

blood

2002 100: 230-237
doi:10.1182/blood.V100.1.230

Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6

Marina Ratta, Francesco Fagnoni, Antonio Curti, Rosanna Vescovini, Paolo Sansoni, Barbara Oliviero, Miriam Fogli, Elisa Ferri, Gioacchino Robustelli Della Cuna, Sante Tura, Michele Baccarani and Roberto M. Lemoli

Updated information and services can be found at:

<http://bloodjournal.hematologylibrary.org/cgi/content/full/100/1/230>

Articles on similar topics may be found in the following *Blood* collections:

[Immunobiology](#) (4005 articles)

[Neoplasia](#) (4217 articles)

[Immunotherapy](#) (577 articles)

Information about reproducing this article in parts or in its entirety may be found online at:

http://bloodjournal.hematologylibrary.org/misc/rights.dtl#repub_requests

Information about ordering reprints may be found online at:

<http://bloodjournal.hematologylibrary.org/misc/rights.dtl#reprints>

Information about subscriptions and ASH membership may be found online at:

<http://bloodjournal.hematologylibrary.org/subscriptions/index.dtl>

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

Copyright 2007 by The American Society of Hematology; all rights reserved.



Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6

Marina Ratta, Francesco Fagnoni, Antonio Curti, Rosanna Vescovini, Paolo Sansoni, Barbara Oliviero, Miriam Fogli, Elisa Ferri, Gioacchino Robustelli Della Cuna, Sante Tura, Michele Baccarani, and Roberto M. Lemoli

We studied concentration, phenotype, and function of peripheral blood (PB) dendritic cells (DCs) from patients with multiple myeloma (MM). The absolute number of circulating precursors of myeloid and plasmacytoid DCs was significantly lower in MM patients than in healthy subjects. After maturation, PBDCs from MM patients showed significantly lower expression of HLA-DR, CD40, and CD80 antigens and impaired induction of allogeneic T-cell proliferation compared with controls. Remarkably, they were not capable of presenting the patient-specific tumor idiotype to autologous T cells. Conversely, DCs generated in vitro from CD14⁺ monocytes from the same

patients, and PBDCs freshly isolated from healthy donors efficiently stimulated allogeneic and autologous T cells. To clarify the mechanism of PBDC deficiency in MM, we investigated the effects of the main plasma cell growth factor, interleukin-6 (IL-6), on the development of DCs from CD34⁺ cells. IL-6 inhibited the colony growth of CD34⁺ DC progenitors and switched the commitment of CD34⁺ cells from DCs to CD14⁺ CD1a⁻ CD86⁻ CD80⁻ CD40⁺HLA-DR[±] monocytic cells exerting potent phagocytic activity but no antigen-presentation capacity. This effect was reversed by anti-IL-6 antibodies. Growing CD34⁺ cells in the presence of autolo-

gous serum (without IL-6) also suppressed the development of functional DCs. This study demonstrates that PBDCs from MM patients are functionally defective, partially because of IL-6-mediated inhibition of development. This brings into question the advisability of using PBDCs as antigen carriers for immunotherapy trials in MM. The results also suggest a novel mechanism whereby myeloma cells escape immune recognition. (Blood. 2002;100:230-237)

© 2002 by The American Society of Hematology

Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by the expansion of plasma cells and late B cells that are clonally committed to the production and secretion of a specific immunoglobulin that can be considered the tumor-associated antigen. Among the cytokines involved in the proliferation and differentiation of myeloma cells, interleukin-6 (IL-6) is widely recognized as the major plasma cell growth factor,¹ acting through paracrine and autocrine growth-stimulation mechanisms.^{2,3} High levels of serum IL-6 correlate with aggressive disease and poor prognosis not only in MM^{4,5} but also in renal cell carcinoma, melanoma, and colorectal cancer.⁶⁻⁸ Based on the pivotal role of IL-6 in the pathogenesis of MM, therapeutic administration of anti-IL-6 antibodies has been proposed for patients with advanced disease.⁹

Among professional antigen-presenting cells (APCs), dendritic cells (DCs) are the most potent stimulators of T-cell responses, and a growing body of evidence indicates that they play a crucial role in antitumor immunity (reviewed in reference 10). Renal cell carcinoma cell lines inhibit the differentiation of hematopoietic CD34⁺ cells into DCs through an IL-6-mediated mechanism.¹¹ In addition, IL-6 switches the differentiation of monocytes from DCs to

macrophages.^{12,13} Thus, similar to what happens with vascular endothelial growth factor (VEGF),¹⁴ the overproduction of IL-6 may provide neoplastic cells with a mechanism to inhibit antitumor immune responses by suppressing the development and functional maturation of DCs. Phenotypic and functional analysis of peripheral blood (PB) DCs from MM patients has never been performed. This information could be of particular relevance because autologous circulating DCs are used to induce an anti-idiotype (Id)-specific T-cell response in myeloma and lymphoma patients.^{15,16}

Herein, we demonstrate that the number, phenotype, function, and development of DCs are significantly altered in patients with MM. This finding can be partly explained by tumoral overproduction of IL-6. In fact, IL-6 inhibited the colony-forming unit DC (CFU-DC) growth of CD34⁺ cells, redirecting the differentiation of hematopoietic progenitor cells toward the monocyte-macrophage lineage. Thus, the results presented here indicate that the administration of PBDCs may not be suitable for clinical trials on MM. Furthermore, our data help to elucidate one of the potential mechanisms by which myeloma cells escape immune recognition through the IL-6-mediated inhibition of DCs.

From the Institute of Hematology and Medical Oncology, "Lorenzo e Ariosto Seràgnoli," University of Bologna; the Department of Internal Medicine, University of Parma; and S. Maugeri Foundation, Istituto di Ricerca e Cura a Carattere Scientifico (IRCCS), Clinica del Lavoro e della Riabilitazione, Scientific Institute of Pavia, Italy.

Submitted September 20, 2001; accepted February 20, 2002.

Supported by Italian Association for the Research against Cancer, Milan, Italy, Ministero della Università e della Ricerca Scientifica (MURST) (ex 40%), and Consiglio Nazionale delle Ricerche (CNR) (N. 00.00118-ST97).

M.R. and F.F. contributed equally to this paper.

Reprints: Roberto M. Lemoli, Institute of Hematology and Medical Oncology Lorenzo e Ariosto Seràgnoli, Via Massarenti, 9 Bologna, Italy; e-mail: rmllemoli@med.unibo.it.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2002 by The American Society of Hematology

Materials and methods

Purification of CD34⁺, CD3⁺, and CD14⁺ cells

Steady-state or granulocyte-colony-stimulating factor (G-CSF)-mobilized PB mononuclear cells (MNCs) were obtained by gradient centrifugation (Lymphoprep; 1.077 g/mL; Nycomed Pharma, Oslo, Norway) from 25 MM patients and 17 healthy adults. Seven of 25 patients were studied at diagnosis, whereas 17 patients were studied during the course of the disease. Light-density cells were washed twice in phosphate-buffered saline (PBS) with 1% bovine serum albumin (Sigma, St Louis, MO), and CD34⁺, CD3⁺, or CD14⁺ cells were highly purified from MNC fraction by MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.¹⁷ In particular, CD34⁺ cells were obtained from G-CSF-treated patients undergoing PB stem cell collection, whereas CD14⁺ cells were selected from steady state MNCs and were used to generate DCs for comparative studies (see below). Flow cytometric reanalysis of purified cell fractions was performed on a gated population set on scatter properties using FACScan equipment (Becton Dickinson, San Jose, CA) (see below and reference 17). A minimum of 10 000 events was collected in list mode on FACScan software. The purity of the enriched populations was always greater than 90%.

Generation of DCs in liquid culture from CD34⁺ progenitor cells

Suspension cultures of CD34⁺ cells were initiated with Iscove modified Dulbecco medium (IMDM) supplemented with 20% fetal calf serum (FCS; Sera Lab, Crawley Down, Sussex, United Kingdom), L-glutamine, and antibiotics at an initial density of 4×10^4 cells/mL, as previously reported.^{18,19} All cultures were maintained at 37°C in humidified 5% CO₂ atmosphere for 12 to 14 days in the presence of optimized concentrations of the following rh-cytokines: tumor necrosis factor (TNF)- α (10 ng/mL; Innogenetics, Zwijndrecht, Belgium); granulocyte macrophage-colony-stimulating factor (GM-CSF; 50 ng/mL; Novartis, Basel, Switzerland); stem cell factor (SCF; 20 ng/mL; Amgen, Thousand Oaks, CA); and FLT3-L (50 ng/mL; Immunex, Seattle, WA). After 1 week, half the culture medium was replaced by fresh medium and growth factors with IL-4 (50 ng/mL; Schering Plough, Kenilworth, NJ) replacing SCF and FLT3-L.¹⁸ In selected experiments, autologous or AB serum was used in place of FCS.

When indicated, IL-6 (25 ng/mL; Endogen, Woburn, MA) or VEGF (25 ng/mL; R&D Systems, Abingdon, United Kingdom) was added either at the beginning of the culture or at day +7. The concentration of the cytokines was chosen based on a dose-response curve (1-100 ng/mL; data not shown) and on previously reported results.¹⁰ In neutralization experiments, specific anti-IL-6 antibody (5 μ g/mL; Bender MedSystem, Vienna, Austria) or matched-isotype control immunoglobulin was added to the culture.

Generation of DCs from CD14⁺ monocyte precursors

DCs were also generated from PB CD14⁺ cells as previously described.¹⁷ Briefly, 1×10^6 purified CD14⁺ cells were cultured for 5 to 7 days in 1 mL RPMI 1640 supplemented with 10% FCS (Sera Lab), antibiotics, L-glutamine, and 50 ng/mL GM-CSF (Novartis) and IL-4 (Schering Plough). TNF- α (25 ng/mL; Innogenetics) was added to the culture for 24 to 36 hours to induce terminal maturation of DCs.¹⁷ Cultures were maintained at 37°C in 5% CO₂ by replacing the culture medium and cytokines at day +3.

Enrichment of circulating DCs

Mature PBDCs were isolated from steady-state PB from MM patients and healthy donors without the addition of exogenous cytokines, as previously reported,^{16,20-22} with minor modifications. MNCs were obtained by gradient centrifugation (Lymphoprep) and were resuspended in RPMI 1640 with 10% human AB serum or autologous serum. Light-density cells were then incubated overnight (12-24 hours) at 37°C in 10% CO₂, and PBDCs were separated from high-density lymphocytes and most monocytes by sequen-

tial centrifugation over 15% and 14% (wt/vol) metrizamide hypertonic gradients (Sigma). The purity of each enriched PBDC cell preparation, obtained in parallel from patients and control subjects after 2 metrizamide hypertonic gradients and to be tested in T-cell proliferative assays, ranged between 30% and 60%, as evaluated by flow cytometry. The yield was $0.4\% \pm 0.3\%$ of the initial number of nucleated cells. In terms of purity and overall recovery, we did not observe any differences between samples from healthy donors and MM patients. Contaminating cells in enriched PBDC preparations were mainly (more than 90%) activated CD14⁺ monocytes whose contribution to T-cell proliferation induced by PBDCs was reported to be marginal.²⁰⁻²² The total number of stimulators used for inducing the proliferation of T cells was adjusted according to the purity of each PBDC preparation to have a fixed final number of PBDCs in each experiment, as indicated in the figure legends.

Short-term colony assays

Hematopoietic CD34⁺ cells were cultured in semisolid medium as previously described.^{18,19} Briefly, 2000 to 5000 cells were plated in duplicate in culture medium consisting of 1 mL IMDM supplemented with 30% FCS, 10^{-5} M 2-ME (Sigma), and 0.2 mM bovine hemin (Sigma). The final concentration of methylcellulose was 1.1%. Cytokines were added as follows: 10 ng/mL TNF- α ; 50 ng/mL GM-CSF; and 20 ng/mL SCF. CFU-DCs and macrophage CFUs (CFU-M) were recorded as aggregates larger than 50 cells, after 12 to 14 days of incubation at 37°C in a fully humidified 5% CO₂ atmosphere. To confirm the dendritic origin of scored colonies, individual aggregates were plucked from methylcellulose under direct inspection by inverse-phase microscopy. Cells were then resuspended in IMDM-10% FCS, washed twice in the same medium, and cytocentrifuged onto glass slides. Morphology was assessed by May-Grünwald-Giemsa staining.^{18,19}

Flow cytometry analysis

Monoclonal antibodies (mAbs) used in this study for cell surface staining were purchased as directly conjugated either to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP). The following mAbs were purchased from Becton Dickinson: anti-CD80 FITC and PE (L307.4, IgG1); anti-CD123 PE (9F5, IgG1); anti-HLA-DR FITC, PE, and PerCP (L243, IgG2a); and anti-CD34 FITC (anti-HPCA-2). A cocktail of FITC-conjugated mAbs against common lineage markers such as anti-CD3, -CD14, -CD16, -CD19, -CD20, and -CD56 was also purchased from Becton Dickinson. The following mAbs were purchased from Pharmingen (San Diego, CA): anti-CD3 PE (UCHT1, IgG1); anti-CD14 FITC and PE (M5E2, IgG2a); anti-CD16 FITC and PE 3G8, IgG1; anti-CD19 PE (B43, IgG1); anti-CD40 FITC; and anti-CD86 FITC and PE (IT2.2, IgG2b). The following mAbs were purchased from Caltag Laboratories (San Francisco, CA): anti-CD40 PE (14G7, IgM) and anti-HLA class I FITC (TU149, IgG2a). The following mAbs were purchased from Coulter Immunology (Hialeah, FL): anti-CD33 PE (D3HL60.251, IgG1); anti-CD1a PE (T6-RD1, IgG2b); and anti-CD83 PE (HB15, IgG2b). Isotypic controls of irrelevant specificity were purchased from each company and were used for setting limits of nonspecific immunoglobulin cell binding.

Flow cytometry was performed using a FACScan or a FACScalibur flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser and operated with Lysis II or with CellQuest software, respectively (Becton Dickinson). Instrument setting and compensation were adjusted by the acquisition of leukocytes stained by each mAb individually and also by running cells double or triple stained with distinct single, bright positive controls. Limits of negative controls were defined by staining with isotype-matched controls. Viable nucleated cells were gated, acquired, and stored according to linear forward light scatter versus linear side light scatter parameters. For cell-surface staining of PB myeloid and plasmacytoid DCs, 100 μ L EDTA-containing blood freshly drawn was stained by adding 1 μ g mAb per million leukocytes. After 20 to 30 minutes of incubation on ice, red-cell lysis was obtained by adding 2 mL FACS lysing solution (Becton Dickinson) to samples, which then were left for 10 minutes at room temperature in the dark according to the manufacturer's instruction. After 2 centrifugations for 7 minutes \times 400g at 10°C to 12°C

Table 1. Numbers of circulating monocytes and DCs in MM patients and healthy donors

Subjects	WBC	Monocytes	Myeloid DC	Plasmacytoid DC
MM patients at diagnosis (n = 7)	7328 ± 2000*	304 ± 95	4.7 ± 4.2	3.7 ± 3.8
MM patients with advanced disease (n = 18)	7150 ± 3200	284 ± 130	8.9 ± 6.2	3.1 ± 2.9
Normal donors	6552 ± 2300	391.1 ± 94.1†	17 ± 6.3†	12.1 ± 3.2†

Twenty-five MM patients and 17 healthy subjects were analyzed as described in "Materials and methods."

*Results are expressed as the mean number of cells ± SD/ μ L of whole PB.

†Data from the 3 groups were considered significantly different when $P < .05$. $P = .011$ for monocytes, .003 for myeloid DCs, and .0002 for plasmacytoid DCs when healthy donors were compared with MM patients either at diagnosis or with advanced disease. No differences were found when the two groups of patients were compared.

with washing buffer, cells were resuspended in FACS flow (Becton Dickinson) and were finally acquired by the flow cytometer. Percentages and absolute numbers of different populations were obtained as previously described.²³

For staining of cultured DCs, cells were resuspended in PBS containing 1% FCS, 1% human serum, 10% mouse serum, and 0.01% sodium azide and then were stained with combinations of saturating amounts of fluorochrome-conjugated mAbs for 30 minutes on ice in the dark. After staining, cells were washed in cold PBS, fixed with 1% paraformaldehyde, and run through the flow cytometer.

FITC-dextran assay

To evaluate the capacity for uptake of soluble antigens from the culture medium, DCs were incubated with 1 mg/mL FITC-dextran at 37°C or at 0°C for 1 hour. Uptake was stopped by adding ice-cold PBS followed by 4 washes in a refrigerated centrifuge.¹⁹ Cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson).

Allogeneic and autologous T-cell proliferation assay

To test their allogeneic stimulatory activity, DCs were irradiated (3000 cGy) and tested as stimulators in primary mixed leukocyte reaction (MLR).¹⁷⁻¹⁹ Cells were resuspended in RPMI 1640, 25 mM HEPES, antibiotics, and 15% AB human serum that had been inactivated at 56°C for 30 minutes. Allogeneic PB CD3⁺ cells (5×10^4) were mixed with decreasing numbers of stimulators in round-bottomed 96-well plates for 6 days at 37°C in a 5% CO₂ humidified atmosphere. Cells were pulsed with 3.7×10^4 Bq/well (1 μ Ci/well) ³H-thymidine for 18 hours before harvest on day 6. Where indicated, the stimulation index (SI) was calculated for each individual experiment as follows: SI = counts per minute (cpm) (T-cell responders + stimulators)/cpm (T-cell responders).

Autologous MLRs were set up to demonstrate the capacity of DCs to process and present nominal antigens to T cells.¹⁷⁻¹⁹ Briefly, 10^5 PB CD3⁺ T cells were co-incubated with decreasing numbers of autologous APCs without antigens or with 50 μ g/mL keyhole limpet hemocyanin (KLH; Sigma) or 1 μ g/mL tetanus toxoid (TT, Calbiochem, La Jolla, CA). Circulating and monocyte-derived DCs were also incubated with 50 μ g/mL tumor Id purified from the serum of MM patients, as previously described.¹⁷ T-cell proliferation was measured as follows: SI = cpm (T-cell responders + antigen-pulsed stimulators)/cpm (T-cell responders + stimulators).

Statistical analysis

Results are expressed as the mean ± SD of at least 3 different experiments. Results were analyzed with the paired nonparametric Wilcoxon rank sum test, and $P < .05$ was considered significant.

Table 2. Expression of HLA and accessory molecules of mature bulk PBDCs obtained from MM patients and healthy donors

	MM patients	Healthy donors
HLA-class I	551 ± 237.7	570.3 ± 262.5
HLA-class II DR	349.7 ± 107.3*	738.3 ± 124.8
CD40	18.3 ± 11.2*	43.3 ± 10.5
CD80	10.7 ± 4.5*	29.5 ± 12.9
CD86	201 ± 174.5	235.7 ± 208.6

Mature bulk PBDCs were enriched as indicated in "Materials and methods." After 72 hours from the beginning of PBMC culture, bulk DCs were gated as DR (+) lineage markers (–) cells. The phenotype of gated cells was further examined in 3-color flow cytometry analysis by plotting histograms for the expression of each indicated surface molecule. Data are expressed as mean values ± SD of mean fluorescence intensity after subtracting fluorescence intensity given by isotype-matched controls. Values have been calculated from 3 separate experiments conducted in parallel on PBDCs of one normal subject and one MM patient.

*Data from the 2 groups were considered significantly different when $P < .05$.

Results

Evaluation of PBDC precursors in MM patients

Mature DCs can differentiate from PB precursors, including monocytes and PBDCs expressing HLA-DR but lacking common lineage markers such as CD3, CD14, CD16, CD19, CD20, CD34, and CD56. These PBDCs can be further distinguished into plasmacytoid and myeloid lineages based on their reciprocal expression of either CD123 or myeloid markers such as CD11c and CD33. We first compared the number of monocytes and plasmacytoid and myeloid DC precursors in the whole blood of MM patients and age- and sex-matched healthy donors. Furthermore, to assess whether the clinical state of the patients may influence the concentration of PBDCs and their precursors, we analyzed separately MM patients at diagnosis and later during the course of the disease. As shown in Table 1, we found that whereas the white blood cell counts did not differ in the 3 groups, the numbers of the 3 types of DC precursors, including monocytes, were significantly lower in MM patients. However, we did not find any difference between patients studied at diagnosis (n = 7) and patients with advanced disease (n = 18) (Table 1). Interestingly, there was a

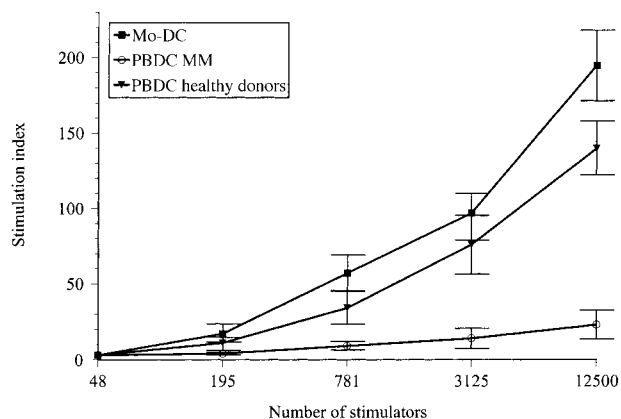


Figure 1. Alloreactivity of circulating DCs (PBDCs) and DCs derived from CD14⁺ monocytes (Mo-DC) from the same MM patients and of PBDCs from healthy subjects. Increasing numbers of DCs were tested for their capacity to stimulate 5×10^4 allogeneic CD3⁺ cells. The negative control, which gave 1800 ± 160 cpm when 12 500 cells were tested, is represented by unmanipulated mononuclear cells. Results report the mean ± SD of 3 different experiments. Statistical analysis demonstrated that both myeloma Mo-DCs and circulating DCs from healthy controls were more efficient stimulators of allogeneic T cells than PBDCs from MM patients ($P < .01$).

trend toward a higher number of PBDCs in MM patients with low tumor burden (stage I disease according to Durie-Salmon classification) compared with patients with stage III disease: 10.2 ± 3.4 versus 6.8 ± 4.5 (myeloid DCs) and 4.1 ± 2 versus 2.8 ± 2.1 (plasmacytoid DCs) ($P = .07$ for both groups).

Furthermore, when enriched PBDCs were analyzed for their expression of surface HLA and costimulatory molecules after the maturation process (Table 2), we found that MM patients showed a significantly lower expression of HLA-DR, CD40, and CD80 antigens.

Decreased antigen presentation capacity of PBDCs

To investigate whether the low expression of HLA-DR, CD40, and CD80 can cause the defective function of myeloma PBDCs, we first examined the ability of these cells to stimulate the proliferation of allogeneic T cells. We also compared the activity of these PBDCs with that of DCs derived from CD14⁺ cells (Mo-DCs) purified from the PB of the same MM patients and with that of PBDCs from healthy donors (Figure 1). In comparison with the strong T-cell stimulation exerted by the Mo-DCs, the activity of the

myeloma PBDCs was remarkably impaired; a nonsignificant decrease in stimulation was also observed in the PBDCs from healthy controls (Figure 1).

To further characterize the functional capacity of Mo-DCs and DCs freshly isolated from the same MM patients, we studied their ability to process and present nominal antigens to autologous cryopreserved T cells (Figure 2). Consistent with previous results,¹⁷ Mo-DCs pulsed with either KLH or with allogeneic Id or TT efficiently induced primary or secondary immune responses, respectively. In contrast, PBDCs from MM patients showed a significantly decreased antigen presentation to T cells (Figure 2A). Remarkably, PBDCs did not stimulate autologous T cells in response to the patient-specific tumor Id (Figure 2B).

Taken together, these results indicate that PBDCs from MM patients are phenotypically and functionally defective compared with DCs generated ex vivo from CD14⁺ precursors and circulating DCs from healthy donors. It should be noted that by comparing Mo-DC and mature DCs from the same patients, we were able to rule out the possibility that functional defects of responder T cells could have influenced the findings.

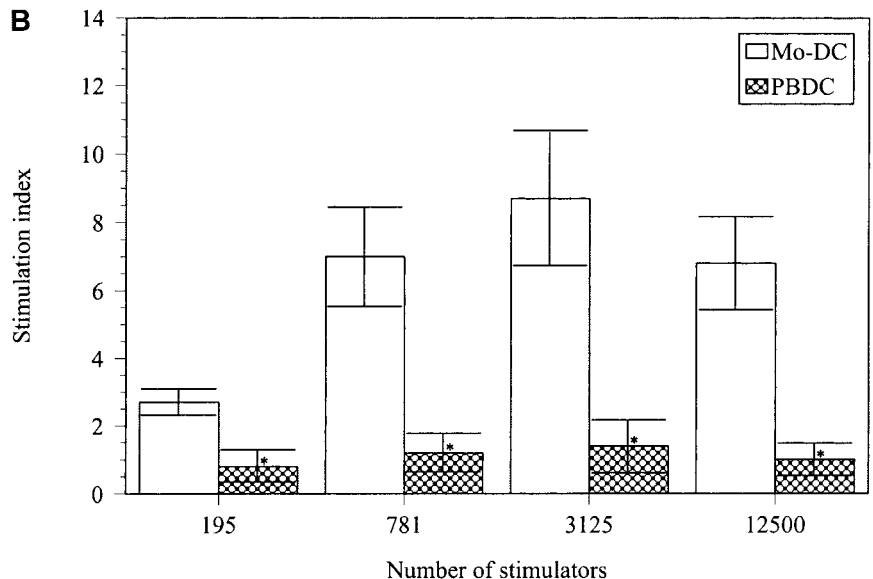
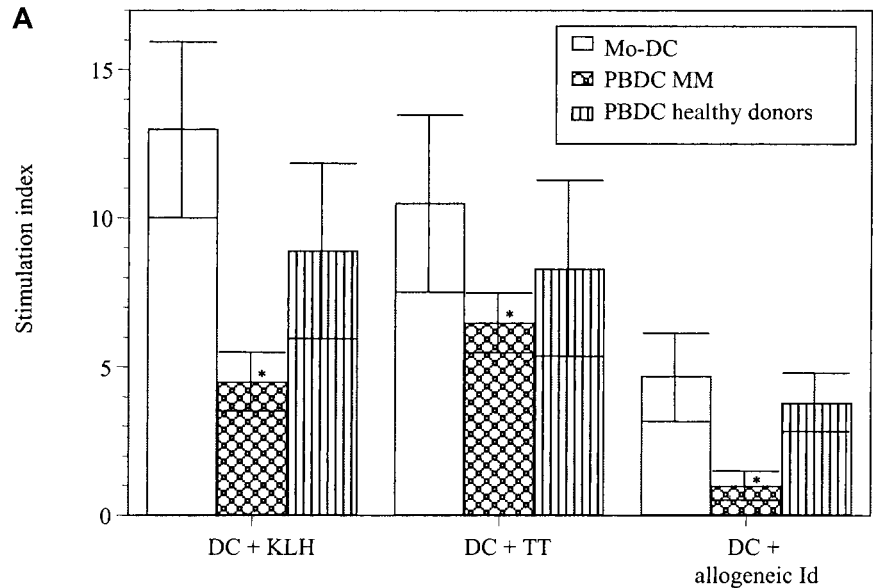


Figure 2. Capability of PBDCs and Mo-DCs from the same MM patients and of PBDCs from healthy subjects to present soluble antigens to autologous T cells. CD3⁺ cells (1×10^6) were incubated with either a fixed number (3000) of DCs (A) or increasing numbers of DCs (B). Co-incubation was performed in the presence or absence of TT, KLH, and allogeneic Id (A) or of autologous, patient-specific Id (B). Results report the mean \pm SD of 10 different experiments for MM patients and of 6 experiments for healthy subjects. Autologous DCs alone (negative control) gave 2800 ± 500 cpm when 12 500 cells were tested. Statistical analysis showed that PBDCs from MM patients were less efficient than Mo-DCs in stimulating autologous T lymphocytes in response to KLH, TT, and allogeneic Id ($P < .01$) and less efficient than PBDCs from healthy subjects in presenting allogeneic Id ($P < .05$). When autologous, patient-specific Id was tested, we did not observe any proliferative response of T cells co-incubated with PBDCs (B). By contrast, Mo-DCs were strong stimulators of T-cell proliferation ($P < .01$). * $P < .05$.

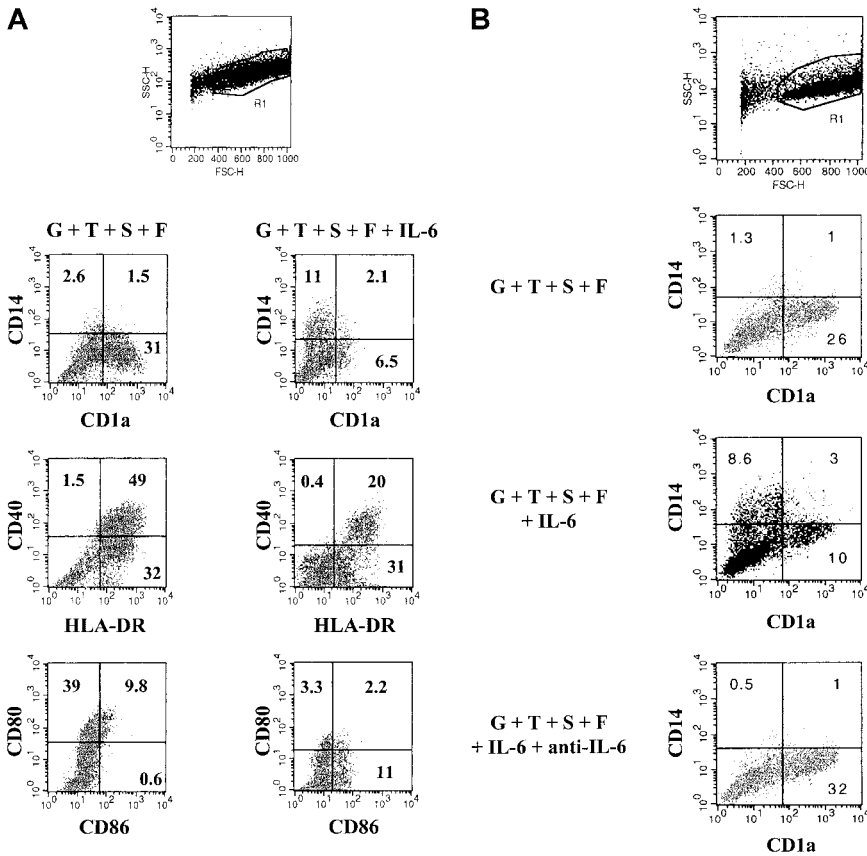


Figure 3. Differentiation of DCs derived from CD34⁺ cells is inhibited by IL-6. CD34⁺ progenitor cells were incubated with GM-CSF, TNF- α , SCF, and FLT3-L (G+T+S+F) with or without IL-6. Medium with cytokines was replaced at day +7 with IL-4 used in place of the early-acting growth factors SCF and FLT3-L to stimulate terminal differentiation of DCs. At day +14, the cells were labeled with CD14-PE/CD1a-FITC, HLA-DR-PE/CD40-FITC, or CD86-PE/CD80-FITC and were analyzed on a FACScan (A). (B) Antibodies to anti-IL-6 were used to neutralize the inhibitory effect of the cytokine. Results are from one representative experiment of 5 different samples analyzed. IL-6 up-regulated CD14 and down-regulated CD1a, HLA-DR, CD40, and CD80 antigens (A). The effects of IL-6 on CD14/CD1a phenotype were reversed by anti-IL-6 neutralizing antibodies (B).

Exogenous IL-6 inhibits DC development from CD34[±] cells

Earlier studies have shown that soluble factors released from cancer cells affect the maturation and function of DCs.^{14,24} We hypothesized that such a mechanism of action may be operative in MM. Therefore, we investigated whether IL-6 and VEGF can induce the blockade of DC generation from CD34⁺ cells. Highly purified hematopoietic progenitor cells were thus differentiated into DCs in the presence and absence of IL-6 and VEGF. Whereas VEGF did not affect the phenotype of DCs (data not shown), IL-6 up-regulated CD14 and down-regulated CD1a, HLA-DR, CD40, and CD80 antigens (Figure 3A). The effects of IL-6 on the CD14-CD1a phenotype were reversed by anti-IL-6 neutralizing antibodies (Figure 3B). Similar results were observed when IL-6 was added to the culture from day +7, during the maturation phase. Conversely, no effect on the terminal differentiation of DCs was observed if IL-6 was removed from the culture at day +7 (data not shown). To further confirm the specific effect of IL-6 on DC precursors within CD34⁺ cell fraction, we analyzed the clonogenic efficiency of CD34⁺ CFU-DC with or without IL-6 (Table 3). The addition of IL-6 reduced the colony-forming activity of GM-CSF and TNF- α and of GM-CSF, TNF- α , SCF, and FLT3-L

Table 3. Cloning efficiency of CD34⁺ cells in the presence and absence of IL-6

Cytokine stimuli	CFU-DC	CFU-M
G + T	0.4 \pm 0.2	0.6 \pm 0.3
G + T + IL-6	0.08 \pm 0.06*	0.7 \pm 0.1
G + T + S + F	1.1 \pm 0.4	0.9 \pm 0.4
G + T + S + F + IL-6	0.1 \pm 0.3*	1.1 \pm 0.3

Results are presented as the mean \pm SD of 5 different experiments. Cloning efficiency is indicated by the number of colonies scored after 14 days of culture \times 100 CD34⁺ cells plated.

*Addition of IL-6 inhibited CFU-DC growth ($P < .05$).

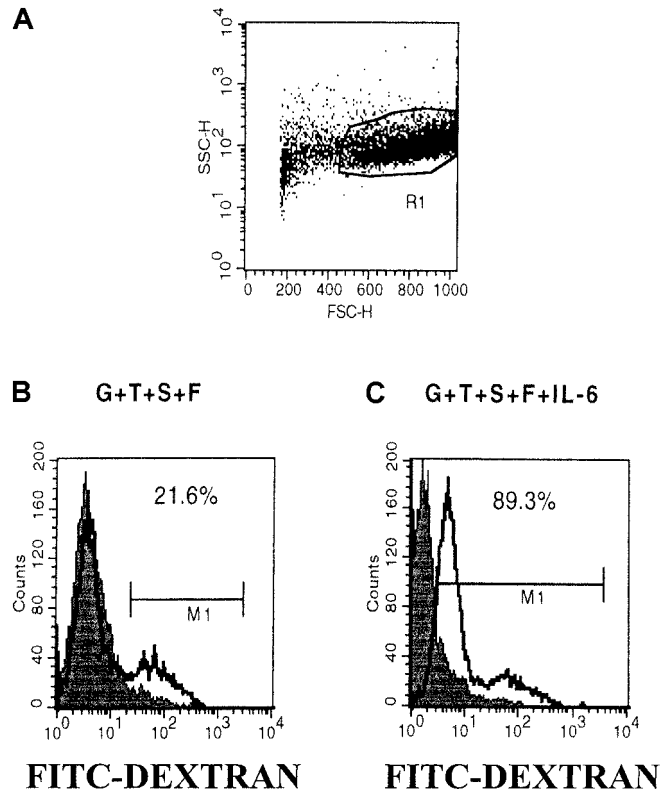


Figure 4. Addition of IL-6 resulted in maintenance of DC high phagocytic capacity. CD34⁺ cells were cultured for 12 to 14 days with GM-CSF, TNF- α , SCF, and FLT3-L (G+T+S+F) with or without IL-6. Their capacity to uptake soluble antigens was then determined by the FITC-dextran assay as described in "Materials and methods." Whereas mature DC lost their phagocytic ability, the addition of IL-6 led to the maintenance of this capacity. Results are from 1 representative experiment of 3 different samples analyzed.

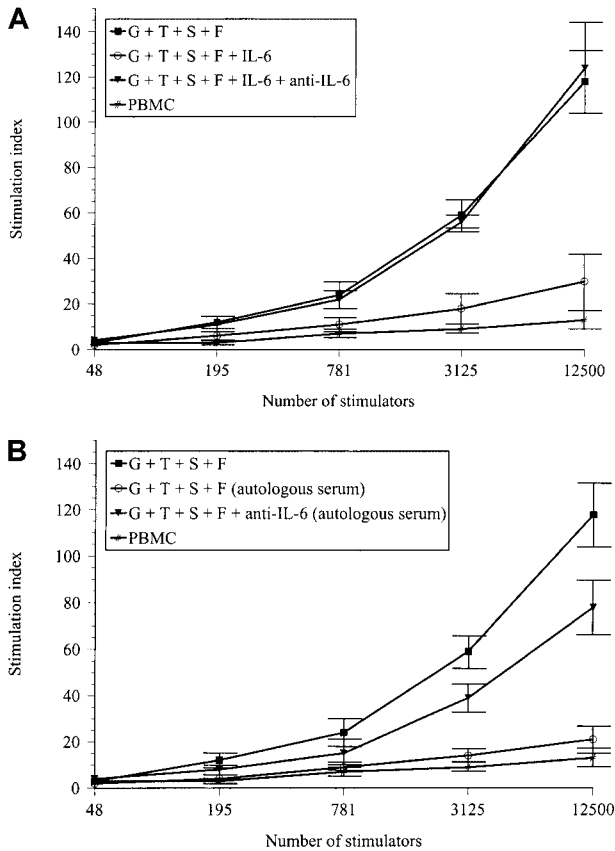


Figure 5. Alloreactivity of myeloma DCs derived from CD34⁺ cells generated in the presence of GM-CSF, TNF- α , SCF, and FLT3-L (G+T+S+F) and grown in FCS (A) or autologous serum (B) with and without IL-6. Increasing numbers of DCs were tested for their capacity to stimulate 5×10^4 allogeneic CD3⁺ cells. Negative controls, which always gave less than 2000 cpm, are represented by unmanipulated mononuclear cells and allogeneic PB mononuclear cells (PBMCs). Results report the mean \pm SD of 5 different experiments. The addition of IL-6 (A) to the culture of CD34⁺ cells significantly inhibited the development of alloreactive APCs ($P < .002$). The inhibitory effect of IL-6 was abrogated by anti-IL-6 antibodies. Similarly, autologous serum prevented the differentiation of functional DCs. However, anti-IL-6 antibodies did not fully reverse the APC ability of DC ($P < .05$ compared to G+T+S+F).

from 0.4 ± 0.2 to 0.08 ± 0.06 and from 1.1 ± 0.4 to 0.1 ± 0.3 , respectively ($P < .05$).

Functionally, the addition of exogenous IL-6 resulted in the maintenance of a high phagocytic capacity of DCs analyzed at day 14 of culture, as demonstrated by the FITC-dextran assay (Figure 4): the mean value of positive cells grown in the presence of IL-6 was $74\% \pm 15\%$ compared with $24\% \pm 6\%$ of control cultures ($P < .03$). Furthermore, as shown in Figure 5, DCs cultured with IL-6 were significantly weaker stimulators of allogeneic T cells than control cell populations in a conventional allogeneic MLR (Figure 5A). Anti-IL-6 antibodies abrogated the inhibitory activity of the cytokine. Of note, when CD34⁺ cells were grown in autologous serum in place of FCS, we observed a similar pattern of reduced proliferation of responder T cells (Figure 5B). In these experiments, however, anti-IL-6 antibodies did not completely reverse the inhibitory effect of autologous serum, suggesting that soluble factors other than IL-6 may also contribute to DC dysfunction. Similarly, exposure of DCs to IL-6 reduced their ability to process and present KLH and TT to autologous T cells to stimulate a primary or secondary immune response, respectively (Figure 6A). Finally, the inhibitory effect of autologous serum was partly counteracted by anti-IL-6 antibodies (Figure 6B). Taken together, these results indicate that IL-6 switches the commitment

of CD34⁺ cells from DCs to CD14⁺CD1a⁻CD86⁻CD80⁻CD40[±]HLA-DR[±] monocytic cells with a potent phagocytic activity but lacking antigen-presentation capacity.

Discussion

Studies have shown that the deficient antigen-presentation capacity of the DCs found in tumor-bearing animals and in patients with advanced breast cancer induces a cellular-immune deficit.^{24,25} This functional impairment of DCs correlates with the production of tumor-derived soluble factors, such as VEGF and IL-6, that affect DC maturation.^{10,14} Conversely, DCs generated in vitro from monocyte precursors are capable of efficient T-cell stimulation.^{17,24,26,27} These studies suggest that circulating PBDCs may not be optimal vehicles for antigen delivery in immunotherapy trials of cancer.

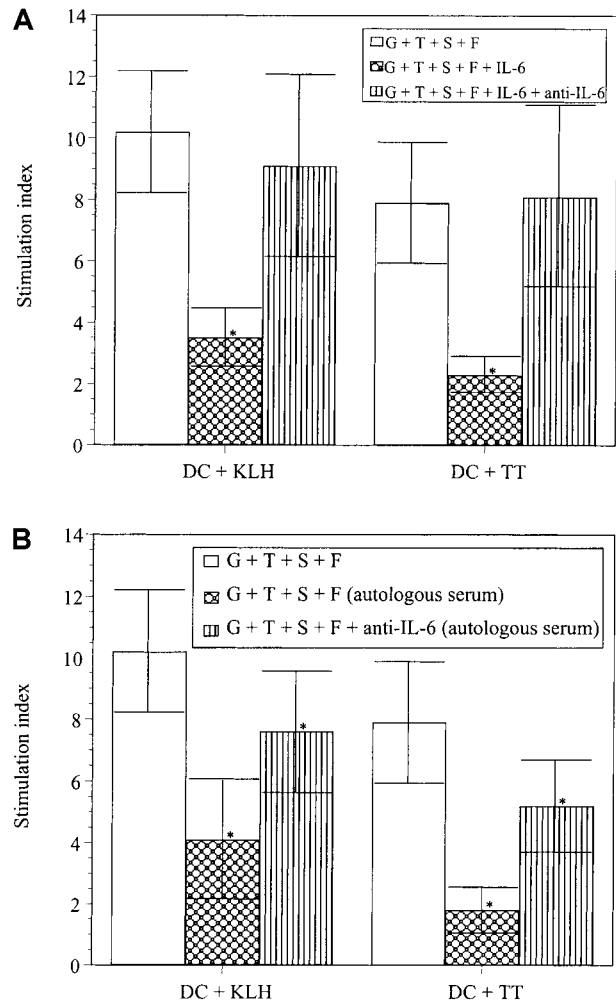


Figure 6. Effect of IL-6 on the antigen-presenting ability (to autologous cells) of myeloma DCs derived from CD34⁺ progenitors in the presence of GM-CSF, TNF- α , SCF, and FLT3-L (G+T+S+F) and grown in FCS (A) or autologous serum (B). CD34⁺ cells (1×10^5) were incubated with a fixed number (3000) of DCs pulsed with TT or KLH. Results report the mean \pm SD of 5 different experiments. Autologous DCs alone gave 2500 ± 400 cpm. The addition of IL-6 (A) to the culture of CD34⁺ cells significantly inhibited the development of functional APCs ($P < .002$). The inhibitory effect of IL-6 was abrogated by anti-IL-6 antibodies. Similarly, the addition of autologous serum in place of FCS significantly reduced the antigen-presentation capacity of DCs ($P < .03$). However, neutralizing experiments with anti-IL-6 antibodies did not fully reverse the APC ability of DCs ($P < .05$ compared with G+T+S+F). * $P < .05$.

When PBDCs were used for anti-Id vaccination in patients with MM or non-Hodgkin lymphoma, a high Id-specific immune response rate was observed in most of the lymphoma patients,¹⁶ but it was observed in only 4 of 26 subjects with MM.¹⁵ Based on these results, we hypothesized that circulating myeloma PBDCs may be defective and that their dysfunction could be caused by MM-specific factors. In the present study, we investigated the number, phenotype, and functional characteristics of PBDCs freshly isolated from MM patients. We then compared their characteristics with those of DCs isolated from healthy donors and of DCs generated in vitro from CD14⁺ precursors from the same MM patients.

Our data demonstrate that the absolute number of circulating monocytes, myeloid DC precursors, and plasmacytoid DC precursors is significantly lower in MM patients than in healthy subjects, regardless of whether the patients were analyzed at diagnosis or during the course of the malignancy. Of note, we found a trend toward a decreased number of PBDCs in patients with high tumor burden. Moreover, myeloma PBDCs showed a lower expression of HLA-DR, CD40, and CD80 antigens on maturation. Decreased expression of MHC class II and costimulatory molecules was responsible for the defective induction of T-cell proliferation. Remarkably, we were able to show that circulating myeloma DCs were not capable of presenting the patient-specific tumor Id to autologous T cells. By contrast, Mo-DCs and PBDCs from healthy controls were potent stimulators of T-cell immunity. Moreover, comparison of Mo-DC and mature PBDCs from the same patients ruled out the possibility that the differences depended on functional defects of responder T cells. These comparative experiments strongly support the hypothesis that tumor-specific factors with inhibitory activity are functional in vivo in patients with MM.

We then looked at the effects of the main plasma cell growth factor, IL-6, on the development of DCs from CD34⁺ cells. We found that IL-6 inhibited the colony growth of CD34⁺ DC progenitors and switched the commitment of CD34⁺ cells from DCs to CD14⁺ CD1a⁻ monocytic cells, exerting potent phagocytic activity but low antigen-presentation capacity. In neutralizing experiments, this effect was reversed by anti-IL-6 antibodies. These findings extend the observation that IL-6 has an immunosuppressive role in cancer patients by inhibiting the development of DCs.¹¹ Interestingly, we found that the defective function of the

DCs persisted even when the CD34⁺ cells were cultured in the presence of autologous serum. However, the concentration of exogenous recombinant IL-6 required to block DC differentiation (20 ng/mL) was much higher than that reported in the serum of MM patients.^{28,29} In this study, the serum levels of IL-6 in MM patients ranged from 0 to 500 pg/mL. Although there was a tendency in patients with active stage III disease to have IL-6 levels higher than 10 pg/mL and in patients with stable stage I disease to have IL-6 levels lower than 10 pg/mL, the small number of patients studied did not allow a strong correlation to be found between disease stage, IL-6 level, and DC impairment. Moreover, the addition of anti-IL-6 antibodies did not completely restore the functional activity of the DCs. This suggests that other inhibitory factors are probably present in the serum of MM patients. Although we found that VEGF, which is produced by plasma cells,³⁰ did not show any effect on DC development, other candidate cytokines could be IL-10 and transforming growth factor- β_1 , which are expressed and produced by human myeloma cells^{31,32} and have been reported to inhibit the differentiation and function of DCs.^{13,32}

An alternative explanation for the finding that circulating DCs are specifically defective in stimulating T-cell proliferation in response to Id in vitro (present findings) and in vivo¹⁵ could be that the serum paraprotein may itself down-regulate the immune response by inducing anergy or deletion of Id-specific T cells.³³ This hypothesis is supported by data from DC-based anti-Id vaccination trials in MM.³⁴ Few patients with circulating paraprotein showed an Id-specific cytotoxic T-cell response despite the development of B- and T-cell reactivity to KLH.

In summary, our study supports the concept³² that circulating DCs isolated from MM patients have an impaired capacity for T-cell stimulation. Moreover, we found that this deficiency is partly caused by IL-6-mediated inhibition of DC development. These results question the advisability of using freshly isolated PBDCs in clinical immunotherapy trials as a means of stimulating T cells. They also suggest a novel mechanism whereby myeloma cells can escape immune recognition.

Acknowledgment

We thank Robin T. Cooke for editing the manuscript.

References

- Klein B, Zhang X-E, Lu Z-Y, Bataille R. Interleukin-6 in human multiple myeloma. *Blood*. 1995; 85:863-872.
- Klein B, Zhang X-G, Jourdan M, et al. A paracrine but not autocrine regulation of plasma cell growth and differentiation by interleukin-6. *Blood*. 1989; 73:517-526.
- Kawano M, Hirano T, Matsuda T, et al. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myeloma. *Nature*. 1988;332:83-85.
- Bataille R, Jourdan M, Zhang X-G, Klein B. Serum levels of interleukin-6, a potent myeloma cell growth factor, as a reflection of disease severity in plasma cell dyscrasias. *J Clin Invest*. 1989;84: 2008-2011.
- Ludwig H, Nachbaur DM, Fritz E, Krainer M, Huber H. Interleukin-6 is a prognostic factor in multiple myeloma. *Blood*. 1991;77:2794-2795.
- Blay JY, Negrier S, Combaret V, et al. Serum level of interleukin-6 as a prognosis factor in metastatic renal cell carcinoma. *Cancer Res*. 1992;52:3317.
- Tartour E, Blay JY, Dorval T, et al. Predictors of clinical response to interleukin-2-based immunotherapy in melanoma patients: a French multi-institutional study. *J Clin Oncol*. 1996;14:1697.
- Deehan DJ, Heyes SD, Simpson WG, Broom J, Franks C, Eremin O. In vivo cytokine production and recombinant interleukin-2 immunotherapy: an insight into the possible mechanisms underlying clinical responses. *Br J Cancer*. 1994;69:1130.
- Bataille R, Barlogie B, Lu ZY et al. Biologic effects of anti-interleukin-6 murine monoclonal antibody in advanced multiple myeloma. *Blood*. 1995;86: 685-691.
- Bancherou J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1988;392:245-252.
- Menetrier-Caux C, Montmain G, Dieu MC, et al. Inhibition of the differentiation of dendritic cells from CD34⁺ progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood*. 1998;92:4778-4791.
- Chomarar P, Bancherou J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol*. 2000;1:510-514.
- Mitani H, Katayama N, Araki H, et al. Activity of interleukin 6 in the differentiation of monocytes to macrophages and dendritic cells. *Br J Haematol*. 2000;109:288-295.
- Gabilovich DI, Chen HL, Gargis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med*. 1996;2:1096-1103.
- Reichardt VL, Okada CY, Liso A, et al. Idiotype vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma: a feasibility study. *Blood*. 1999;93: 2411-2419.
- Hsu F, Benike C, Fagnoni F, et al. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med*. 1996;2: 52-58.
- Ratta M, Curti A, Fogli M, et al. Efficient presentation of tumor idiotype to autologous T cells by CD83⁺ dendritic cells derived from highly purified circulating CD14⁺ monocytes in multiple myeloma patients. *Exp Hematol*. 2000;28:931-940.
- Ratta M, Rondelli D, Fortuna A, et al. Generation

- and functional characterization of human dendritic cells derived from CD34⁺ cells mobilized into peripheral blood: comparison with bone marrow CD34⁺ cells. *Br J Haematol*. 1998;101:756-765.
19. Curti A, Fogli M, Ratta M, Tura S, Lemoli RM. Stem cell factor and FLT3-ligand are strictly required to sustain the long-term expansion of primitive CD34⁺DR⁻ dendritic cell precursors. *J Immunol*. 2001;166:848-854.
 20. Takamizawa M, Rivas A, Fagnoni F, et al. Dendritic cells that process and present nominal antigens to naive T lymphocytes are derived from CD2⁺ precursors. *J Immunol*. 1997;158:2134-2142.
 21. Takamizawa M, Fagnoni F, Mehta-Damani A, Rivas A, Engleman EG. Cellular and molecular basis of human gamma delta T cell activation: role of accessory molecules in alloactivation. *J Clin Invest*. 1995;95:296-303.
 22. Fagnoni FF, Takamizawa M, Godfrey WR, et al. Role of B70/B7-2 in CD4⁺ T-cell immune responses induced by dendritic cells. *Immunology*. 1995;85:467-474.
 23. Fagnoni FF, Oliviero B, Zibera C, et al. Circulating CD33⁺ large mononuclear cells contain three distinct populations with phenotype of putative antigen-presenting cells including myeloid dendritic cells and CD14⁺ monocytes with their CD16⁺ subset. *Cytometry*. 2001;45:124-132.
 24. Gabrilovich DI, Corak J, Ciernik IF, Kavanaugh D, Carbone DP. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin Cancer Res*. 1997;3:483-490.
 25. Gabrilovich DI, Ciernik F, Carbone DP. Dendritic cells in anti-tumor immune responses, I: defective antigen presentation in tumor-bearing hosts. *Cell Immunol*. 1996;170:101-110.
 26. Pfeiffer S, Gooding RP, Apperley JF, Goldshmidt H, Samson D. Dendritic cells generated from the blood of patients with multiple myeloma are phenotypically and functionally identical to those similarly produced from healthy donors. *Br J Haematol*. 1997;98:973-982.
 27. Raje N, Gong J, Chauhan D, et al. Bone marrow and peripheral blood dendritic cells from patients with multiple myeloma are phenotypically and functionally normal despite the detection of Kaposi's sarcoma herpesvirus gene sequences. *Blood*. 1999;93:1487-1495.
 28. Emile C, Femand JP, Danon F. Interleukin-6 levels in patients with multiple myeloma. *Br J Haematol*. 1994;86:439-440.
 29. Kyrstsonis MC, Dedoussis G, Baxevanis C, Stamatelou M, Maniari A. Serum interleukin-6 (IL-6) and interleukin-4 (IL-4) in patients with multiple myeloma (MM). *Br J Haematol*. 1996;92:420-422.
 30. Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. *Cancer Res*. 1999;59:728-733.
 31. Otsuki T, Yamada O, Yata K, et al. Expression and production of interleukin 10 in human myeloma cell lines. *Br J Haematol*. 2000;111:835-842.
 32. Brown RD, Pope B, Murray A, et al. Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor- β 1 and interleukin-10. *Blood*. 2001;98:2992-2998.
 33. Bogen B. Peripheral T cell tolerance as a tumor escape mechanism: deletion of CD4⁺ T cells specific for a monoclonal immunoglobulin idiotype secreted by a plasmacytoma. *Eur J Immunol*. 1996;26:2671-2679.
 34. Bocchia M, Bronte V, Colombo MP, et al. Antitumor vaccination: where we stand. *Haematologica*. 2000;85:1172-1206.