



## Dendritic cells: specialized antigen presenting cells

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### ABSTRACT

Renewing interest in cancer immunotherapy reflects the excellent results that have been obtained in animal models and the promising results in early clinical trials with dendritic cell (DC) based approaches. The central role that DCs play in the initiation of an immune response raises the possibility of using them to trigger specific anti-tumor immunity. In addition, deeper knowledge of DC biology will allow better understanding of the mechanism(s) underlying allergic and autoimmune diseases as well as tolerance phenomena. These crucial issues were critically reviewed during a workshop organized by the Italian Society for Experimental Hematology in Florence, Italy, on March 18<sup>th</sup>, 1999. The chairmen have prepared this report for the readers of Haematologica.

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The induction of an efficient immune response depends on appropriate T-antigen presenting cells (APC) interaction(s), such as the ability to efficiently prime naive T-cells in order to drive clonal proliferation and functional differentiation of antigen-specific T-cells as well as to prevent antigen-specific T-cell anergy.<sup>1</sup> In this context, a growing number of tumor antigens, including tissue-specific genes, transformation related genes, and gene mutations occurring in neoplastic cells, have recently been identified as being recognized by T-lymphocytes and are capable of provoking an antitumor immune response.<sup>2,3</sup> However, tumors frequently lack the expression of co-stimulatory molecules that drive clonal expansion of T-cells, production of cytokines, and development into cytotoxic T-cells.<sup>4-6</sup>

Dendritic cells (DCs) are now recognized as being specialized or *professional* APC which play a key role in generating primary and secondary immune responses against specific antigens,<sup>1</sup> and thus provide a means of solving these challenges. DCs isolated from tumor patients<sup>7</sup> have shown functional impairment as revealed by reduced T-cell stimula-

tion capacity. As shown by Gabrilovich *et al.*,<sup>7</sup> this defect can be dependent on vascular endothelial growth factor (VEGF) production by human tumor cells which can inhibit the functional maturation of DCs. This evidence provides one of the potential explanations for the failure of the immune system to control tumor growth once the neoplastic cells have acquired the ability to block DC maturation. Nevertheless, DCs differentiated *in vitro* from patients with a tumor have been shown to be functional APCs that can be used not only for *in vitro* T-cell activation but also for *in vivo* transfer into patients after antigen loading in the attempt to boost the immune response to tumor antigens. In the human, the DC system represents a heterogeneous group of APCs differing at the level of precursor cells, factors influencing growth and maturation, phenotype, and APC function. DCs are found in virtually all tissues of the body and in the peripheral blood, where they represent 0.1% of leukocytes.<sup>8,9</sup> They capture and process antigens and migrate to lymphoid organs, the spleen and the lymph nodes, where they interact with activated antigen-specific T-cells. Recently, it has also been demonstrated that different subsets of DCs provide different cytokine microenvironments that determine the differentiation of either type-1 T-helper (T<sub>H</sub>1) or T<sub>H</sub>2 cells.<sup>10</sup> At the same time, a negative feedback loop from the mature T-helper by production of different cytokine(s) may selectively enhance or reduce DCs maturation.<sup>10</sup> In addition, the recent development of simple methods to isolate DC precursors from bone marrow,<sup>11</sup> peripheral blood<sup>12</sup> or cord blood<sup>13</sup> and the expansion of these cells *ex vivo* to yield potent APCs has enabled their clinical use in cancer immunotherapy.

### Generation of dendritic cells

Two types of DC (monocyte-derived and CD34<sup>+</sup>-derived) become the most frequently used APCs for both *in vitro* and *in vivo* studies. Most investigators have used either monocytes or CD34<sup>+</sup> progenitors cultured in the presence of growth factor cocktails. Differentiation of DCs from these precursors can be traced easily by monitoring changes in some key surface molecules such as CD1a (acquired by DCs) and CD14 (expressed by monocytes and lost by DCs). Furthermore expression of other co-stimulatory molecules such as CD40, CD80 and CD86, as well as HLA antigens, can be used to evaluate the stage of differentiation and the degree of maturation of DCs during *in vitro* culture. In this context, two new markers, CD83 and p55, have shown to be selectively

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expressed by a small subset of mature DCs differentiated *in vitro* culture conditions.<sup>14,15</sup> CD83 and p55 are uniformly expressed at high levels in mature cells but are absent from fresh blood monocytes and peripheral blood CD34<sup>+</sup> cells.

Human monocytes have generally been cultured in the presence of GM-CSF and IL-4, following the protocol originally reported by Sallusto and Lanzavecchia.<sup>16</sup> The application of this method for the generation of large amounts of functional DCs is hampered by two major obstacles. First, production of significant number of DCs is dependent on the use of fetal calf serum (FCS).<sup>17</sup> Second, when the cytokines are removed, the cells revert to an adherent and less stimulatory state.<sup>17</sup> For these reasons, several authors<sup>1,16,18</sup> have proposed a protocol for the generation of DCs, starting from peripheral blood monocytes, that includes two consecutive steps: monocytes are cultured with GM-CSF and IL-4 for 7 days and then the final maturation of DCs is obtained by the addition of TNF- $\alpha$  or CD40L that leads to increased HLA-class II and accessory molecule expression that parallels increased T-cell stimulation capacity. In addition, CD83 and p55 are expressed only at low levels by monocytes that are primed with GM-CSF and IL-4, but are upregulated following culture with TNF- $\alpha$  or CD40L. Other studies have indicated that maturation, migration and immunostimulatory capacity of monocyte-derived DCs can be promoted by cytokine cocktails containing not only TNF- $\alpha$  and IL-1 $\beta$ , but even a conditioned medium (CM) produced by culturing T-depleted blood mononuclear cells on immobilized human gammaglobulin.<sup>17,19</sup> Additional factors of the TNF family are involved in DC survival. One of these factors, TRANCE (tumor necrosis factor-related activation-induced cytokine), has been found to inhibit *in vitro* apoptosis of monocyte-derived DCs.<sup>20</sup> As TRANCE is produced by T-cells, it has been proposed that T-cell-derived factors can contribute to DC survival. It is also to be remembered that DC survival depends on T-DC interaction(s) mediated by the CD40 receptor ligand system.<sup>21</sup> Thus, both soluble factors as well as cell surface molecules on T cells contribute to DC survival. Furthermore, the T-DC interaction(s) also has a cytokine component that profoundly affects the outcome of immune responses by shifting the TH1/TH2 balance towards one or other of the two main CD4 T cell subsets. This cytokine-mediated interaction is based on opposite signals dependent on IL-12 and on IL-10.<sup>21</sup>

Data presented at the *Workshop of the Italian Society for Experimental Hematology* (SIES; held in Florence, March 18<sup>th</sup>, 1999) by Ricciardi Castagnoli *et al.* showed that DC activation induced by bacteria or by lipopolysaccharide (LPS) can be separated into two distinct processes: maturation leading to upregulation of MHC and co-stimulatory molecules, and rescue from immediate apoptosis following withdrawal of growth factors (survival). The results of this study indicate that ERK and NF- $\kappa$ B regulate different aspects of LPS-induced DC activation: ERK regulates DC survival while NF- $\kappa$ B is responsible for DC maturation.

In addition, Corinti *et al.* in the same SIES Workshop confirmed that bacteria were very effective inducers of DC maturation by upregulating the

expression of membrane molecules and reducing both phagocytic and endocytic activities. In particular, recombinant *Streptococcus gordonii* expressing the C-fragment of tetanus toxin (TTFC) on the surface was tested as an antigen delivery system for human monocyte-derived DCs. DCs incubated with recombinant *S. gordonii* were much more efficient than DCs pulsed soluble TTFC at stimulating specific CD4<sup>+</sup> T-cells. However, bacterial vectors enhanced the capacity of DCs to activate specific CD4<sup>+</sup> T-cells at concentrations that did not stimulate DC maturation.

In contrast to monocyte-derived DCs, CD34<sup>+</sup> progenitors can generate DCs when cultured with GM-CSF and TNF- $\alpha$ ,<sup>11,13,12,22</sup> a mixture allowing fully mature APCs characterized by efficient stimulatory activity for allogeneic cells to be obtained. More recently, it has clearly been shown that both stem cell factor (SCF) and FLT3 ligand are able to augment DC yield.<sup>23-26</sup> SCF probably acts by expanding the precursor cells, while FLT3 ligand by inducing DC differentiation.<sup>27</sup> Notably, these results can be achieved utilizing autologous recovery-phase serum instead of FCS. In view of the *ex vivo* manipulation of DCs for clinical use, it is essential to avoid T-cell responses to xenogenic antigens of FCS. The pool of DCs generated from CD34<sup>+</sup> progenitor cells comprises about 10% of cells that are indistinguishable from cutaneous Langerhans cells.<sup>23,28</sup>

Growth factors utilized for *ex vivo* generation of DCs have already been used *in vivo* as adjuvants for anti-cancer vaccination. The induction of immune responses to foreign proteins as well as peptides derived from a self tumor antigen is due to the activation of functional DCs. GM-CSF was proven to be effective in enhancing peptide-specific immune reactions by amplification of dermal peptide-presenting DCs.<sup>29,30</sup> However, the role played by GM-CSF as an adjuvant in peptide-pulsed vaccination is still controversial.<sup>31,32</sup> In contrast, the administration of FLT3 ligand both in animals and in humans results in a reversible accumulation of functionally active DCs in both lymphoid and non-lymphoid tissues.<sup>26,33,34</sup> In murine models it was demonstrated that FLT3 caused the regression of various tumors.<sup>35,36</sup> These data support the suggestion that DC may be directly involved in the antitumor effect of FLT3 ligand. Recently, Peron *et al.*<sup>37</sup> showed that FLT3 ligand induces a strong augmentation of natural killer (NK) cells in the spleen and blood. NK depletion significantly abrogated the antitumor effect of FLT3 ligand in the murine tumor model, suggesting that NK cell activity was necessary for the inhibition of tumor growth induced by FLT3 ligand in a model of early therapy. A similar mechanism has been observed by the same authors for the IL-12-based therapy. FLT3 ligand may act similarly to IL-12 or may stimulate IL-12 production by immune cells, in particular by DCs. The authors hypothesized that the interaction between DC and NK cells within the tumor microenvironment may also play an important role in the effector phase of antitumor immune responses.

The identification and study of subsets of CD34<sup>+</sup> cell precursors of DCs with different potential functions was discussed by Rondelli at the SIES Workshop. The data that he presented showed that constitutive expression of CD18 and rapid induction of

co-stimulatory molecules identify a subset of CD34<sup>+</sup> cells capable of presenting alloantigen, whereas progenitor cells failing to express these molecules might be exploited in allogeneic transplant settings since they contain committed and early precursors and are non-immunogenic.

### *Ex vivo* manipulation of dendritic cells

The generation of large numbers of functional DCs has allowed protocols to be designed for their clinical utilization depending on their stage of maturation. In fact, more immature DCs derived from peripheral blood monocytes might be used for the construction of RNA/DNA based-vaccines when optimal antigen processing function is needed. In contrast, mature DCs derived from CD34<sup>+</sup> progenitors or from GM-CSF + IL-4 primed-monocytes subsequently cultured with TNF- $\alpha$  may be better suited for the peptide-pulsed approach. The possibility of extending these principles to immune intervention in many human tumors has been made stronger by the recent identification of several peptides recognized in association with HLA class I antigens by autologous CTLs.<sup>2</sup> Furthermore, both synthetic peptides corresponding to known tumor antigens and tumor-eluted peptides could be used for DC-mediated antigen presentation. While synthetic peptides represent only the limited antigenic repertoire of the presently known tumor antigens, tumor-eluted peptides may contain still unknown highly immunogenic epitopes. Even though DCs can be loaded with cocktails of different peptides corresponding to different tumor antigens expressed by the same tumor, a procedure that has been shown to be clinically effective,<sup>38</sup> it is nevertheless possible that the synthetic peptide-approach will limit patient selection, on the basis of the HLA phenotype, and will prevent the possibility of activating both CD4 and CD8 T-cells directed to different epitopes of the same antigen. An alternative to the use of synthetic or tumor-eluted peptides would be the loading of DCs with tumor lysates. This procedure has been shown to be effective in mouse models as well as in one human trial of vaccination.<sup>37</sup> The principle is that proteins (or even RNA) extracted from tumor cells may be used to load DCs and lead to intra-cellular processing and presentation of tumor-derived peptides. The advantage of this approach is that it can lead to peptide presentation by both HLA class I and II antigens expressed by DCs and thus enable the activation of both CD4<sup>+</sup> helper and CD8<sup>+</sup> cytotoxic T-cells. However, a possible limitation of this approach is the complete lack of control on the nature of the antigens that are being presented by the DCs, since any cellular protein not necessarily being a tumor antigen may be processed and presented by DCs without any advantage for the anti-tumor response. In addition, the theoretical, although not yet proven, possibility exists of inducing autoimmunity against self proteins commonly expressed in several tissues.<sup>39</sup>

A further possible strategy derives from the expression of whole genes in DCs by means of appropriate expression vectors. DCs can be modified to express the whole gene coding for a tumor associated antigen (TAA), thus potentially leading to presentation of

a wider array of T-cell epitopes in comparison to the currently known peptides from each TAA. The transduction of TAA genes into DCs can be achieved by different means including viral vectors. The advantages of recombinant viral vaccines include their ability to infect a broad range of cell types, high efficiency in delivery of antigens, and induction of both humoral and cell-mediated responses.

Retrovirally transduced-DCs should be able to constitutively express and process TAA to produce long-term antigen presentation *in vivo*.<sup>40,41</sup> Limitations to the use of retroviruses include: a) difficulty of obtaining viral supernatant with a sufficiently high titer as that required for clinical applications; b) low efficiency of transduction (15-20%); c) theoretical risk of oncogenic transformation of infected DCs; d) ability to transduce only actively replicating cells.

Adenoviral vectors are another tool for efficient delivery of foreign genes into mammalian cells. These vectors are an attractive choice because they infect both replicating and non-replicating cells, are easy to handle, and allow the production of high titer supernatant. *Ex vivo* transduction of murine or human monocyte-derived DCs by the Melan-A/adenovirus vector elicited a specific CTL response against the transduced TAA<sup>42,43</sup> and several murine studies have shown that immunization with recombinant adenovirus containing a tumor antigen results in tumor rejection.<sup>44,45</sup> The use of adenoviral vectors has been limited by the potential immunogenicity of concomitant adenoviral gene expression.

Vaccinia virus vectors can efficiently infect DCs and lead to expression of reporter genes.<sup>46</sup> Vaccinia virus is a member of the poxvirus family and seems to be a good tool for TAA-gene transduction into human DCs because is not oncogenic, does not integrate into the host genome, is easy to manipulate and capable of accepting large fragments of heterologous DNA.<sup>47</sup> Recombinant vaccinia virus (Rvac) has been used for several years in experimental systems aimed at inducing a T cell response after direct virus inoculation. In phase I clinical trials, Rvac, modified to encode HPV proteins<sup>48</sup> and CEA genes,<sup>49</sup> has been injected into patients affected by cervical and colon cancer, respectively. In neither study were significant side effects observed. More interestingly, even though the patients mounted an anti-vaccinia immune response, evidence of enhanced immunity to the HPV gene products and of generation of anti-CEA CTLs was observed in some of the treated patients.<sup>47,50</sup> In agreement with initial data obtained in a phase I trial with a Rvac encoding the HIV gp160 envelope gene,<sup>51</sup> clinical trials in cancer patients have confirmed that even in the human host Rvac can be effective for the priming of T-cells directed to the inserted sequences.

A different method for *in vivo* targeting of DCs was presented by Colombo *et al.* in the SIES Workshop. The authors<sup>52</sup> demonstrated that oral vaccination of mice with an attenuated bacterial vector carrying  $\beta$ -galactosidase gene resulted in the transduction of intestinal APC. A model to deliver a gene into APC by oral administration of attenuated bacterial vectors could be developed to avoid any *ex vivo* manipulation of DC.

As investigated by Gilboa *et al.*,<sup>53,54</sup> it is possible to transfect DCs with RNA encoding antigen(s) and

subsequently induce CTL targeted against tumors. RNA can enter directly into immature DCs, but a transfecting agent, such as a cationic lipid, is necessary to transfect more mature DCs, as measured by their ability to induce CTLs *in vitro*.<sup>51</sup> Using RNA derived from tumor cells may overcome the lack of known tumor antigens. However, there may be an increased risk of autoimmune reactivity, particularly if this approach is combined with other immune-enhancing strategies such as simultaneous administration of cytokines. In fact, DCs are transfected with RNA encoding normal as well as tumor proteins.

Acquired genetic abnormalities identified in hematologic malignancies can be recognized by specific T-cells. In fact, intracellular proteins can be processed and presented on the cell surface by MHC molecules indicating the possibility that leukemia-specific genetic abnormalities may be targets for cytotoxic T-cells. Normal peptide-pulsed DCs have been used for the generation of cytotoxic, BCR-ABL-specific T cells. It has been shown that T-cells generated from a normal donor after stimulation with autologous DCs primed with a 16mer peptide spanning the b3a2 breakpoint of BCR-ABL, lysed cells from the peripheral blood of chronic myelogenous leukemia patients.<sup>55</sup> The generation of functional monocyte-derived DCs carrying the specific genetic lesion has been reported for both acute myelogenous leukemia as well as chronic myelogenous leukemia.<sup>56,57</sup> Current protocols for *ex vivo* DC generation from CD34<sup>+</sup> cells does not allow the production of large numbers of *leukemic* DCs, probably due to a defective proliferative and/or maturative capacity of transformed CD34<sup>+</sup> cells. Recently, a protocol which allows the optimal generation of BCR/ABL-positive DCs from CML-derived CD34<sup>+</sup> cells has been reported.<sup>58</sup>

DCs are also deeply implicated in the initiation or exacerbation of dangerous immune reactions, including autoimmune disorders, transplant rejection and allergic diseases. This issue was illustrated at the SIES Workshop by Girolomoni *et al.* This author has demonstrated that cutaneous DCs are intimately involved in the initiation of allergic contact dermatitis to haptens as well as in the propagation of IgE-dependent immune responses associated with atopic dermatitis. Thus, *ex vivo* generation of DCs inducing anergy instead of activation of specific lymphocytes could be helpful for the therapy of autoimmune diseases.

### Clinical studies

Initial studies have been performed in melanoma patients because melanoma is one of the few human cancers in which host immune responses can be reproducibly demonstrated. As reported by Nestle *et al.*,<sup>37</sup> vaccination with peptide- or tumor lysate-loaded DCs led not only to positive delayed type hypersensitivity to peptide-loaded DCs in 11/15 patients but also to objective responses in 5/16 evaluable patients. Responses included regression of metastases in skin, soft tissue, lung and pancreas, suggesting that DC-mediated vaccination can lead to a systemic response effective in inducing tumor regressions at lesions in several organs. By contrast administration of melanoma peptides, without DCs, failed to elicit clinical-

ly significant responses. These were observed only when high doses of IL-12 were added to the peptide vaccination.<sup>59</sup>

The vaccination approach by peptide-pulsed DCs has also been attempted in other neoplastic diseases, such as non-Hodgkin's B cell lymphoma (NHL), prostatic cancer and multiple myeloma.<sup>60-62</sup> Hsu *et al.* demonstrated that vaccination of NHL patients with DCs loaded with the tumor-specific idiotype protein led to objective tumor regressions including one complete response.<sup>58</sup> A phase I clinical study was performed in 51 patients with hormone-refractory prostatic cancer to evaluate the efficacy of vaccination with autologous DCs pulsed with prostate specific membrane antigen (PSA) peptides.<sup>59</sup> Seven partial responses were observed. By contrast, in the control group treated by injection of peptides alone, no tumor regressions were observed. Recently, a feasibility study of DC-based anti-idiotype vaccination after autologous peripheral blood stem cell transplantation in multiple myeloma patients was performed by Reichardt *et al.*<sup>60</sup> This study showed that, despite high-dose chemotherapy, patients mounted a strong anti-keyhole limpet hemocyanin (KLH) immune response, and more important few patients also showed an anti-idiotype CTL-response.

The clinical efficacy of cancer therapy by vaccination with tumor antigen-loaded APC may be improved by understanding whether tumor growth is associated with development of immunity to tumor peptides and whether this leads to evidence of T-cell-mediated tumor regression in metastatic lesions. This important issue was discussed by Anichini *et al.* during the Florence SIES Workshop. Anichini *et al.* evaluated the frequency of cytotoxic T-cell precursors (CTLp) against a Melan-A/Mart-1 peptide in the blood of patients with metastatic melanoma. They found that some patients possessed a high frequency of CTLp within the memory (CD45RO<sup>+</sup>) subset, while others only possessed a low frequency repertoire in the naive (CD45RA<sup>+</sup>) T-cell subset. In the latter group of patients, professional APC (DCs) were necessary for T-cell activation, since no Melan-A/Mart-1-specific CTL could be generated when using non-professional APCs, such as monocytes. The observation of Anichini *et al.* is relevant because knowledge about the immune status of a patient may facilitate the choice of tumor antigens to be used in vaccination attempts employing DCs, as patients with a higher percentage of CTLp will generate higher numbers of specific CTL.

In conclusion, despite a large body of evidence strongly supporting the existence of DC-mediated antitumor effects, many issues related to DCs need to be understood in more detail to allow successful manipulation of the immune system.<sup>63-65</sup> In particular, the role of different DC subsets, including their effects on B-cells and tolerance should be elucidated. In fact, besides the role of DCs in anti-cancer immunity, emerging evidence points towards a role for DCs in central and peripheral tolerance phenomena. It is, therefore, essential to pursue basic research on DCs more vigorously in the fields of allergy, transplantation and autoimmunity.

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