant glioma HLA-A2⁺ patients with the recurrent disease receive intra-nodal injections of α DC1s loaded with HLA-A2 binding peptides EphA2 (883–891) (182), IL-13R α 2 (345–353:1A9V) (183), YKL-40 (202–211) and GP100 (209–217: 2M) at two-week intervals and receive twice weekly injections of poly-ICLC. To date, 19 participants have completed at least 4 vaccinations with no major adverse events. In our interim analyses, increased CD8⁺ cells reactive to HLA-A2.1-EphA2 (883–891) or HLA-A2.1-IL-13R α 2 (345–353) tetramers in post-vaccine peripheral blood mononuclear cells (PBMC) was associated with 6-month progression-free survival, suggesting a possible correlation between tetramer-detected immune responses and clinical response. Interestingly, several of these patients also showed up-regulation of a CXCR3 on CD8⁺ PBMC following vaccines, indicating that this vaccination regimen induced can induce a similar receptor chemokines of tumor-specific T cells, as observed in our preclinical mouse (138) and human *in vitro* (137) studies.

Recent clinical studies of current cancer vaccines including the “first-generation” DC-based vaccines, have suggested that, despite their limited activity in inducing regression of established cancer, they may promote a models but significant prolongation of the overall survival of cancer patients. Recent advances in the area of DC biology allow the development of new means to generate fully-mature DCs with elevated, rather than suppressed ability to produce IL-12p70 and other pro-inflammatory cytokines and chemokines, helps to design next generations of DC-based vaccines (Figure 2), exploiting not only the classical functions of DCs as carriers of “signal 1” and “signal 2”, but also the ability of DCs to deliver “signal 3” and “signal 4”, helping tumor-specific T cells to acquire desirable effector functions and the ability to enter tumor tissues. Moreover, the demonstration of stable differences in chemokine profiles of the DCs maturing in different environments, may allow to preferentially deliver DCs to the immune cells mediating the effector arm of immune responses without hyper-activating *Treg* cells, and possibly to modulate the tumor-infiltrating DCs, in order to direct the vaccine-induced effector cells to tumors.

Acknowledgments

The author thank Drs. R. Muthuswamy, P. Watchmaker, E. Berk, T. Reinhart, L. Geskin, J. Kirkwood, K. Chatta and David Bartlett, for stimulating discussions and for sharing unpublished data. This work was supported by the NIH grants CA095128, CA114931, CA101944, CA121773, EA055944, CA137214, NS055140, and NS40923.

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Figure 1. Unique tasks of therapeutic vaccines against cancer

In contrast to protective vaccines, therapeutic vaccines need to function in cancer patients in spite of the dysfunction of endogenous DCs and the presence of tumor-induced suppressive immune cells, such as regulatory T_{reg} cells. Even more importantly, their role needs to go beyond the induction of long-lived memory cells, the key task of protective vaccines. Since in contrast to infections, cancer is a poor source of pro-inflammatory alarm signals capable of inducing effector functions and peripheral homing potential in Ag-specific T cells, the effectiveness of therapeutic vaccines may require provision of such signals by vaccines themselves or by additional factors used in combination with vaccines. Since some tumors show limited production of the chemokines capable of attracting effector cells (CTLs, Th1- and NK cells), instead overproducing T_{reg} -attracting chemokines, the immunotherapies of such tumors may benefit from the combination of vaccines with the additional modulation of the production of effector cell-attracting versus T_{reg} -attracting chemokines within tumor tissues.

Figure 2. Unique features of polarized DC1s, as compared to non-polarized mature DCs (used in “second-generation” DC-based vaccines) and immature DCs (used in the “first generation” DC-based vaccines). While all the above DC types can provide T cells with antigen (“signal 1”) and different levels of costimulation (“signal 2”) needed for activation and expansion of tumor-specific T cells. Polarized DCs are particularly capable of providing an polarizing “signal 3”, promoting the development of type-1 effector mechanisms in naïve/resting immune cells (63) and “signal 4” allowing the differentiating cells to acquire the expression of peripheral homing receptors, including CCR5 and CXCR3. In addition, to preferentially inducing the desirable immune effector cells, unique chemokine production profile of polarized DC1s may also help them to preferentially interact with pre-existing (or induced by previous cycles of vaccination) effector and memory cells, rather than with T_{regs}.