

## State-of-the-Art Review

# Effects of Cytokines on the Culture and Differentiation of Dendritic Cells In Vitro

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### ABSTRACT

The ability to culture dendritic cells (DC) in vitro has been integral to the dramatic increase in research in the area of immunotherapy. Over time, a number of methods for generating these cells have been developed. This article will provide an overview of the isolation and generation of DC and will give a detailed description of the role specific cytokines play in this process from the mobilization of precursors to the final maturation of DC.

### INTRODUCTION

**D**ENDRITIC CELLS (DC) are highly potent antigen-presenting cells (APC), which are integral to the initiation of T cell immunity. They have a characteristic morphology, are potent stimulators of mixed lymphocyte reactions, and can function at very low cell number compared with other APC. Dendritic cells also have unique migratory capability. These cells are bone marrow derived, but whether they are of lymphoid or myeloid lineage has long been debated (1,2). More recently, however, there has been evidence to support the existence of both DC lineages (3,4), which then depend on distinct molecular signals for their formation (5). Over the past decade, many investigators have focused on studying the development and function of DC. The ability to culture these cells in vitro has further stimulated this field of research; consequently, dendritic cell immunotherapy is being aggressively investigated as a mechanism to enhance immune responses for a variety of diseases, including malignancies. This paper will serve to provide an overview of DC isolation and generation with a focus on the role cytokines play in this process.

### CLINICAL USE OF DENDRITIC CELLS

#### *Why use them?*

Dendritic cells are considered to be the most potent APC, and therefore are well suited for utilization in vitro. Through the use of a variety of cytokines (described below), their availability in sufficient numbers and in the appropriate functional state is feasible. Dendritic cell-based therapy provides potentially numerous advantages over other developments in treatment of chronic infections and a variety of malignancies. For instance, several antigens have recently become targets of monoclonal antibodies that are used as specifically targeted treatment options for subsets of non-Hodgkin's lymphoma and breast cancer (6–8). Although very effective, such antibody-based therapy means passive immunity with a definite half-life of the molecule making repeated infusions necessary, sometimes until progression of disease. Such repeated infusions are associated with continuing inconvenience to the patient, potential side effects, and complications, as well as substantial cost. The theoretical advantage of DC-based therapy over the use of antibodies

is the induction of cellular-based and active immune response with potentially better cytotoxicity and sustained effector cell effect. Finally, when tailored to the autologous tumor antigen, it has the potential to lack any cross immunity to normal tissues, with tissue specificity exerting reduced toxicity.

### *Possible uses in cancer*

A number of human cancers carry a specific and unique antigen pattern on the cell surface, e.g., CD19 and CD20 in B cell lymphomas, CD5 in some T cell lymphomas and chronic B cell leukemia, human epidermal growth factor receptor-2 (HER-2) in one-third of breast cancers, and carcinoembryonic antigen (CEA) in many adenocarcinomas, like gastrointestinal cancers. Ideally, no normal tissues should express any of the potential targeted antigens, so no tissue damage occurs. This is the case for some (e.g., HER-2) but not other antigens (e.g., CD20). Because a sustained and effective immunization is the goal of any immunotherapy, the choice of appropriate antigen(s) and a corresponding malignancy is paramount for success. Moreover, it seems that at time of minimal residual disease (MRD) (e.g., in a state of clinical complete remission) immunotherapy has the best potential to be effective. Therefore, it seems logical, to explore this approach in patients who undergo high-dose chemotherapy (HDCT) and hematopoietic cell transplantation for two reasons: (1) after such treatment, many patients are in remission and therefore only MRD needs to be targeted by the immunotherapy; (2) moreover, the bone marrow aspirate or apheresis performed prior to the HDCT gives the investigator an easy access to the appropriate cell populations from which autologous DC can be generated. In certain circumstances, the apheresis product (AP) or bone marrow cell suspension contains autologous malignant cells that actually will become the target of the DC-based vaccine and can be used as autologous antigen.

### *Where/when to get DC?*

In the last few years, many potential sources of DC have been identified: peripheral blood, bone marrow, lung, liver, cord blood, tonsils and adenoids, and skin (9–12). More recently, cytokine- and/or chemotherapy-mobilized peripheral blood has been used for hematopoietic rescue after HDCT for a variety of diseases. The collection by apheresis makes this source of hematopoietic cells readily available. It has been shown that a subset of the AP, the CD34<sup>+</sup> cells, are an excellent source of precursor cell that can be driven into differentiation of a variety of cells. Expansion of CD34<sup>+</sup> in vitro, maturation of the CD34<sup>+</sup> population, and cultivation of DC are only a few examples of “graft engineering” (13,14). In our

own laboratory, we have shown that induction of differentiation into functional granulocytes with successful infusion to patients was possible (15), and, more recently, generation of DC can successfully be performed with better yields than unmobilized peripheral blood (16). In our hands we observed consistently greater yields of monocyte-derived DC from mobilized granulocyte colony-stimulating factor (G-CSF) as opposed to unmobilized peripheral blood (16). Therefore, our data and reports from other groups indicate that the AP may be a very convenient (as opposed to bone marrow) and effective (as opposed to unmobilized peripheral blood) source of DC for in vitro cultivation and differentiation (16,17).

## **HISTORICAL OVERVIEW**

Historically, DC were difficult cells to investigate. Studies were restricted by the low frequency of these cells (they account for less than 0.1% of blood leukocytes (18), and the lack of a truly specific DC marker. A variety of isolation/enrichment techniques have been developed to allow for assessment of these cells. These include the use of physical separation techniques, which base isolation upon adherence and/or buoyancy (18,19), and rigorous immunodepletion procedures (20–23). Although such freshly isolated DC allow for the examination of the DC population actually present in vivo, the paucity of DC in peripheral blood makes such isolations labor intensive and the yield poor, thereby limiting experimental investigations. Early observations suggested that DC could be generated in vitro by exploiting appropriate “progenitor” cells. Peters and colleagues were the first to describe monocyte differentiation into dendritic-like cells (24,25).

## **CYTOKINE-DRIVEN DC GENERATION**

Cellular manipulation with cytokines has made it possible to generate pure DC in sizeable numbers, although it is still unknown whether such cytokine-driven ontogeny occurs in vivo. Two general techniques for ex vivo expansion of DC exist. Central to such protocols is the careful selection of a cocktail of cytokines and the source of initiating cells. The first method uses CD34<sup>+</sup> cells to generate DC. CD34 is a surface protein expressed on early lympho-hematopoietic stem and progenitor cells. The CD34<sup>+</sup> cell population contains most myeloid and lymphoid progenitors. When these cells from bone marrow, cord blood, or peripheral blood are cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) cells with DC morphology, phenotype and function are produced (26–28). In a second method, a cocktail of GM-CSF and inter-

leukin-4 (IL-4) is used to develop DC from circulating monocytes (14,28). Both techniques generate large numbers of functional DC. Some studies suggest that a lower outgrowth of cells occurs when monocytes are used as compared with protocols that use CD34<sup>+</sup> cells. Other investigations suggest that monocyte-derived DC have weaker expression of co-stimulatory molecules such as intercellular cell adhesion molecule-1 (ICAM-1), B7-1, and B7-2 compared with CD34<sup>+</sup>-derived DC (29,30). Further comparisons between monocyte-derived and CD34-derived DC have been described. Monocyte-derived and CD34<sup>+</sup> derived DC have highly similar morphology, phenotype, antigen uptake, and presentation ability (31), however, monocyte-derived DC have a more mature differentiation stage. These two DC populations have also been noted to have comparable cytokine mRNA expression and expression of peptide antigen (32). Monocyte-derived DC have been described as functioning better in a mixed lymphocyte reaction (MLR) (32). Other groups, however, do not note such differences (33), or have found that CD34<sup>+</sup> derived DC are more potent (34), indicating that subtle differences in cytokine selection, concentration, and administration can have an impact on function. Ferlazzos et al. (33) has shown that CD34<sup>+</sup> derived DC have a preferential ability to activate antigen-specific CD8 T cells. Mortarini and colleagues found that CD34<sup>+</sup> and monocyte-derived DC are not functionally equivalent. CD34<sup>+</sup> derived DC were found to be required for the activation of low-frequency peptide-specific cytotoxic T lymphocytes (CTLs) in melanoma patients (30). Increases in costimulatory molecules on CD34<sup>+</sup> derived DC may contribute to their enhanced function (34). Monocyte-derived and CD34<sup>+</sup> derived DC are also described as being similar when generated from cancer patients. On top of being phenotypically comparable, both populations can process and present antigen and induce CTL-dependent IFN production (33). Because CD34<sup>+</sup> cells are only found in trace numbers in peripheral and cord blood, a system exploiting monocyte differentiation is still attractive to researchers. On the other hand, many patients will be treated with HDCT and mobilized peripheral blood, which could serve as an alternative cellular source for the generation of DC. In many circumstances, patients fail to mobilize or have low CD34 counts; in such situations exploitation of monocytes and culturing of these cells may be very advantageous.

## CYTOKINES

As discussed above, central to all protocols for ex vivo expansion of DC is the use of a panel of cytokines. The remainder of this paper will examine the role of specific cytokines in DC expansion.

### *Granulocyte colony-stimulating factor*

GM-CSF is the central mediator for the generation of DC in vitro (35,36), and was one of the first cytokines identified to have an effect on DC. GM-CSF is a 20- to 30-kDa glycoprotein, which is synthesized by lymphocytes, monocytes, fibroblasts, and endothelial cells. Its major function appears to be to prolong cellular survival. It promotes the differentiation of monocytes to large macrophage-like cells, increasing their metabolism and function as APC by enhancing MHC II expression (37).

Initial studies revealed that mouse blood or marrow cultures supplemented with GM-CSF could generate DC (35,36,38). Similar studies using human peripheral blood were less successful (28). It appeared that other factors were required to produce DC successfully. Culturing monocyte-enriched blood mononuclear cells with GM-CSF induces macrophages with a different morphology and CD14/1a expression (39), but in concert with either IL-4 or TNF- $\alpha$  GM-CSF has the potential to generate a large number of DC. GM-CSF also increases the expression of CD1 on monocytes (40). Along with this role in the generation of DC, GM-CSF also promotes DC survival, and affects the morphology and viability of DC isolated from peripheral blood (41). GM-CSF has also been used to transduce autologous tumor cells for their use as vaccines (42) with the GM-CSF recruiting APC to the site of the tumor.

### *Interleukin-4*

IL-4 is an 18- to 20-kDa glycoprotein that is produced primarily by activated T cells. It is felt to be the principal factor controlling the differentiation of monocytes into DC, as monocytes cultured in the presence of IL-4 acquire a macrophage-like DC morphology (43). Upon exposure to IL-4, cells increase in size and develop extensive processes (43). In general, IL-4 appears to suppress overgrowth by macrophages (28), allowing for the generation of human DC from peripheral blood (14,28). Specifically, IL-4 inhibits macrophage colony formation and allows extensive DC growth and maturation, whereas GM-CSF alone induces the formation and survival of aggregates of DC progenitors (14,28). It appears that progenitors in peripheral blood have the ability to develop into macrophages unless their monocyte differentiation potential is suppressed by IL-4. IL-4 also reduces CD14 expression, a hallmark of differentiation into a dendritic cell (4).

Together, GM-CSF and IL-4 have the capability of inducing a large pool of potent APC from peripheral blood monocytes or adherent apheresis cells (14,28,45). Numerous groups have employed various regimens of GM-CSF and IL-4 to produce functional APC for a variety of clinical applications, most significantly cancer im-

munotherapy. Together these cytokines act to up-regulate DC properties and function. Addition of both growth factors to blood monocyte cultures induces their differentiation into immature DC. These cells, which efficiently capture and process antigen, express MHC I and II, CD1, B7 but not CD14 (14,28).

### *Tumor necrosis factor- $\alpha$*

TNF- $\alpha$  is another cytokine used to induce production of DC, but is used on CD34<sup>+</sup> cells as opposed to monocytes. It is a 17-kDa protein produced primarily by macrophages. Culturing CD34<sup>+</sup> cells in the presence of GM-CSF results in adherent and nonadherent cells. Without TNF- $\alpha$ , both these cell types differentiate into monocytes, macrophages, and granulocytes; however, under the influence of TNF- $\alpha$ , cells display DC features (26). TNF- $\alpha$  has been suggested to diminish granulocyte production, and to enhance the cell's responsiveness to GM-CSF (46,47). Addition of TNF- $\alpha$  to GM-CSF on cultures of CD34<sup>+</sup> cells resulted in a 10- to 20-fold increase of viable CD1a<sup>+</sup> DC (48). TNF- $\alpha$  also delivers a critical signal for maturation of monocytes into DC, because DC in the presence of GM-CSF and TNF- $\alpha$  showed only low-accessory cell capacity.

TNF- $\alpha$  has different effects, depending upon the initial cell population. Cord blood requires only the depletion of erythroid progenitors and the addition of GM-CSF and TNF- $\alpha$  to observe aggregates and the production of typical DC progeny (28). When blood was taken from patients receiving CSFs after chemotherapy for malignancy, GM-CSF and TNF- $\alpha$  also generated DC. In contrast, blood from healthy adult donors when exposed to the same cytokines only generated small DC aggregates, which then seemed to become monocytes. If IL-4 was used to suppress monocyte development, addition of GM-CSF led to the production of DC aggregates within 5–7 days. Thus, TNF- $\alpha$  appears to be useful, when one uses cord blood or blood obtained from patients receiving CSF therapy, for generating DC (39).

### *Granulocyte colony-stimulating factor, stem cell factor, and Flt3 ligand*

Other cytokines that have been used in DC culturing technology include G-CSF, stem cell factor (SCF), and Flt3 ligand (FL). Many investigators have focused on such cytokines to expand DC progenitors, in the hopes of exploiting the potential of traditionally rare CD34<sup>+</sup> stem cells. G-CSF and SCF are factors employed to mobilize bone marrow progenitor cells into the peripheral blood. G-CSF is used to regulate the production of neutrophils and enhances their maturation. It is widely used for patients with neutropenia after chemotherapy. More recently, G-CSF has been shown to mobilize T helper 2

preferentially, inducing dendritic cells (DC2) over those that induce T helper1 cells (49).

SCF, or c-kit ligand, enhances the mobilizing effects of G-CSF (50). Signaling through c-kit and flk2 (FL receptor) ensures the proper production or expansion of hematopoietic progenitors, and may be used to expand the CD34<sup>+</sup> progenitor pool (46,51,52). Binding of SCF to its receptor recruits progenitor cells into mitosis and renders them responsive to both the proliferative and differentiating effects of other cytokines (52,53). Szabolcs and colleagues have demonstrated that c-kit ligand enhances expansion of immunostimulatory DC from CD34<sup>+</sup> bone marrow cells when combined with GM-CSF and TNF- $\alpha$  (52). DC generated from mobilized CD34<sup>+</sup> cells are enhanced by either FL or SCF, and further enhanced by a combination of these growth factors (29,54).

FL is a member of a small family of growth factors. It stimulates proliferation of hematopoietic cells and mobilizes stem and progenitor cells to peripheral blood (55). FL can increase the absolute numbers of mature myeloid precursor-derived DC generated from cultured CD34<sup>+</sup> human bone marrow progenitors (56). Alone, it weakly stimulates proliferation of hematopoietic progenitor cells, when added with other hematopoietic growth factors it has some synergistic effects, allowing for great production of hematopoietic progenitor cells. Injection of mice with the human FLT3 ligand gene caused a substantial increase in proportion of DC in the lymph nodes and spleen (57). Many studies have examined the use of FL in conjunction with GM-CSF, IL-4, and TNF- $\alpha$  DC generation (58). Furthermore, FL appears to potentiate SCF, inducing increased proliferation of DC (59). FL, c-kit, and G-CSF all have a role in allowing for maximal production of DC.

### *Interleukin-3 and interleukin-13*

IL-3 and IL-13 have also been used in a variety of DC expansion protocols. IL-3 is a 20-kDa protein secreted by activated CD4<sup>+</sup> T cells. It has been shown to improve the viability of DC precursors (60). Activated T cells produce IL-13, which is closely linked to IL-4. Some groups have used IL-13 in concert with GM-CSF to induce monocytes into DC (61,62). IL-13 appears to share many characteristics with IL-4, and it appears that it can substitute for IL-4, resulting in DC with many of the same characteristics including extensive dendrites and high levels of MHC class II and CD86 (63,64). IL-13 has also been shown to have a role in inducing the functional maturation of monocyte-derived DC (65), as evidenced by an increase in HLA-DR, CD83, and CD86 expression, along with a down-modulation of endocytic capacity. The use of IL-13 to generate DC has also been shown to produce DC that were highly effective in an allogenic MLR and that could activate antigen-specific CD8 T cells (64).

### Interleukin 10

IL-10 is a well-described immunosuppressive cytokine. It is an 18-kDa polypeptide expressed by a variety of cells, including T lymphocytes, monocytes, and activated B cells. It has been reported to inhibit the differentiation pathway, preventing monocytes from developing DC morphology or inducing MHC class II expression (62). When DC are differentiated in the absence of IL-10, IL-10 will then cause a modest reduction in MHC class II expression, reducing antigen presentation of the cells while inducing endocytic activity (62). IL-10 has also been reported to reduce the expression of B7-1 on Langerhans cells, and B7-2 on blood DC (66,67). IL-10 can further effect DC function by inhibiting the production of cytokines such as IL-12 by these cells (68). IL-10 has also been observed to have effects on endocytic activity by down-regulating the expression of the mannose receptor (69).

### Transforming growth factor- $\beta$

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is another suppressive cytokine. This cytokine almost totally blocks DC maturation from mouse bone marrow (70). DC generated in the presence of TGF- $\beta$  are immature with respect to CD86 expression and cell shape, but TGF- $\beta$  did not appear to inhibit the commitment of progenitor cells to the DC lineage (71). This cytokine has been reported to inhibit expression of adhesion and costimulatory molecules, and reduce T cell-stimulating activity of DC (72). Both TGF- $\beta$  and TNF- $\beta$  inhibit the effects of FL (73). TGF- $\beta$  in the presence of GM-CSF and IL-4 can induce the differentiation of blood monocytes into Langerhans cells (74). TGF- $\beta$  prevents final maturation of Langerhans cells, specifically causing a reduction in CD80, CD86, and CD83 expression, resulting in a loss of antigen uptake (75).

the lymphatic system and begin their maturation process. Finally, they activate appropriate T cells in the various secondary lymphatic organs to generate potent T cell responses.

TNF- $\alpha$ - and monocyte-conditioned media are both mechanisms that have been employed to mature cultured DC (45,77). DC cultured in this fashion have an immature phenotype and can efficiently take up and process antigens; however, they are unstable. Over time, in the absence of continuous cytokines in the liquid culture, these cells lose their DC phenotype (77). Maturation of DC with monocyte-conditioned media or TNF- $\alpha$ , however, results in a stable phenotype and a potent immunostimulatory cell (45).

GM-CSF and IL-4 may help to develop DC of a more immature phenotype so one can enhance their antigen-presenting capacity, uptake, and processing. Subsequent maturation can be induced allowing for further up-regulation of their immunostimulatory capabilities. Pathogenic molecules like lipopolysaccharide (LPS) or cytokines such as TNF- $\alpha$  and GM-CSF have also been shown to induce maturation of DC (14,26,78), resulting in cells with a highly immunostimulatory phenotype.

Zhou and Tedder (79) have demonstrated that TNF can induce differentiation into mature CD83<sup>+</sup> DC, which display increased APC function. CD83 is a member of the immunoglobulin superfamily that appears to have a role in signal transduction (80). Treatment with TNF or CD40L also resulted in increased surface expression of MHC classes I and II, and B7, all characteristic of mature DC. These cells have increased T cell stimulatory capacity in a mixed lymphocyte reaction (MLR) (79). IL-13 has also been used to mature monocyte-derived DC functionally, so they are more potent in MLR, and, in concert with TNF- $\alpha$ , down-modulated endocytic capacity of the cells and induced expression of CD83 (65). Finally Jonuleit and colleagues (81) have demonstrated that

## CYTOKINE DRIVEN MATURATION

Laboratories interested in DC culture are also interested in exploiting cytokine-based technology to develop cells of the appropriate maturation state for particular applications. Dendritic cells have the ability to acquire, process, and present antigens to T cells to generate CTL and T helper responses. As DC mature, both their phenotype and function change. Progenitor, or immature, DC have increased endocytic capacity, lower MHC class II expression, and reduced immunostimulatory ability (38) as compared to their mature counterparts (14,76). DC enter the bloodstream in an immature form from the bone marrow. They then migrate into tissues whereupon, encountering a variety of danger signals, they migrate into

TABLE 1. DIFFERENCES IN DC YIELD FROM PERIPHERAL BLOOD AND MOBILIZED PERIPHERAL BLOOD IN PATIENTS WITH LYMPHOMA OR BREAST CANCER AND NORMAL VOLUNTEERS

|          | Blood                          | Apheresis                     |        |
|----------|--------------------------------|-------------------------------|--------|
| Lymphoma | 2.45 $\pm$ 0.72<br>(0.75–5.0)  | 2.06 $\pm$ 0.11<br>(1.31–3.9) | NS     |
| Breast   | 1.42 $\pm$ 0.31<br>(0.75–2.2)  | 2.53 $\pm$ 0.14<br>(2.25–2.7) | 0.036  |
| Normal   | 2.1 $\pm$ 0.34<br>(0.95–4.2)   | 4.85 $\pm$ 0.28<br>(4.5–5.4)  | 0.0013 |
| Mean     | 1.89 $\pm$ 0.24<br>(0.75–2.73) | 2.8 $\pm$ 0.34<br>(1.8–5.4)   | 0.04   |

Values are given as number of DC  $\times 10^6 \pm$  SEM. Three to nine experiments were performed for each group.

a cocktail of cytokines containing IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and prostaglandin E2 (PGE2) induces full DC maturation. Monocyte-conditioned medium that contains substantial amounts of all these cytokines has also been used to mature DC, with the advantage of meeting good manufacturing procedure (GMP) quality (82).

## OUR EXPERIENCE

In our laboratory, we have performed extensive experiments culturing DC from peripheral blood and apheresis product using adherent cells as a starting population. In our hands, standard concentrations of GM-CSF (800 U/ml) and IL-4 (1,000 U/ml) worked best at producing high yields of pure DC. Added manipulations, such as replenishing cytokines during the culture, extending incubation periods, or resting cells prior to cytokine introduction, did not enhance the yield or purity of the resultant cells. These simple techniques in T25 tissue culture flasks provided the highest yield of highly pure DC. This is very attractive, as this methodology is the least invasive and will be useful while making the protocols more clinically relevant. We also observed differences between peripheral blood-derived and AP-derived DC obtained from patients or normal donors (Table 1).

## SUMMARY

A variety of cytokines can contribute to the ex vivo generation and maturation of DC. The possibilities are numerous. Due to the advances in culturing DC and their potential in immunotherapy, a number of groups have recently reported on procedure modifications to create more clinically relevant protocols (77,82). The exact cytokine repertoire required will depend upon the specific requirements and circumstances of how the cells will be utilized.

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