

Role of hematopoietic growth factors/*flt3* ligand in expansion and regulation of dendritic cells

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Dendritic cells (DCs) are hematopoietic cells that initiate immune responses by presenting antigen to T cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a primary growth factor for DCs *in vitro*, but recently it was recognized that other factors including *flt3* ligand (FL) and G-CSF expand various DC subsets *in vivo*. DCs undergo a complex series of maturation and activation steps after they acquire antigen and before they can activate resting T cells. In addition, they must traffic to T-cell-rich areas of lymph nodes (LN) to achieve this. Each of these steps is tightly regulated, and in the last year progress has been made in identifying some of the key molecules involved in each of these steps. This progress will further the efforts underway to develop DCs as vaccine adjuvants. *Curr Opin Hematol* 2001, 8:149–154 © 2001 Lippincott Williams & Wilkins, Inc.

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Abbreviations

APC	antigen-presenting cells
BM	bone marrow
CCR	CC chemokine receptor
DC	dendritic cell
GM-CSF	granulocyte macrophage colony-stimulating factor
GMP	good manufacturing practices
IL	interleukin
LC	Langerhans cells
LN	lymph nodes
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MIP3	macrophage-inflammatory protein 3
MLR	mixed leukocyte reaction
mono-DCs	monocyte-derived dendritic cells
NK	natural killer
TLR	toll-like receptors
TRANCE	tumor necrosis factor-related activation-induced cytokine

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Dendritic cells (DCs) are antigen-presenting cells (APC) that can present antigen to and activate naive T cells, and in so doing initiate an immune response [1,2•]. DCs are a heterogeneous group of bone marrow (BM)-derived hematopoietic cells that are present at a low frequency in peripheral tissues. DCs reside at peripheral sites in an immature state. Upon exposure to antigen and pro-inflammatory cytokines, *eg*, IL-1 β and TNF- α or microbial products, *eg*, endotoxin, they take up antigen and migrate to draining LN. The antigen is processed to peptides that are presented on class I (through a process referred to as cross-presentation [3]) or class II major histocompatibility complex (MHC) molecules to T cells.

During the process of maturation molecules are upregulated that co-stimulate T-cell activation, and cytokines are produced that promote the generation of antigen-specific effector T cells. DCs are not restricted to presenting microbial antigens, and recently much attention has been focused on the ability of these powerful cells to present tumor-associated antigens to T cells, and in so doing to potentially generate effective immune responses to tumors in oncology patients [4•]. This review will highlight recent progress made in understanding the biology of these cells and in developing DCs as vaccine adjuvants for oncology and infectious disease patients.

Generating dendritic cells *in vivo* and *in vitro*

The growth factors that control the production of DCs from BM-derived progenitor cells *in vivo* are not well defined. DCs are generated *in vitro* when progenitors are cultured with granulocyte macrophage colony-stimulating factor (GM-CSF) [5] and this has become the basis of all DC culture systems. In addition, administration of soluble polyethylene-modified GM-CSF to mice expands a subset of myeloid-related DCs (CD11c⁺, CD8 α ⁺, CD11b⁺) *in vivo* [6•]. The role of GM-CSF in DC development *in vivo* is unclear, because GM-CSF knockout mice do not lack DCs and common β receptor (GM-CSF, IL-3, IL-5) knockout mice have only a small reduction in LN DCs [7]. Recently a GM-CSF-independent culture system was described. After culture for 7 to 9 days in the presence of FL alone murine BM cells develop into DCs [8•], in agreement with the ability of FL to induce DC expansion *in vivo* [9]. Endogenous IL-6 but not GM-CSF is required in the culture. FL knockout

mice have reduced numbers of myeloid-related and lymphoid-related (CD11c⁺, CD8 α ⁺, CD11b⁻) DCs in the spleen, LN, and thymus, but Langerhans cell (LC) (DCs present in the epithelial layers) numbers are normal [10•]. Conversely TGF- β knockout mice lack LC but not other DCs [11].

Little is known about the signals that attract DC and LC precursors from the BM to their respective sites. A recent report suggests that macrophage-inflammatory protein 3 α (MIP3 α) specifically recruits LC precursors, which express the MIP3 α receptor CC chemokine receptor 6 (CCR6), to the epithelia [12•,13•]. In addition, the DC molecule DC-SIGN (CD209) may be involved with trafficking of immature DCs from the peripheral blood (PB) to the tissues [14•].

In humans DCs represent less than 1% of PB mononuclear cells, making the study of these cells difficult. Human DCs can be generated *in vitro* from CD34⁺ progenitors or from PB monocytes [15]. When PB monocytes that are CD14⁺ are cultured with GM-CSF plus IL-4 for 7 days, the cells become CD14⁺, Class II⁺, CD11c⁺ and have the functional properties of DCs. Large numbers of relatively immature monocyte-derived DCs (mono-DCs) are generated this way, are responsive to activating signals like TNF- α , CD40L, IFN γ and microbial products, and can be pulsed with protein or peptide antigens. A systematic study to optimize the production of mono-DCs under good manufacturing practices (GMP) conditions was described. It was concluded that GM-CSF+IL-4-cultured monocytes could be optimally matured in the absence of fetal bovine serum with TNF- α + IL-1 β + IL-6 + prostaglandinE2, loaded with antigens (proteins or peptides), and then cryopreserved for use as vaccines [16].

Success has been reported in the last year at generating large numbers of DCs *in vivo* by administering hematopoietic cytokines to healthy human volunteers. Administration of FL led to a 40- to 50-fold expansion of CD11c⁺, MHC Class II⁺, IL-3R α ⁺ (CD11c⁺) DCs in the PB resulting in DC counts of 0.9 to 1.8 $\times 10^6$ /mL [17•,18•]. The FL-DCs are immature, (CD80⁻, CD83⁻, CD86^{low}), and can acquire protein antigen, process it, and present it to naive and memory T cells. Because DC expansion takes 9 to 10 days of daily administration before maximal increases are observed, it is likely that the expansion is being driven by progenitor cells and does not represent mobilization of DC already present at peripheral sites. In humans, in addition to CD11c⁺ DCs (DC1), a second less-frequent subset has been described that contains DC precursors (DC2), also referred to as plasmacytoid T cells or plasmacytoid monocytes [19]. This cell expresses MHC Class II, CD40, CD4, and high levels of the IL-3R α (CD123)

chain, but not CD11c or CD8 α . The counterpart to this cell in the mouse has not been described. The CD11c⁻, IL-3R α ⁺ DCs are expanded 12- to 13-fold *in vivo* by FL to 1.9 to 3.9 $\times 10^5$ /mL [17•,18•]. Administration of G-CSF to humans also increases the number of CD11c⁻, IL-3R α ⁺ DCs, but not CD11c⁺ DCs in the PB [17•,20•]. After 5 days of G-CSF administration the CD11c⁻, IL-3R α ⁺ DCs are expanded 5-to 7-fold to 0.2 to 2.1 $\times 10^5$ /mL. Without activation these cells do not stimulate allogeneic T-cell proliferation in a mixed leukocyte reaction (MLR), but upon stimulation with TNF α [20•] or CD40L + IL-3 [17•] they acquire this APC function. The T cells that are activated appear to be skewed toward a T helper 2 (Th2) phenotype, as previously described for this DC subset [21], producing increased amounts of IL-10 and decreased amounts of IFN γ . Conversely the T cells activated by the CD11c⁺ DCs are skewed toward a Th1 phenotype, producing IFN γ and decreased amounts of IL-10 [17•]. However, both subsets induce production of the Th2 cytokine IL-4. The role of specific DC subsets controlling Th skewing is controversial. There are reports showing that the ratio of CD11c⁺ DCs to T cells [22], the different subsets within the CD11c⁺ DCs [23], and environmental influences [24] can influence the outcome.

Circulating DCs are expanded in cancer patients treated with a combination of low-dose GM-CSF plus IL-4 [25•]. DCs identified as MHC Class II⁺, CD83⁺ increased to 4.5 $\times 10^5$ /mL by day 7. Conversely, administration of GM-CSF plus TNF- α resulted in a modest expansion of epidermal LC but not PB DCs [26]. In both studies, GM-CSF alone had no effect on increasing DCs in the PB, in contrast to its effects in mice [6•].

Regulation of dendritic cell function: the role of microbes, cytokines, and chemokines

To initiate an immune response, DCs must respond to signals that induce uptake and processing of antigen, activation, trafficking to LN, and interaction with T cells. Immature DCs can acquire intact antigen via macropinocytosis or endocytosis, and these functions are down-regulated as DCs mature. A Rho GTPase Cdc42 may control DC endocytic activity, though precisely how this occurs is unclear [27•]. Immature DCs express aquaporins (AQP3 and AQP7), which regulate cell volume during macropinocytosis. As DCs mature, aquaporin expression is downregulated [28•]. Once antigen is acquired it undergoes processing to peptides, becomes associated with MHC class II in lysosomes, and is transported to the cell surface. Transfer of the complexes to the cell surface occurs via non-lysosomal MHC class II vesicles [29••]. DCs are activated by bacterial products *eg*, lipopolysaccharide (LPS), which bind to Toll-like receptors (TLR). Human mono-DCs express TLR-2 and TLR-3 [30,31].

Trafficking of DCs from distal sites to LN is induced by inflammation at the site, and chemokines have an important role in this process. In addition, chemokines direct the subsets of DCs to specific sites within the LN [32•]. LC express CC chemokine receptor 2 (CCR-2) and trafficking to the LN is dependent on this expression [33•], but other factors including CD40L [34•] and CpG nucleotide sequences [35] can influence trafficking. The initial interaction between DCs and resting T cells in the LN appears to be mediated by DC-SIGN on the DCs and ICAM-3 on the T cells [36••].

Dendritic cells upregulate co-stimulatory molecules and produce cytokines in response to activation by CD40L [37]. CD40L is particularly important for inducing production of the bioactive form of IL-12 (IL-12p70), which activates natural killer (NK) cells and Th cells, skewing the Th cells toward Th1 differentiation. Th cells upregulate membrane-bound CD40L upon activation, and in turn further activate DCs. In addition, CD40L activation of DCs induces production of IL-15 that augments cytolytic T-cell expansion [38]. Mono-DCs are responsive to CD40L activation *in vitro*, but produce even higher levels of IL-12p70 upon addition of IFN γ or IFN γ + LPS to CD40L [39]. *In vivo* a microbial signal is required in addition to CD40L to induce activation of DC and subsequent IL-12p70 production [40•].

A surprising role for IL-4 as a co-factor for IL-12p70 production was recently described [41–43••]. IL-4 is typically produced from Th2 cells and yet IL-12p70 favors Th1 development. Human Th2 cells interacting with mono-DCs induce IL-12p70 production, and as a result convert to a Th0/1 phenotype. IL-4 was as effective as IFN γ at acting as a cofactor for CD40L-induced production of IL-12p70 [41••]. Similarly, IL-4 is a cofactor for IL-12p70 production and down-regulates production of the antagonistic IL-12p40 homodimer *in vivo* and *in vitro* in the mouse [42••]. IL-4 induces up-regulation of co-stimulatory molecules on APC, and blocking mixed leukocyte cultures with anti-IL-4 antibodies inhibits T-cell activation. Prolonged allogeneic skin graft survival was noted in mice treated with blocking IL-4 antibodies [43••]. Collectively, the results suggest that IL-4 plays a critical role in DC activation and IL-12p70 production.

Dendritic cells as vaccine adjuvants: pre-clinical models

Numerous approaches are being taken in an effort to generate immunity to microbial or tumor antigens using DCs as antigen delivery vehicles. In a study designed to examine the potential use of the enzyme telomerase as a universal tumor antigen, mice were successfully immunized with DCs transfected with telomerase RNA

[44••]. Telomerase is typically expressed in tumor cells but not somatic tissues, making it an attractive vaccine candidate. DCs transfected with CD40L and injected intra-tumorally were also successfully used as a tumor vaccine in a mouse model [45•]. The power of this strategy was further demonstrated when mice lacking CD4⁺ Th cells were immunized with DCs transfected with CD40L and pulsed with *Pseudomonas* antigens. Mice generated humoral immunity to the bacteria, demonstrating that DCs expressing CD40L could substitute for the lack of *in vivo* T-cell help [46•]. A comparison of transfecting DCs and tumor cells with tumor antigens or cytokines and then immunizing mice led to the conclusion that DCs are more effective vaccines than tumor cells, and that additional transfection of the DCs with GM-CSF, TNF- α , or CD40L augmented tumor immunity [47]. A recently described growth factor, tumor necrosis factor-related activation-induced cytokine (TRANCE) [48], also referred to as RANK ligand [49], may improve immunization success with *ex vivo*-pulsed DCs. When DCs were cultured in the presence of TRANCE during peptide or protein pulsing, increased DC survival and trafficking to the draining LN resulted in increased immunity [50•]. Type I interferons activate mono-DCs and may also improve immunization success. Mono-DCs produce IL-15 and promote Th1 responses after stimulation with type I interferon [51•].

Although murine studies are invaluable for testing immunization strategies, they do not mimic the situation in humans where tumor growth is relatively slow and patients are often immunologically tolerant of their tumors. In an effort to model the human situation, mice transgenic for tumor antigens have been generated [52,53]. Mice transgenic and therefore tolerant for the papillomavirus E7 oncoprotein were successfully immunized with DCs pulsed with E7 peptides, demonstrating that tolerance to a 'self' tumor antigen can be broken [54].

Dendritic cells as vaccine adjuvants: clinical trials

Numerous studies have been undertaken since the first efforts to immunize patients against their tumors using DCs [4•]. For the most part they have met with limited success; however, these early trials have demonstrated that DC vaccinations are well tolerated and that few adverse reactions are associated with them. Impressive results were achieved with a group of 17 patients with metastatic renal cancer who were immunized with fusions of their tumor cells and DCs [55]. Mono-DCs expressing CD80, CD86, and CD83 generated from allogeneic healthy donors were used, serving to activate Th cells. Patients received 2 to 7 subcutaneous immunizations. With a mean follow-up

of 13 months, four of the 17 patients had a complete response and an additional three had a partial response. Eleven patients developed a delayed hypersensitivity response to challenge with their own tumor cells, demonstrating that a Th response had developed. Similarly, fusion of breast carcinoma cells to autologous mono-DCs induced CTL expansion *in vitro* whereas tumor cells alone did not [56]. Eleven patients with advanced stage IV melanoma were immunized with mono-DCs pulsed with a melanoma peptide [57•]. CTL expansion was noted in eight patients, and regression of individual metastases were noted in six. CD8⁺ T cells were associated with regressing metastases. However, no complete responses were noted.

Conclusions

Dendritic cells are present throughout the body, acting as sentinels. Upon contact with infectious agents, these cells migrate to draining LN, undergo maturation and activation, and induce naive T-cell activation, resulting in the generation of an antigen-specific T-cell response.

This powerful adjuvant activity has made them attractive as vehicles for antigen delivery for immunization of patients with cancer and infectious diseases. Unfortunately, these cells are rare in the PB. Two successful approaches are available to bypass this problem. PB monocytes can be converted to cells with DC properties after culture with GM-CSF + IL-4, or DCs can be expanded *in vivo* with FL, G-CSF, or GM-CSF + IL-4 administration. Though sufficient DCs can now be generated for use as vaccines, the next challenge will be to determine how best to use these cells to achieve successful immunization.

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This paper demonstrates the generation of CTL effector function in patients with melanoma after immunization with mono-DC pulsed with melanoma peptides. A high response rate was noted in this population of patients with advanced disease.