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REVIEW

Dendritic cell biology and the application of dendritic cells to immunotherapy of multiple myeloma

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Dendritic cells (DCs) are extremely efficient antigen-presenting cells that are potent stimulators of both B and T cell immune responses. Although DCs are normally present in extremely small numbers in the circulation, recent advances in DC biology have made it possible to generate DCs in culture. DCs can be generated *in vitro* from various cellular sources including bone marrow, cord blood and peripheral blood. Although culture conditions are extremely diverse, the majority of protocols grow DCs in GM-CSF and either TNF-alpha and/or IL-4. The addition of other growth factors such as SCF and Flt-3 ligand can dramatically enhance DC recovery. It is important to appreciate that DC subsets have been identified. Thus, DC at different stages of maturation, based on phenotype and capacity to capture antigen, can be obtained depending on culture conditions. For clinical applications, DCs can be generated in serum-free media and cryopreserved for future clinical applications. The ability to obtain DCs in numbers suitable for manipulating immune responses has pushed DC-based immunotherapies into the spotlight for treatment of various malignancies, including multiple myeloma, a B cell malignancy that is presently incurable. Although high-dose chemotherapy and transplantation have improved complete remission rates and overall survival in myeloma, immunotherapeutic strategies are needed for the additional cytoreduction needed to achieve a cure. Because DCs specialize in antigen capture and are extremely potent at stimulating T cell responses, they are ideally suited for generating anti-myeloma T cell responses *in vivo*. Several studies have demonstrated that myeloma protein, also called idiotype (Id), is sufficiently immunogenic and can be used to generate *in vivo* T cell responses in myeloma patients. Clinical trials using Id-pulsed DCs as a vaccine to treat minimal residual disease or relapsed myeloma are currently underway. Feasibility studies indicate that antigen-pulsed autologous DCs can be used to elicit *in vivo* Id-specific T cell responses. Additional studies are needed to optimize current DC vaccination protocols and determine clinical benefits associated with this approach. It is hoped that, following conventional therapies, a combination of adoptive

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immunotherapeutic modalities such as DCs together with myeloma-specific T cells may lead to improved clinical responses in multiple myeloma, and ultimately lead to complete remission and cure.

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Introduction

Recent studies examining the role of cellular immunity in tumor rejection have focused on T cell mediated recognition of tumor antigens.[1] Tumor immunity appears to be due to the development of major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL). Generation of CTL requires cooperation between several elements of an intact immune system. It is thought that antigen-presenting cells (APCs), CD4+ T cells and CD8+ T cells play a crucial role in anti-tumor immune responses. Dendritic cells (DCs) are a unique subset of APCs that have been shown to play an important role in generating anti-tumor cell responses. DCs are extremely efficient at antigen capture and presentation of processed antigens to naive T cells.[2] The discovery that DCs could be expanded ex vivo in large numbers[3,4] has made DC-based immunotherapeutic approaches attractive for treatment of various malignancies. It has been shown that DCs can be derived from both proliferative and nonproliferative precursors in culture using a combination of granulocyte-macrophage colony-stimulating factor (GM-CSF) and either IL-4 with/without TNFα.[3,4] The addition of other cytokines such as Flt-3 ligand and SCF can dramatically increase the number of DCs generated *in vitro*.[5] Two distinct DC precursors give rise to either myeloid or lymphoid DCs that differ in their ability to control T helper cell differentiation.[6] Several clinical studies are currently underway exploring the efficacy of DCs for treating a variety of malignancies, including multiple myeloma. In this review we discuss general characteristics of DCs, followed by methods for cultivation and *ex vivo* expansion from various cellular sources for use in clinical trials. The final part of this article will focus on DCs and vaccination protocols for treatment of multiple myeloma. Despite recent progress and improved long-term survival following myeloablative therapy and transplantation, multiple myeloma is still an incurable disease.[7] Therefore, novel immunotherapeutic strategies utilizing DCs to elicit T cell mediated recognition of myeloma tumor cell antigens offers promise for eradicating residual disease following conventional therapies.

Features of dendritic cells

DCs were identified in 1973 as a weakly adherent cell population in the spleen of mice.[8] Human interstitial DCs were later described in 1981.[9] In human bone marrow, a minor subset of hematopoietic progenitor cells has been identified that is phenotypically distinct (CD34+, Lin-, CD45RA+, CD38+, Thy-1-, c-kit-) and gives rise to T cells, B cells, NK cells and DCs, but does not produce myeloid and erythroid cells.[10] DCs reside in both lymphoid and nonlymphoid tissues and differ functionally and phenotypically depending on their stage of activation.[11–13] The observation that DCs are extremely efficient at presenting antigens to naive T cells has pushed DCs in the spotlight as an ideal antigen presenting cell for generating tumor-antigen specific cytotoxic T cell responses. Although DCs are extremely diverse depending on their stage of differentiation/activation, the following properties are attributable to DCs: (a) the ability to stimulate primary T cell responses; (b) spontaneous and rapid clustering with T cells; (c) marked cell motility and the ability to migrate/home to T cell areas within lymphoid tissue;[12] (d) specialized phagocytic activity and antigen capture by receptor-mediated uptake and macropinocytosis;[14] and (e) a phenotype that is distinct from other cell types. For instance, cultured dendritic cells (that is, activated/differentiated) do not express classical T cell, B cell, or monocyte/macrophage markers[15] but are positive for DC specific molecules (CMRF-44, CMRF-56, CD83, S100), costimulatory molecules (CD40, CD80, CD86), adhesion molecules (CD11a, CD11c, CD44, CD50, CD54, CD58, CD102), leukocyte common anti-

gens (CD45RA, CD45RO), MHC antigens (HLA-ABC, HLA-DP, HLA-DQ, HLA-DR), and activation markers such as CD25. DCs are further distinguished from monocytes/macrophages since DCs do not contain myeloperoxidase activity and express only low levels of 5′ nucleotidase, dipeptidyl peptidase and cathepsin B activity. Although DCs were named based on their distinctive morphology, this feature is not sufficient for identification since other cells such as B cells and fibroblasts can adopt a very similar appearance.[12]

The activation status and *in vivo* migratory capacity is an essential aspect of DC biology that needs to be carefully considered when optimizing cancer vaccines and immunotherapeutic protocols involving DCs. DC progenitors derived from the bone marrow travel by way of the blood to various nonlymphoid tissues, such as skin epidermis and lung. Since blood-derived DCs migrate and localize in tissue, they are present in the circulation in extremely small numbers (0.1% of all peripheral blood mononuclear cells). DCs isolated from the blood and nonlymphoid tissues are referred to as 'immature' DCs, and are extremely efficient at capturing and processing antigens.[16] Inflammatory mediators such as TNFα, IL-1 and LPS induce DC maturation and subsequent migration to T cell areas (paracortical regions) of secondary lymphoid tissue.[17] Maturation of DCs results in reduced capacity to endocytose antigens and increased levels of adhesion and costimulatory molecules for optimal presentation of peptide−MHC complexes to T cells.[18] In view of the migratory patterns of human DCs, an important aspect of immunotherapy is whether *in vitro* expanded DCs will retain migratory properties and localize to T cell areas of draining lymph nodes following adoptive transfer. A recent study by Barratt-Bozes *et al* examined the migratory capacity of *in vitro* generated DCs after adoptive transfer into chimpanzees.[19] They found that fluorescent-labeled DCs migrated to T cell areas of lymph nodes draining the site of immunization following subcutaneous injection. The DCs persisted in the parafollicular zone surrounding B cell follicles for 5 d and retained expression of large amounts of CD86, CD40 and MHC class II molecules. In contrast, labeled DCs could not be observed in draining lymph nodes following intravenous injection.[19] These data, together with results using murine models,[20,21] illustrate that the route of immunization can play a major role in the homing properties of *in vitro* generated DCs.

In vitro dendritic cell generation

Numerous protocols have been described for *in vitro* generation of human DCs using various cellular sources such as bone marrow,[22−24] neonatal cord blood,[23,25,26] mobilized peripheral blood progenitor (stem) cells,[27−31] and peripheral blood mononuclear cells.[3,4,32−34] Two distinct DCs precursors arise from CD34+ progenitor cells based on expression of CD1a and CD14. The CD1a+, CD14- subset differentiates in culture to yield epidermal DC whereas the CD1a−, CD14+ subset produces either macrophages or interstitial/dermal DCs, depending on stimulation conditions.[11,35,36] Peripheral blood can also be used to generate DCs either from CD34+ precursor cells or the more abundant CD34− precursor population, depending on culture conditions.[37,38] When DCs are generated from CD34− precursor cells, removal of non-adherent cells can significantly reduce the level of contaminating lymphocytes.[3,4] Human CD14+ monocytes can also be used as the starting population and will differentiate into DCs under appropriate stimulation conditions.[35] Generation of DC from monocytes is radioresistant and occurs in the absence of DNA synthesis, verifying that the DCs are derived from monocytes and not contaminating progenitor cells.[35,39] As illustrated in Table 1, a variety of protocols have been developed to generate DCs in culture under different stimulation conditions using cells obtained from a variety of anatomic sites.

Although culture conditions for generating DCs are extremely diverse, GM-CSF appears to be essential for DC expansion regardless of the starting cell population. GM-CSF has been shown to induce DC progenitor expansion[37] and promotes differentiation and survival of DCs for up to 6 weeks.[33] IL-4 is often used together with GM-CSF for DC generation, especially when the cell source is nonadherent peripheral blood mononuclear cells or peripheral blood monocytes. IL-4 inhibits macrophage colony formation[40] and induces DC growth and maturation.[37] When bone marrow or cord blood cells (CD34+ progenitor cells) are used to generate DCs, TNFα is used together with GM-CSF in place of IL-4.[31,37,41] TNFα presumably acts by reducing granulocyte production and upregulating

Table 1 Culture conditions for generating dendritic cells from various cellular sources

Cellular source	*Culture conditions*	*Medium/additive*	*Reference*
Bone marrow	SCF, GM-CSF, TNFα	IMDM	Saraya *et al*[23]
	SCF, GM-CSF, TNFα	IMDM/20% FCS	Szabolcs *et al*[36]
Cord blood	SCF, GM-CSF, TNFαCSF, TNFα, Flt-3, IL-4, IL-13	RMPI/10% FCS	Saraya *et al*[23] Rosenzwajg *et al*[102]
PBSC	GM-CSF, TNFα	RPMI/10% FCS	Bernhard *et al*[27]
	SCF, GM-CSF, IL-4, IL-3, IL-6	RPMI/10% FCS	Herbst *et al*[28]
PBMC	GM-CSF, IL-4	RPMI/10% FCS	Sallusto *et al*[38]
	GM-CSF, IL-4, TNFα	RPMI/10% FCS	Zhou *et al*[53]
	GM-CSF, IL-4, MCM	RPMI/1% plasma	Bender *et al*[3]

FCS: fetal calf serum; PBSC: peripheral blood stem cells; PBMC: peripheral blood mononuclear cells; MCM: macrophage conditioned medium.

GM-CSF beta chain expression on progenitor cells making them more responsive to cytokine signaling.[26,42] Chen *et al* investigated the role of TNFα in generating DCs from peripheral blood in serum-free media and found that the addition of TNFα to cultures at day 7 resulted in a 2-fold increase in DC yield and a markedly enhanced capacity to present soluble antigens to T cells.[43] The obligatory role of GM-CSF in generating DCs has recently come into question in light of the observation that a combination of IL-3 and TNF alpha was found to be sufficient for growing DCs from cord blood CD34+ cells.[25] CD40 ligand (CD40L) can also promote differentiation of adherent blood monocytes into functional DCs in the absence of other growth factors.[44] Numerous other cytokines besides IL-4 and TNFα have been evaluated for their ability to generate large numbers of DCs with enhanced stimulatory capacity such as IL-13,[29,45] stem cell factor (SCF)[24,46] and TGFβ1.[47] When Flt-3 ligand was added to cultures of CD34+ bone marrow cells stimulated with GM-CSF, TNFα and IL-4, a 5-fold increase in DC yield was observed, and was further augmented by the addition of SCF.[48] The use of a continuous flow perfusion culture system further enhanced expansion of DCs from CD34+ cells when cultured with GM-CSF, TNFα, SCF, Flt-3 ligand, and TGFβ[49] IL-10 abrogates differentiation of DCs and converts immature DCs into tolerogenic APCs. Such an approach might be a useful tool in the therapy of autoimmune or allergic diseases.[50] Another strategy for generating DCs involves a two-stage culture system that permits cells to be exposed to different cytokines at specified times during culture. Ye *et al* cultured CD34+ bone marrow cells for 5 d in GM-CSF, SCF and TNFα followed by replacement of TNFα with CD40L and IL-4 for an additional 5 days to increase DC yield.[51] Another group stimulated CD34+ peripheral blood cells with SCF, IL-3 and IL-6 for 7 d followed by GM-CSF and IL-4 for an additional 3 weeks for generating DCs.[28]

It is important to appreciate that the starting cell population, choice of cytokines and culture variables can dramatically alter the yield, phenotype, and function of DCs that are generated in culture. Since DCs differ in their ability to endocytose, process, and present antigens depending on their maturational stage, DCs can be extremely heterogeneous with respect to functional capacity. This can become extremely important when utilizing DCs as immunotherapy for cancer patients. In addition, recent studies have indicated two distinct subsets of DCs that differ in their ability to stimulate T helper cell immune responses.[6] One subset is derived from peripheral blood monocytes and gives rise to immature myeloid DCs (designated DC1) when cultured in the presence of GM-CSF and IL-4.[18,38] The other subset is derived from blood or tonsil CD4+, CD3− and CD11c− plamacytoid cells and develop into lymphoid DCs (designated DC2) when cultured with IL-3.[16] Although stimulation with CD40L induces maturation of both DC subsets, only mature DC1 produce IL-12.[6,52] Another major difference is that DC1 induce T helper cells (CD4+) to differentiate into Th1 type cells whereas DC2 direct Th2 development.[6] This can lead to completely different antigen-driven immune responses. For instance, myeloid DCs are potent stimulators of T cell responses and would be beneficial when encountering foreign and/or tumor antigens. Conversely, lymphoid DCs may not be

stimulatory and might play an important role in inducing tolerance, a potential benefit in certain autoimmune and allergic diseases.

Mature or terminally differentiated DCs express abundant costimulatory activity and are the most potent at inducing naive T cell responses.[4,12] Mature DCs can be identified phenotypically by CD83 antigen expression.[4,12] Either TNFα, LPS or CD40L can be used to induce differentiation of DCs when peripheral blood mononuclear cells are cultured with GM-CSF and IL-4 in media containing 10% fetal calf serum (FCS).[38,53] However, differentiation of DCs by TNFα (CD83+ cells) was found to be transient when T cell-depleted peripheral blood mononuclear cells are cultured with GM-CSF and IL-4 in media containing 1% human plasma.[54] Other investigators have demonstrated that the addition of monocyte-conditioned medium to DC cultures resulted in the highest yield of terminally differentiated DCs (CD83+ cells) with the greatest T cell stimulatory activity.[3,4,55] Furthermore, the addition of monocyte-conditioned medium resulted in DCs displaying a mature phenotype after removal of cytokines, unlike DCs grown in TNFα that acquire macrophage features (plastic adherence and CD14 expression) when subsequently cultured in cytokine-free media.[3,4,55] CD40 probably also plays a critical role in DC maturation *in situ* since ligation of CD40 results in high levels of IL-12 production by DCs and increased T cell stimulatory activity.[52,56,57] These data indicate that optimal maturation of DCs is a complex process involving a plethora of growth factors, some of which are currently poorly defined.

In summary, a variety of cellular sources can be used to generate DCs from either CD34+ or CD34− precursors depending on culture conditions. The combination of GM-CSF and IL-4 results in generation of DCs that are immature and express low levels of accessory molecules such as CD25, CD40, CD80, CD83, and CD86.[3,4] Immature DCs are extremely efficient at antigen uptake, processing, and formation of MHC peptide complexes.[14] Addition of TNFα or CD40L results in DC maturation, and monocyte-conditioned medium can induce terminally differentiated DCs that maintain expression of mature phase markers following removal of cytokines.[3,4,54] Mature DCs do not trap and process antigens efficiently, but express increased levels of class II molecules, and adhesion/costimulatory molecules that facilitate priming of naive T cells.[14,38,43] Subsets of DCs have recently been identified (DC1 and DC2) that differ phenotypically in their ability to induce helper T cell differentiation. *In vivo* maturation of DCs during encounters with foreign antigens is critical for eliciting immune responses, and the process of DC maturation is regulated by cytokines produced within the local microenvironment.

DC generation for clinical use

Cell source and DC yield

From a practical point of view, peripheral blood can easily be obtained in large enough quantities for generating adequate numbers of DC for clinical trials. Because the frequency of DC precursors is relatively rare in peripheral blood,[41] many procedures enrich for DC precursors prior to *in vitro* stimulation. There are numerous techniques for enriching for CD34+, CD34− or CD14+ DC precursors by either positive or negative selection.[13] Since the majority of cells in peripheral blood mononuclear cell preparations are T cells, several techniques have been used to enrich for DC precursors by eliminating T cells, such as density gradient separation, panning, nylon wool columns, immunomagnetic beads and rosetting with neuraminidase-treated sheep red blood cells. When either monoclonal or polyclonal antibodies are used, a cocktail of antibodies is often used to remove not only T cells, but also B cells and natural killer cells. An alternative approach is to directly isolate the desired precursor cell by positive selection, using antibodies that recognize either CD34 or CD14 antigen. Positive selection for CD34 cells has also been used when DCs are generated from bone marrow samples. Unfortunately, all of these separation techniques are extremely labor intensive and expensive when used in clinical settings. The generation of DCs from lymphocyte-depleted (T cell with/without B cell depletion) and monocyte-enriched (CD14+) peripheral blood mononuclear cells is illustrated in Table 2. The yield of DCs from peripheral blood mononuclear cells can be increased an additional 6-fold and 30-fold by pretreating donors with G-CSF or Flt-3 ligand, respectively.[4,5] Leukapheresis products from cancer patients following anticancer therapy contain greater numbers of CD34+ cells and can be used to generate large numbers of DCs in culture (mobilized PBMC; Table 2). Small amounts of bone marrow can also yield large numbers of DCs when

stimulating CD34+ cells with SCF, GM-CSF and TNFα in culture (Table 2).

A simplified approach to enrich for DC precursors involves selecting for weakly adherent progenitor cells (CD34−) by removing cells not adhering to plastic after 2 h of culture. When adherent cells are cultured in GM-CSF and IL-4, between 3 and 8×10^6 DCs could be obtained from 40 ml of blood.[37] This method of generating DCs is easy to perform and can be used to generate large numbers of DCs despite the fact that removal of nonadherent cells is difficult to standardize, and lymphocyte contamination is variable. In our experience, enrichment of DC precursors by plastic adherence using mobilized peripheral blood stem cells is adequate for generating sufficient numbers of DCs for clinical trials.

Choice of media

Different types of media have been tested for growth of human DCs such as RPMI-1640, AIM-V, X-VIVO 10/15/20, Hybricare and Iscove's. Human serum and plasma (pooled and autologous) have also been tested in place of FCS and fetal bovine serum (FBS) in order to avoid presentation of xenogeneic antigens by DCs. Although X-VIVO 20 and AIM-V media supplemented with 1% autologous nonheat inactivated plasma could be used to generate mature DCs from lymphocyte-depleted peripheral blood, the use of RPMI-1640 resulted in a higher yield of DCs.[3,4] The highest yield of DCs was obtained using RPMI-1640 supplement with 10% FCS. Tarte *et al* compared unsupplemented X-VIVO 15 medium to RPMI containing 10% FCS. Peripheral blood stem cells cultured in X-VIVO 15 with GM-CSF and IL-4 yielded a lower percentage of CD1a+ cells that were not as effective at stimulating allogeneic T cell proliferation compared to cells grown in RPMI-1640 containing 10% FCS.[58,59] However, the addition of TNFα to DCs grown in either X-VIVO 15 and RPMI-1640 supplemented with 10% FCS resulted in a similar phenotype and stimulatory capacity.[58,59] We have compared AIM-V to RPMI-1640 for culturing peripheral blood stem cells using GM-CSF and IL-4, and found that the yield and percentage of CD1a+ cells is highest using RPMI-1640 containing 10% FBS (unpublished data). Thus the generation of DCs in serum-free media is possible and sufficient; however, addition of FCS results in a higher yield of DCs.[3] Whenever possible, FCS or FBS should be avoided when generating DCs for clinical trials in order to prevent eliciting an unwanted immune response against foreign serum proteins.

Cryopreservation of DCs and DC precursors

DCs can be generated from peripheral blood mononuclear cells that have been cryopreserved in dimethyl sulfoxide containing either human serum albumin or FCS.[4,60] Apheresis cells with/without stem cell mobilization and mononuclear cells isolated from whole blood (routine venipuncture) can be frozen and used later for generating DCs. Makino and Baba compared cryopreserved and fresh peripheral blood mononuclear cell preparations from the same donors and obtained similar numbers of DCs after *in vitro* culture in GM-CSF and IL-4.[4,58,60] DCs generated from frozen peripheral blood mononuclear cells expressed costimulatory molecules and stimulated an allo-MLR similar to that observed when DCs were generated from fresh cells.[60] Optimal cryopreservation was obtained by freezing DC precursor cells in 12% dimethylsulfoxide containing 25–30% FCS[60] Although DCs are extremely sensitive to freezing and thawing procedures.[61] this procedure results in minimal loss of DCs with

Table 2 Dendritic cell recoveries from peripheral blood and bone marrow precursor cells

Cellular source	*Amount of starting material*	*Dendritic cell yield*	*Ratio of DCs to input cells*	*Reference*
PBMC (E-rosette neg)	40 mL blood	$1.5-3.8\times10^6$	74–96%	Bender *et al*[3]
PBMC ($CD3^-$, DR^-)	40 mL blood	$4-8\times10^6$	60–80%	Romani[37]
PBMC ($CD2^-$, $CD19^-$)	40 mL blood	$0.8-3.3\times10^6$	30–80%	Romani[4]
PBMC ($CD14^+$)	100 mL blood	$1.2-1.5\times10^7$	70%	Herbst *et al*[28]
Mobilized PBMC (leukapheresis)	2×10^5CD34+	8×10^6	40-fold increase	Siena *et al*[103]
Bone Marrow ($CD34^+$)	1 mL	1.7×10^6	75-fold increase	Szabolcs *et al*[24]

PBMC: peripheral blood monnuclear cells.

viabilities normally >90%.[60] If terminally differentiated DCs are required, and DCs are frozen and administered at a later date, it is probably best to freeze optimal numbers of immature DCs and differentiate DCs in culture using cryopreserved DCs.[4]

Antigen pulsing of DCs

As previously indicated, immature DCs are extremely efficient at capturing antigens by various mechanisms involving phagocytosis of particles and microbes, extracellular fluid sampling by macropinocytosis, and receptor mediated endocytosis involving lectin and immunoglobulin Fc receptors.[14] Immature DCs are rich in late-endosomal structures containing large amounts of MHC class II molecules that enhance class II-peptide complex formation. These peptide complexes are then delivered to the cell surface where they remain for several days.[62] DCs can also process exogenous antigens by additional pathways resulting in presentation of MHC class I-peptide complexes to CD8+ T cells. It is estimated that only several hundred MHC−antigenic peptide complexes out of the millions of surface molecules found on DCs, are required to elicit T cell responses.[63,64] Because DCs are extremely active at uptake, processing and antigen presentation, immature DCs probably require only a brief *in vitro* exposure to antigen(s) to be capable of stimulating antigen-specific T cell responses. This is further supported by studies examining DC uptake of FITC-labeled IgG and IgA idiotype (Id) proteins indicating that DCs are capable of internalizing approximately 2×10^5 molecules of Id protein within a 1 h period (Butch *et al*, unpublished data).

Studies in murine models indicate that *in vivo* tumor mediated immune responses can be generated by pulsing DCs with peptides, tumor cell lysates or isolated membranes, as well as lymphoma associated IgM Id protein.[12] Transfer of nonpulsed DCs to tumor bearing mice can also generate tumor-specific CTL responses; however, a more vigorous response was observed following adoptive transfer of tumor antigen pulsed DCs.[64] The ability of nonpulsed DCs to elicit an antitumor response has also been observed in patients with chronic myelogenous leukemia (CML). Choudhury and investigators generated DCs in culture from the peripheral blood of CML patients and found that the DCs (Philadelphia chromosome positive) were capable of inducing cytotoxic T cell activity against CML cells in the absence of exogenous antigen.[65] It is interesting to note that antigens can be transferred between DCs and that antigens are shed from DCs in a form capable of activating T cells.[66] Zitvogel *et al* demonstrated that DCs secrete antigen presenting vesicles, called exosomes, derived from endosomes that are rich in assembled peptide-MHC class I and II complexes. Adoptive transfer of exosomes isolated from tumor peptide-pulsed DC cultures resulted in complete regression or reduced growth of several murine tumors *in vivo*.[67] Furthermore, exosomes were more effective than peptide-pulsed DCs in eradicating tumor growth, indicating the potential use of cell-free vaccines for immunotherapy of cancer.[60]

DC vaccination

The optimal route for administration of DCs to cancer patients is presently unclear. The goal is to introduce DCs into the body at a site that will allow DC localization to areas where tumor cells reside. The optimal route for DC administration may vary depending on the type of malignancy. For solid tumors, DC vaccination by the subcutaneous route may be the most efficacious in view of a recent study demonstrating that DCs localize to regional lymph nodes when administrated subcutaneously to chimpanzees.[19] One can only speculate as to the optimal route for metastatic disease and hematological malignancies. At present, intravenous and subcutaneous routes of DC administration have been utilized for hematological malignancies.[68–70] Another variable that may influence *in vivo* localization of DCs is expression of various surface molecules facilitating tissue interactions such as integrins, ICAM, CD31, CD44, and CD68.[12] Because expression of these molecules by DCs may fluctuate depending on their stage of maturation, it is important to consider this variable when deciding on culture conditions and route of vaccination, based on the aims of the clinical trial.

Numbers of DCs that should be administered to adequately stimulate *in vivo* T cell responses is largely unknown. It is well established that extremely small numbers of DCs can induce proliferation of T cells to soluble antigens or alloantigens.[2,38] In mixed lymphocyte cultures, DCs can elicit proliferation of allogeneic T cells at ratios of DC:T cells as small as 1 : 3000.[2] In clinical trials, repeated doses of DCs ranging from

1×10^6 to 3×10^7 have been administered either intravenously, subcutaneously or directly into an uninvolved lymph node, without any adverse reactions.[68–70] Although the potential for eliciting an autoimmune response following DC vaccination is a possibility, most clinical trials vaccinate patients with numbers of antigen-pulsed DCs that can be readily generated *in vitro* under routine culture conditions.

Another important factor to consider when devising vaccination protocols is that interactions between DCs and T cells are bidirectional and may be influenced by factors produced within the microenvironment where DCs encounter T cells. It has been observed that DCs from different tissues induce T cells to produce distinct patterns of cytokines, that may reflect differences in DC maturation.[71] Signaling through the TRANCE/RANK receptor on DCs by TNF proteins expressed on activated memory T cells can promote DC survival and release of cytokines and/or chemokines such as IL-12, IL-8, MIP-1α.[72,73] The recently identified chemokine, named human macrophage-derived chemokine, is produced by DCs, and may also play a role in T cell activation by inducing an influx of DCs to T cell areas within lymph nodes.[74] Furthermore, DCs have been shown to produce IL-12, a cytokine that is important for T helper cell development and T cell production of Th1 cytokines such as IFNγ.[75] Thus, depending on conditions within the microenvironment, DCs may stimulate a certain T cell subset(s) resulting in a multitude of immune outcomes, including the induction of a tolerant state.

As noted earlier, DC subsets have been identified that are phenotypically distinct. Myeloid DCs (DC1) are CD11c+, $CD4^{low}$, express high GM-CSF and low IL-3 receptor levels, and prime T cells to produce Th1 type cytokines such as IFNγ.[6,76] Lymphoid DCs (DC2) express the opposite phenotype and induce T cells to produce Th2 type cytokines such as IL-4.[6,76] Interestingly, treatment of donors with G-CSF increases the number of DC2 in pheresis products by a full log without altering the number of DC1.[76] Todd *et al* examined various mobilization strategies and found that treatment of normal donors with G-CSF, GM-CSF or a combination of G-CSF and GM-CSF resulted in a 2.5–3 fold increase in DCs in the pheresis product.[77] However, only GM-CSF or a combination of GM-CSF and G-CSF induced the activation marker CD80 to be expressed by DCs.[77] The significance of this finding in relation to graft versus host disease in allogeneic transplants remains to be established. From these studies and others, it is becoming increasingly clear that DCs are extremely diverse cells whose development can be manipulated, both *in vivo* and *in vitro*, setting the stage for immune therapies using DCs in order to augment T cell responses against various pathogens and tumor-associated antigens.

Vaccination strategy for multiple myeloma

Multiple myeloma is a B cell malignancy that cannot be cured with currently available therapies. Repeated cycles of high-dose chemotherapy and autologous stem cell support have progressively increased complete remission rates, event-free survival, and overall survival.[7,78] Unfortunately, relapse remains a problem and is most likely due to tumor cells that survive the chemotherapy. Thus, to maintain long-term complete remission, and possibly a cure, it is necessary to elicit an *in vivo* immune response that can effectively eradicate residual tumor cells following cytoreduction.

Idiotype as a target for immunotherapy

Multiple myeloma is a clonal B cell malignancy that produces a single monoclonal immunoglobulin with a unique heavy and light chain variable region, that can serve as a specific tumor cell marker.[1] This tumor-specific antigen, called Id protein, can be isolated from the plasma of myeloma patients and used to elicit *in vivo* antigen-specific immune responses. *In vitro* studies have shown that T cells from the peripheral blood of myeloma patients proliferate and produce cytokines in response to autologous Id protein, but not against isotype-matched allogeneic Id protein.[79] Interestingly, the highest T cell responses were observed using $F(ab')_2$ and heavy chain fragments.[79] These findings indicate that myeloma Id protein is immunogenic and can elicit immune responses *in vivo*. Although myeloma cells do not express Id protein on their surface, clonally related B cell populations expressing surface Id are present, and eradication of these myeloma cell precursors may be critical for successful treatment of minimal residual disease.[80]

Various vaccination strategies using Id protein isolated from myeloma patients have been investigated in

the clinical setting. Kwak *et al* successfully elicited an Id-specific immune response in a myeloma patient by transplanting allogeneic cells after vaccinating the donor with the patient's Id protein coupled to keyhole limpet hemocyanin (KLH).[81] More recently, a combination of autologous Id-KLH and GM-CSF was used to vaccinate 5 myeloma patients that were in remission after autologous transplantation. All 5 patients displayed both an *in vitro* antibody and T cell proliferative response to KLH. Four of the 5 patients displayed a proliferative response against Id protein and 1 patient developed an Id-specific antibody response.[82] A similar clinical study induced an Id-reactive T cell response by vaccinating myeloma patients in complete remission with Id-KLH followed by IL-2 and GM-CSF.[83,84] Additional preclinical studies are underway using both antigen-encoding DNA and noncoding nucleotides as a component of the vaccines for generating antitumor responses.[85]

A major question and potential limitation of all vaccination protocols is whether Id protein is sufficiently immunogenic for generating an *in vivo* antitumor cell response of such a magnitude that it can be identified by clinical parameters. Investigators from the Karolinska Hospital vaccinated 5 myeloma patients with autologous Id protein in the absence of a foreign carrier protein such as KLH and observed a weak *in vitro* anti-Id T cell response in 3 of 5 patients, based on production of IFNγ and IL-4. None of the 5 patients exhibited a clinical response.[86] In a subsequent trial, this same group augmented the vaccination regiment by adding GM-CSF, a cytokine that can promote *in vivo* antigen presentation by DCs.[87] In this study, all 5 patients developed a sustained Id-specific T cell response, based on IFNγ/IL-2 production, and one patient had a significant reduction in the concentration of circulating Id protein. Taken together, these studies demonstrate that Id protein is sufficiently immunogenic for generating *in vivo* T cell responses in myeloma patients.

Dendritic cells vaccination for multiple myeloma

Because DCs are extremely potent antigen-presenting cells, they may further enhance Id induced anti-tumor cell responses *in vivo* when used as part of a vaccination protocol. This approach became feasible after studies of peripheral blood from patients with multiple myeloma were shown to be suitable for generating functionally active DCs in culture. Pfeiffer *et al* found that the combination of GM-CSF, SCF, Flt-3 ligand and IL-4 could generate functionally active DCs in culture from weakly adherent mononuclear cells isolated from the blood of myeloma patients. DCs generated in this manner were phenotypically and functionally identical to DCs produced from the blood of apparently healthy donors.[88] DCs could also be generated from the blood of myeloma patients receiving chemotherapy with either melphalan/prednisone, or high-dose cyclophosphamide and G-CSF, and from patients on interferon-alpha maintenance therapy.[34,58] More recently, the CD34 $-$ cells in a leukapheresis product from a G-CSF-primed myeloma patient could be separated and used for generating functional DCs in culture, allowing the CD34 $+$ enriched fraction to be saved for peripheral blood stem cell transplantation.[89] Thus, the same leukapheresis product can be used for both peripheral blood stem cell transplantation and DC-based vaccination strategies for treatment of myeloma.

Clinical trials are currently underway using Id-pulsed DC vaccination strategies for treating patients with myeloma and are summarized in Table 3. Although these trials are in the early stages of evaluation, preliminary results are encouraging, and no adverse reactions to either intravenous or subcutaneous administration of DCs have been noted. Investigators from Stanford used Id-pulsed DCs as a vaccine to control residual disease in 12 myeloma patients following high-dose chemotherapy and autologous peripheral blood stem cell transplant.[70] Patients received two monthly intravenous infusions with freshly isolated DCs ($1-11\times10^6$ DCs per infusion) that were pulsed with autologous Id protein, followed by 5 subcutaneous vaccinations with Id-KLH. Two of the 12 patients developed an Id-specific proliferative response and 1 of 3 patients developed an Id-specific CTL response against autologous fibroblasts expressing the relevant immunoglobulin genes.[70] In another study, seven relapsed myeloma patients were immunized subcutaneously with Id peptide-pulsed DCs (1 - 3×10^7 DCs generated from CD34 $+$ peripheral blood stem cells) and GM-CSF at biweekly intervals, for a total of 3 vaccinations.[68] There was a marked increase in anti-Id antibodies after immunization, and 3 of 7 patients had a

Table 3 Clinical trials using dendritic cells to treat patients with multiple myeloma

DC precursor source	*Vaccination schedule*	*DC dose*	*Route*	*Id-specific response*	*Reference*
Mobilized PBSC	DCId×2 KLH-Id×5	$1-11\times10^6$	iv sq	2 of 12 patients	Reichardt *et al*[70]
Mobilized PBSC	DC-Id peptide×3 Id peptide×3 GM-CSF×3	$1-3\times10^7$	sq sq sq	Yes	Bohlen *et al*[68]
PBMC	DC-Id×3	?	iv	5 of 6 patients	Lim *et al*[90]
PBMC	DC-Id×1−4	$1\times10^6/m^2$	iv	2 of 15 patients	MacKenzie *et al*[92]
PBMC	DC-Id×4	?	iv	1 of 1 patient	Cull *et al*[104]

PBSC: peripheral blood stem cell; PBMC: peripheral blood mononuclear cell.

significant decrease in their serum and urine Id protein concentrations.[68] Lim *et al* vaccinated six patients (intravenously) a total of three times with Id-pulsed DCs generated from weakly adherent peripheral blood mononuclear cells. In 5 of 6 patients, an in vitro T cell proliferative response to autologous Id protein was observed, and T cells from 2 of the patients produced IFNγ in response to Id protein.[90] Although the circulating Id protein decreased by 6 g/L in 1 patient after the first vaccination, there was rapid disease progression and the Id protein increased by 12 g/L five weeks after the first vaccination.[91] Interesting, an Id-specific T cell responses was still detectable after the patient received high-dose chemotherapy for relapse, suggesting that long-lasting immune responses can be generated against Id protein that are not abrogated by chemotherapy.[91] At our institution in Little Rock, we have vaccinated 18 myeloma patients with DCs following high-dose chemotherapy and tandem peripheral blood stem cell transplants. Patients received three intravenous vaccinations with DCs two weeks apart, and received between 1 and 150×10^6 DCs at a time. Although it is too early to evaluate clinical responses, we found that patients receiving at least 30×10^6 total DCs often developed an *in vitro* anti-Id T cell response, as measured by T cell proliferation and/or cytokine production (unpublished data). Taken together, results from these studies are encouraging and additional follow-up is needed to determine the efficacy of DCs in eliciting long-term clinical responses in multiple myeloma.

A slightly different approach is currently being used in a phase I/II trial for patients with advanced, refractory multiple myeloma. In this study, dendritic cell precursors were isolated from peripheral blood of myeloma patients and cultured with autologous Id protein for 40 hours in the absence of cytokines that promote DC growth (92). There were no treatment associated adverse events and fifteen patients received 1 to 4 cell infusions at 4 week intervals. The mean number of cells infused was $1027\pm512\times10^6/m^2$ (mean$\pm$SD). *In vitro* studies revealed that 2 of 10 patients developed an anti-Id specific T cell response, suggesting that Id-pulsed DC precursor cells can induce antigen-specific immune responses in myeloma patients with refractory disease.[92]

There are several reports indicating that allogeneic graft versus myeloma (GVM) responses are achievable without myeloablative chemotherapy using donor lymphocyte infusions after allogeneic bone marrow transplantation.[93–95] The response to infusion of donor lymphocytes appears to be very strong, with a 50−60% response rate and a 30% complete response rate. Unfortunately, severe adverse effects lead to donor lymphocyte infusion-related mortality in approximately 15−20% of patients.[93,94] In view of this, various immunotherapeutic approaches have been used to induce an autologous graft versus tumor effect with the potency of an allogeneic response. Treatments such as IL-2 with/without lymphocyte-activated killer cells (LAK cells),[96,97] IFNγ and/or cyclosporine A[98] and IL-2 activated autologous grafts[99–101] have all been tried for treatment of hematological malignancies. Unfortunately, none of these approaches have produced a consistent clinical benefit. It is thought that the main limitation associated with IL-2 and/or LAK cell immunotherapy is the lack of an effective cytotoxic response that can recognize and kill target cells *in situ*. DCs might be the key component that is lacking and necessary for generating a vigorous *in vivo* anti-tumor cell response. Thus, a vaccine comprised of *in vitro* expanded DCs (either pulsed with antigen or non-

pulsed) and myeloma specific T cells might lead to the development of an *in vivo* effector cell response capable of inducing so-called autologous GVM with the effectiveness of an allogeneic GVM response without any adverse effects. At the present time it is unknown whether a correlation exists between *in vitro* parameters that are currently monitored following DC vaccination and the presence of an unequivocal clinical response. Careful monitoring of patients during and after immunotherapy is required to better understand the molecular basis of T cell mediated antitumor responses in the clinical setting.

Concluding remarks

Immunotherapy is a particularly attractive approach for improving the prognosis in multiple myeloma. In particular, strategies aimed at elimination of residual disease following high-dose chemotherapy and transplantation are desperately needed for improvement of event-free and overall survival. Unfortunately *in vitro* parameters measuring the efficacy of adoptive transfer may not always translate into improved response rates and prolongation of event-free survival. Although preclinical trials using antigen-pulsed DCs are encouraging, additional studies are needed to determine the effectiveness of this approach. Because DCs are extremely potent at presenting antigens to naive T cells, they might be the key factor in eliciting strong and long-lasting anti-tumor cell responses *in vivo*. Improvements in the generation of DCs will continue to open new avenues for immunotherapeutic strategies for treatment of multiple myeloma and other hematological malignancies. It is hoped that these new modalities of adoptive immunotherapy will result in improved clinical responses in multiple myeloma, with the ultimate goal of a cure in the near future.

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