Clinical Significance of Defective Dendritic Cell Differentiation in Cancer[1]

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ABSTRACT

Defective dendritic cell (DC) function has been described previously in cancer patients and tumor-bearing mice. It can be an important factor in the escape of tumors from immune system control. However, the mechanism and clinical significance of this phenomenon remain unclear. Here, 93 patients with breast, head and neck, and lung cancer were investigated. The function of peripheral blood and tumor draining lymph node DCs was equally impaired in cancer patients, consistent with a systemic rather than a local effect of tumor on DCs. The number of DCs was dramatically reduced in the peripheral blood of cancer patients. This decrease was associated with the accumulation of cells lacking markers of mature hematopoietic cells. The presence of these immature cells was closely associated with the stage and duration of the disease. Surgical removal of tumor resulted in partial reversal of the observed effects. The presence of immature cells in the peripheral blood of cancer patients was closely associated with an increased plasma level of vascular endothelial growth factor but not interleukin 6, granulocyte macrophage colony-stimulating factor, macrophage colony-stimulating factor, interleukin 10, or transforming growth factor-β and was decreased in lung cancer patients receiving therapy with antivascular endothelial growth factor antibodies. These data indicate that defective DC function in cancer patients is the result of decreased numbers of competent DCs and the accumulation of immature cells. This effect may have significant clinical implications.

INTRODUCTION

A defective host antitumor immune response is an important mechanism allowing tumors to evade immune system control. Induction of an effective antitumor response requires the active participation of host bone marrow-derived APCs[4] responsible for the presentation of tumor-specific antigens (1, 2). The importance of APCs is underscored by the fact that defects in the function of tumor-infiltrating lymphocytes in cancer patients and T cells from tumor-bearing mice can be completely reversed when effective antigen presentation and exogenous IL-2 is provided (3–5). DCs are the most potent APCs. They play a central role in antitumor immunity by taking up tumor antigens and stimulating antigen-specific T cells. In recent years, several groups have described the defective function of DCs in tumor-bearing mice and in cancer patients (6–9). The major finding of these studies was the lack of expression of co-stimulatory molecules in tumor-associated DCs, consistent with the phenotype of immature, nonactivated DCs. A population of DCs isolated from the PB of patients with breast and head and neck cancer demonstrated significantly reduced ability to cluster and stimulate allogeneic and antigen-specific T cell responses (10, 11). These cells have a substantially lower level of expression of MHC class II (HLA-DR) and co-stimulatory molecules than DCs isolated from control donors. In agreement with these reports, DCs isolated from tumor-bearing mice also had a decreased expression of B7-2 and MHC class II, as well as some adhesion molecules. These cells were unable to induce effective peptide-specific and antitumor cytotoxic immune responses and were ineffective as a tumor vaccine (5). Previous data suggest that tumors might affect DC maturation from progenitors. Mature DCs, however, were functionally competent (12). This was consistent with the fact that functionally competent DCs can be generated in the absence of tumor-derived factors from bone marrow progenitor cells in tumor-bearing mice and from PB progenitors in cancer patients (11, 13). Thus, the population of DCs in tumor-bearing hosts is functionally defective. It is likely that the effectiveness of cancer vaccine strategies, even those involving the infusion of antigen-loaded *in vitro* activated DC, will be impaired by deficient endogenous DC function. To address this problem, an understanding of the mechanism of these defects and their association with tumor features and

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[4] The abbreviations used are: APC, antigen presenting cell; CCM, complete culture medium; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; HNSCC, head and neck squamous cell carcinoma; LN, lymph node; M-CSF, macrophage colony-stimulating factor; MLR, mixed leukocyte reaction; PB, peripheral blood; PE, phycoerythrin; TT, tetanus toxoid; VEGF, vascular endothelial growth factor.

Table 1 Stage distribution of the patients

Cancer type	Stage I	Stage II	Stage III	Stage IV
HNSCC	3	11	13	26
Non-small cell lung carcinoma	1	2	2	8
Breast	2	10	3	8

antitumor therapies is essential. To address these questions, we have studied the phenotype and function of DCs isolated from PB and LNs from 93 patients with different types and stages of cancer, before and after therapy. Here, we report a significant decrease in the number and proportion of DCs in the PB of cancer patients, which was associated with appearance of a population of immature cells. We showed that their presence is associated with both decreased immune function and clinical tumor status.

PATIENTS AND METHODS

Patients. Ninety-three patients, 32–79 years of age, with histologically confirmed cancer were enrolled in this study. Of these 93 patients, 53 had HNSCC, 13 had non-small cell lung carcinoma, and 23 had breast adenocarcinoma. The vast majority of patients were newly diagnosed, but a few had recurrent disease with no prior therapy for at least 1 year. Staging was performed in accordance with the American Joint Committee on Cancer criteria, and the data are presented in Table 1. Fourteen healthy volunteers served as controls for the PB DC study. LNs were obtained during tumor resection in HNSCC patients, and only that part of the LNs not needed for medical decision-making was used for the isolation of DCs. Control tonsil tissues were obtained from nine age-matched individuals undergoing tonsillectomy for sleep apnea indications without a history or recent evidence of infection. The three patients shown in Table 4 were treated on a three-arm randomized trial of chemotherapy with carboplatin and Taxol in combination with humanized anti-VEGF antibody (Genentech, VCC THO9806). After chemotherapy, all three patients studied received the antibody alone at either 15 or 7.5 mg/kg every 3 weeks for at least 3 months before the blood was assessed. Informed consent was obtained from all individuals.

Reagents. CCM included RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS and antibiotics. Ficoll-paque was obtained from Amersham Pharmacia Biotech (Upsalla, Sweden). PE-, FITC-, or Quantum Red-conjugated anti-HLA-DR, CD3, CD14, CD19, and CD57 antibodies and isotype-matched mouse immunoglobulin were purchased from Sigma Chemical Co. (St. Louis, MO). FITC-conjugated anti-CD86 and anti-CD40 antibodies were obtained from PharMingen (San Diego, CA), and goat antimouse IgG antibody conjugated with magnetic beads was from Dynal (Lake Success, NY). Sheep RBCs were obtained from Cocalico (Reamstown, PA), and metrizamide was from Neygaard (Oslo, Norway) and Sigma. Collagenase was obtained from Sigma. ELISA kits were purchased from R&D Systems (Minneapolis, MN).

Cell Isolation from PB. DCs and T cells were isolated from PB as described (14) with some modifications. Briefly, mononuclear cells obtained after centrifugation of PB over a Ficoll-paque gradient were incubated with 2-aminoethylisothiouronium bromide (Sigma)-treated sheep RBCs (R). Cells that adhered to the red cells (R+) and those that did not (R–) were separated on a Ficoll-paque gradient. R– cells were then incubated for 24–36 h in CCM. Nonadherent cells were centrifuged over a metrizamide gradient (7.25 g of metrizamide in 50 ml of CCM) to obtain an enriched fraction of DCs. For all functional tests, DCs were further enriched using a magnetic bead separation technique. Briefly, the DC fraction isolated as described above was treated at 4°C with a mixture of monoclonal antibodies: anti-CD3, anti-CD14, anti-CD19, and anti-CD57/HNK. After a 30-min incubation, cells were washed and labeled with goat antimouse IgG antibody conjugated with magnetic beads (Dynal), followed by magnetic separation. In control individuals, the resulting cell population contained more than 95% DCs, as estimated by flow cytometry.

R+ cells were further processed to obtain an enriched T cell fraction by osmotic lysis of the red cells followed by overnight incubation in CCM at 37°C. More than 90% of nonadherent cells were T cells, as estimated by flow cytometry.

Cell Isolation from LNs. For head and neck cancer patients, one-third to one-half of uninvolved cervical LNs were obtained at the time of planned surgery. For controls, one-quarter to one-half of tonsillectomy specimens were obtained at the time of surgery. Specimens were cleaned of adipose and connective tissue, finely minced, and incubated in 400 units/ml of collagenase for 30 min. Cells were then passed through a 70 μM cell strainer and washed in PBS before being placed over Ficoll-paque. The remainder of the DC isolation was the same as for PB DCs described above.

MLR and Antigen-specific T-cell Proliferation. The ability of DCs to stimulate allogeneic T cells was tested in MLR. Because the strength of the response in allogeneic MLR depends on mismatch in HLA, occasionally, a low level of proliferaion was detected even in response to the donor's DCs. Although these low values did not change the overall results, they significantly increased a variation of the data and complicated the analysis. To minimize the effect of differences in HLA between individuals on allogeneic T cell proliferation, DCs from each patient and control individual were tested against allogeneic T cells from three control individuals, and only maximal values of responses were used. Fifty thousand T cells were plated in each well of 96-well round-bottomed plates, and DCs and T cells were cultured at ratios of 1:20, 1:40, 1:80, and 1:160 for 5 days. One μCi of [^{3}H]thymidine was added to each well 18 h prior to harvesting the cells. [^{3}H]Thymidine uptake was counted in a liquid scintillation counter (Beckman, Palo Alto, CA).

Antigen-specific T cell response was measured using TT. DCs were cultured with autologous T cells in the presence of 1.0 μg/ml TT. [^{3}H]Thymidine was added after 4 days of culture, and uptake was counted 18 h later in a liquid scintillation counter. Background levels of T cell proliferation (with no TT) were subtracted.

Flow Cytometry. Cells were labeled with PE-, FITC-, or Quantum Red-conjugated antibodies by incubation on ice for 30 min followed by washing with PBS. Data acquisition and analysis were performed on a FACSCalibur flow cytometer (Becton Dickson, Mountain View, CA) using Cell Quest software.

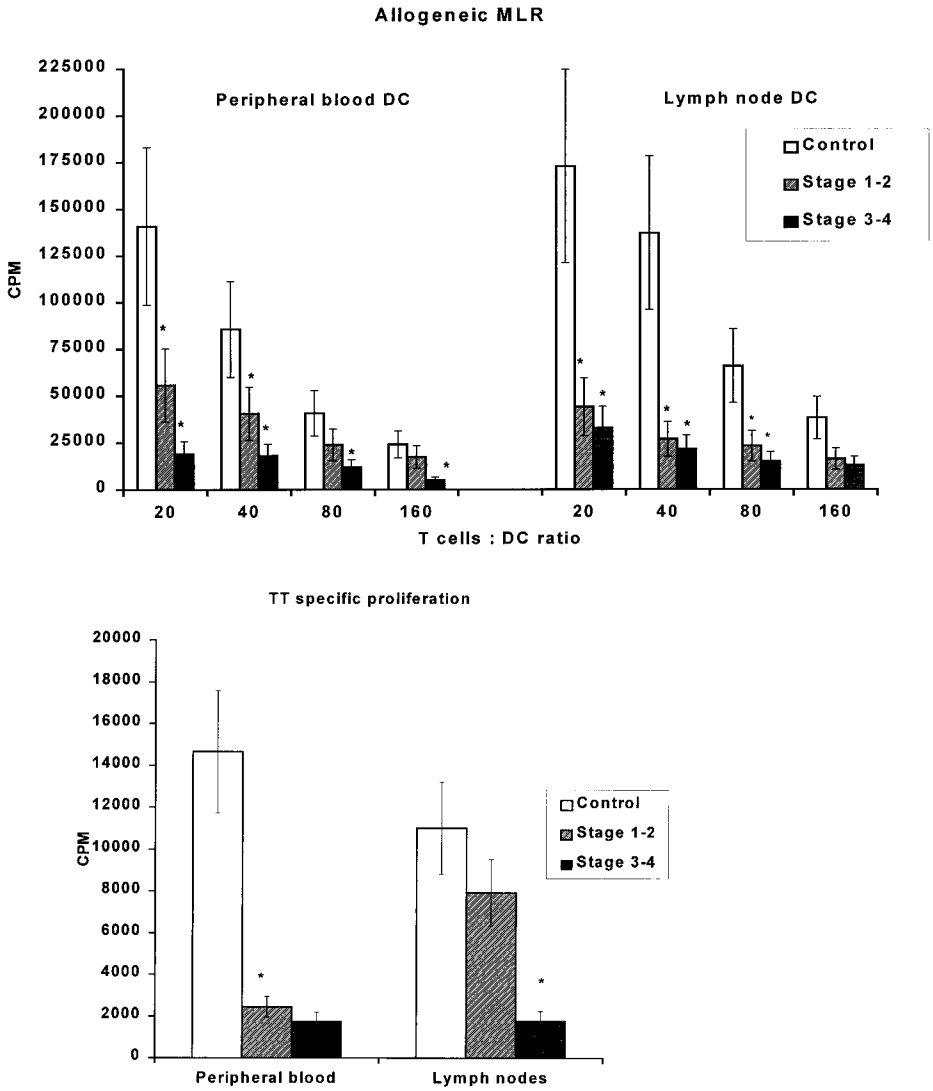

Fig. 1 Decreased functional activity of DCs isolated from PB and LNs of cancer patients. PB and LN DCs were isolated from 18 patients with HNSCC as described in "Patients and Methods." Five patients had stage 1–2, and 13 had stage 3–4 of disease. Control values were obtained from nine healthy individuals. T cells were obtained from PB of the control individuals. In allogeneic MLR, DCs from each patient were tested against T cells from three donors and only the maximum response was scored. One μCi of [^{3}H]thymidine was added 18 h before cell harvesting. In TT-specific T cell responses, DCs were cultured with autologous T cells in the presence of 1 μg/ml TT. The level of spontaneous T cell proliferation (without the presence of TT) was subtracted in each experiment. One DC:T cell ratio (1:20) is shown. T cell proliferation was measured as described in "Patients and Methods." *, statistically significant differences from the control ($P < 0.05$).

Assay for the Presence of Growth Factor and Cytokines in PB. Patient and control plasma samples were obtained by centrifugation of PB at 500 × *g* for 10 min. To minimize nonspecific binding to lipids, all samples were obtained after 3–4 h of fasting and spun down at 10,000 × *g* for 30 min prior to testing. Plasma samples were stored at –20°C. Concentrations of VEGF, TGF-β1, IL-6, IL-10, GM-CSF, and M-CSF were measured using ELISA kits (R&D Systems) and assayed on a spectrophotometer. Data were analyzed using DeltaSoft software.

Statistical Analysis. Statistical analysis was performed using parametric and nonparametric methods and JMP statistical software (SAS Institute Inc., Cary, NC).

RESULTS

Functional Activity of DCs Isolated from PB and LNs of Cancer Patients. To address the question of whether the effect of tumor on DC function is localized or general, we compared the function of DCs isolated from PB and from local draining sentinel LNs in 18 patients with HNSCC. LNs and PB were collected at the time of surgical resection as described in "Patients and Methods." Patients' PB DCs demonstrated significantly reduced ability to stimulate allogeneic control T cells and antigen (TT)-specific proliferation of autologous T cells. (Fig. 1). This was consistent with previously reported data from breast cancer patients (11). DC activity in patients with advanced disease (stages 3–4) was significantly lower than that in patients with early stages of cancer (stages 1–2; $P < 0.05$). The activity of patients' LN DCs was also severely impaired (Fig. 1). No statistically significant differences were seen between the activity of PB DCs and LN DCs (Fig. 1). To exclude the possible effect of especially low values on the average values of DC activity in the groups, we compared the percentage of patients with decreased LN DC and PB DC functional activity. As a control level in this case we used the level corresponding to 1 SD below the median response of DCs from healthy control individuals. The function of PB DCs was decreased in 12 of 18 patients (66.6%), and LN DC function was decreased in 14 of 18

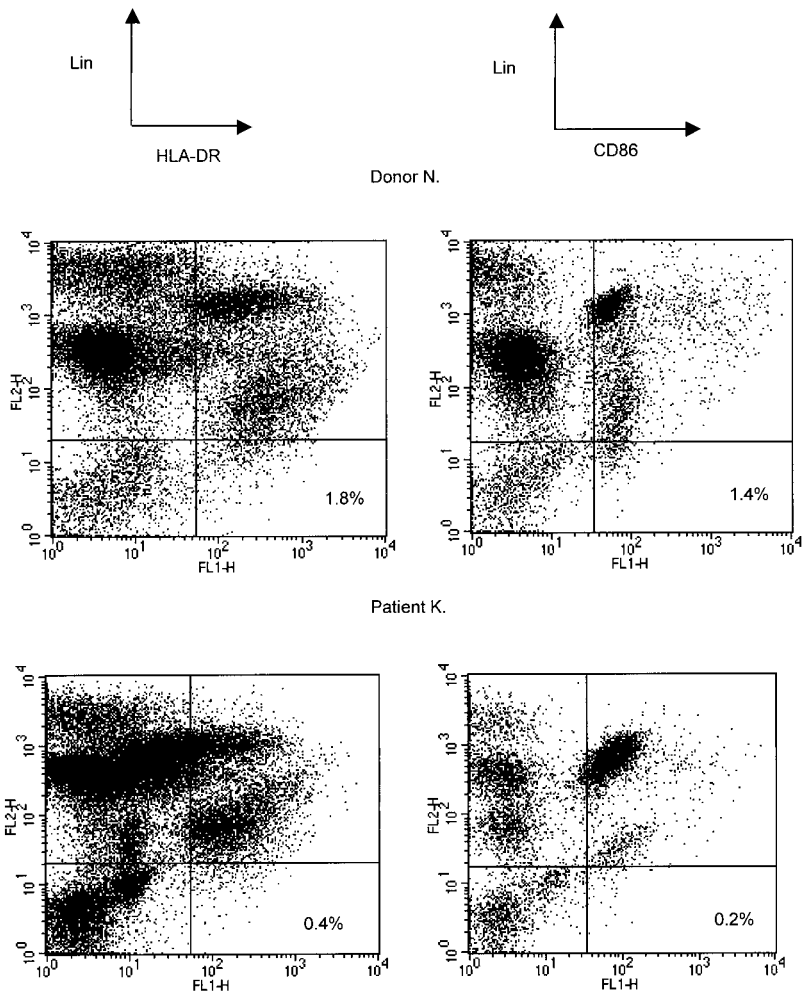

Fig. 2 Presence of DCs in freshly isolated PB mononuclear cells. Freshly isolated mononuclear cells were labeled with PE-conjugated anti-CD3, CD14, CD19, and CD57 antibodies and FITC-conjugated anti-HLA-DR or CD86 antibodies. In each sample, 200,000 cells, in all, were analyzed. Typical profiles of one healthy volunteer and one cancer patient are shown.

patients (77.7%). There was no statistically significant difference between PB and LN DC function. Thus, the function of PB DCs and regional LN DCs appears to be equally affected in patients with HNSCC. This suggests that DC defects in cancer patients are a systemic phenomenon rather than a local effect of tumor-derived factors on regional LN DCs.

Decreased Numbers of DCs in the PB of Cancer Patients. We next asked what could cause these DC defects. We and others have demonstrated previously that tumor-derived factors affect the normal process of DC maturation *in vitro*, resulting in the generation of immature cells (13, 15–17). To test whether tumor-derived factors affect DC differentiation *in vivo*, we have determined the yield of DCs from the PB of patients with HNSCC. Freshly isolated PB mononuclear cells were labeled with a mixture of PE-conjugated lineage-specific antibodies (anti-CD3, CD14, CD19, CD57) and FITC-conjugated anti-HLA-DR or anti-CD86 (B7-2) antibody. Cells were analyzed directly using flow cytometry. Lin^- $HLA\text{-}DR^+$ DCs represented only a small proportion of mononuclear cells in the PB of healthy control individuals. The proportion of mature Lin^- $B7\text{-}2^+$ DCs was smaller (Fig. 2). Cancer patients had a significantly lower percentage of these cells. Typical results are shown in Fig. 2. To calculate the proportion and absolute numbers of DCs in control donors and cancer patients, DCs were enriched by magnetic bead separation of freshly isolated mononuclear cells using anti-CD3, CD14, CD19, and CD57 antibodies. Negatively selected enriched populations of DCs were labeled with PE-conjugated lineage-specific antibodies (anti-CD3, anti-CD14, anti-CD19, and anti-CD57 antibodies) and FITC-conjugated anti-HLA-DR antibody. Lineage-negative, HLA-DR-positive cells were scored as DCs. Sixteen healthy volunteers and 23 patients with HNSCC were studied. As shown in Table 2, the proportion of DCs in PB was significantly decreased in patients with stages 1–2 HNSCC. This resulted in a 2-fold decrease in the total number of DCs. In patients with advanced stages of HNSCC, the proportion of DCs was decreased even further, with a 4-fold decrease in the total number of these cells (Table 2). Thus, the number of DCs was dramatically reduced in the PB of cancer patients, and the degree of reduction was associated with the stage of disease.

Decreased Presence of DCs Correlates with Clinical Parameters. In healthy individuals, the majority of DCs isolated from PB are relatively immature cells. These cells require 24–36 h of culture to up-regulate expression of MHC class II

Table 2 Proportion and the number of dendritic cells in peripheral blood of patients with HNSCC

Individual values for each experiment and mean ± S.E. are shown. *P*, statistical difference from control; P_1, statistical difference between the groups of the patients.

Healthy volunteers		HNSCC stage 1–2		HNSCC stage 3–4	
%	$\times 10^3$/ml	%	$\times 10^3$/ml	%	$\times 10^3$/ml
3.99	52.5	0.7	11.7	0.015	0.4
3.05	39.1	0.51	10.6	0.57	11.6
1.34	23.4	1.46	43.3	0.38	8.3
3.32	50.3	0.58	8.8	0.01	0.07
8.65	90.0	0.49	8.3	0.06	0.56
5.43	19.0	0.59	14.7	0.95	29.9
0.81	8.7	1.2	19.6	0.59	7.2
1.87	17.2	1.29	47.3	0.28	33.7
0.24	9.4			0.27	6.6
0.78	13.5			0.3	14.0
1.17	8.9			0.73	25.3
1.51	148.6			0.04	0.25
2.47	29.0			0.52	12.2
2.99	47.8			0.005	0.19
2.57	60.0			0.77	6.3
0.9	32.4				
2.57 ± 0.53%		0.85 ± 0.14%		0.37 ± 0.08%	
		$P < 0.01$		$P < 0.01$	
				$P_1 < 0.05$	
40.6 ± 9.1 × 10^3/ml		20.5 ± 5.6 × 10^3/ml		10.4 ± 2.8 × 10^3/ml	
		$P < 0.05$		$P < 0.01$	
				$P_1 < 0.05$	

and co-stimulatory molecules. It is possible that the observed effects in cancer patients were the result of an increased presence of relatively immature DCs, and this could potentially be reversed by *in vitro* culture. To investigate this possibility, PB mononuclear cells depleted for T cells were cultured for 24–36 h and then labeled with a PE-conjugated mixture of lineage-specific antibodies and FITC-conjugated anti-HLA-DR, B7-2, or CD40 antibodies. We calculated the percentage of HLA-DR^-, B7-2^-, and $CD40^-$ cells in the population of Lin^- cells. Control values were determined in 11 healthy volunteers. To assure interexperimental reproducibility of the results, three volunteers were tested twice, and one was tested three times. In all cases, the variability of the results was less than 20%. Fig. 3*A* illustrates a typical profile in one of the control donors. More that 95% of Lin^- cells also expressed HLA-DR and thus can be classified as DCs. More than 80% of these cells expressed the B7-2 molecule, and more than 60% expressed CD40, both of which are markers of activated DCs. The proportion of these cells was dramatically reduced in cancer patients because of the accumulation of Lin^- HLA-DR^-, B7-2^-, or $CD40^-$ cells (Fig. 3*B*). Thus, *in vitro* culture of cells obtained from PB of cancer patients did not restore the presence of DCs. This indicates that the observed decrease of DCs in PB from cancer patients was not a transitory phenomenon. This decrease was associated with marked increase in the presence of cells lacking markers of mature cells. We refer to these marker-negative cells as "immature."

To confirm the functional significance of these findings, we analyzed the correlation between functional activity of the total fractions of PB DCs from patients with HNSCC studied in experiments shown in Fig. 1 and the presence of DCs in these fractions. Correlation was calculated between an ability of DCs to stimulate allogeneic T cells obtained from control individuals and the proportion of $Lin^-HLA\text{-}DR^+$ or $Lin^-B7\text{-}2^+$ cells. As expected, the presence of Lin^- HLA-DR^+ and Lin^- B7-2^+ cells was closely correlated with proliferation of allogeneic T cells in response to stimulation by DCs ($r = 0.72$; $P < 0.01$).

To investigate the possible correlation between the presence of the immature cells and clinical parameters, patients with three different types of cancer were studied. Patients with each type of cancer were divided into two groups: patients with advanced disease (stages 3–4) and those with early stage disease (stages 1–2). In 11 healthy volunteers, the proportions of cells were as follows: Lin^- HLA-DR^-, 4.0 ± 1.2%; Lin^- B7-2^-, 15.0 ± 2.0%; Lin^- $CD40^-$, 39.7 ± 5.0%. Patients with breast cancer from both groups demonstrated a dramatic increase in the proportion of immature cells. In patients with advanced disease, the proportion of Lin^- HLA-DR^- cells was increased almost 10-fold, and Lin^- B7-2^- was increased more than 4-fold as compared to control individuals (Fig. 4). The proportion of $Lin^-HLA\text{-}DR^-$ cells was significantly higher in patients with advanced disease than in patients with early stage disease (Fig. 4). An even more profound effect was seen in patients with lung cancer. The proportion of Lin^- B7-2^- cells was increased almost six times, and Lin^- $CD40^-$ cells were increased more than 2-fold as compared to controls (Fig. 4). As in the case of patients with breast cancer, patients with advanced disease had a significantly higher proportion of Lin^- HLA-DR^- cells (Fig. 4). The proportion of Lin^- $CD40^-$ cells was significantly higher than in controls only in patients with advanced disease. The same trend was evident in the patients with HNSCC (Fig. 4). Thus, the proportion of immature cells was dramatically increased, and this increase was closely associated with the stage of disease.

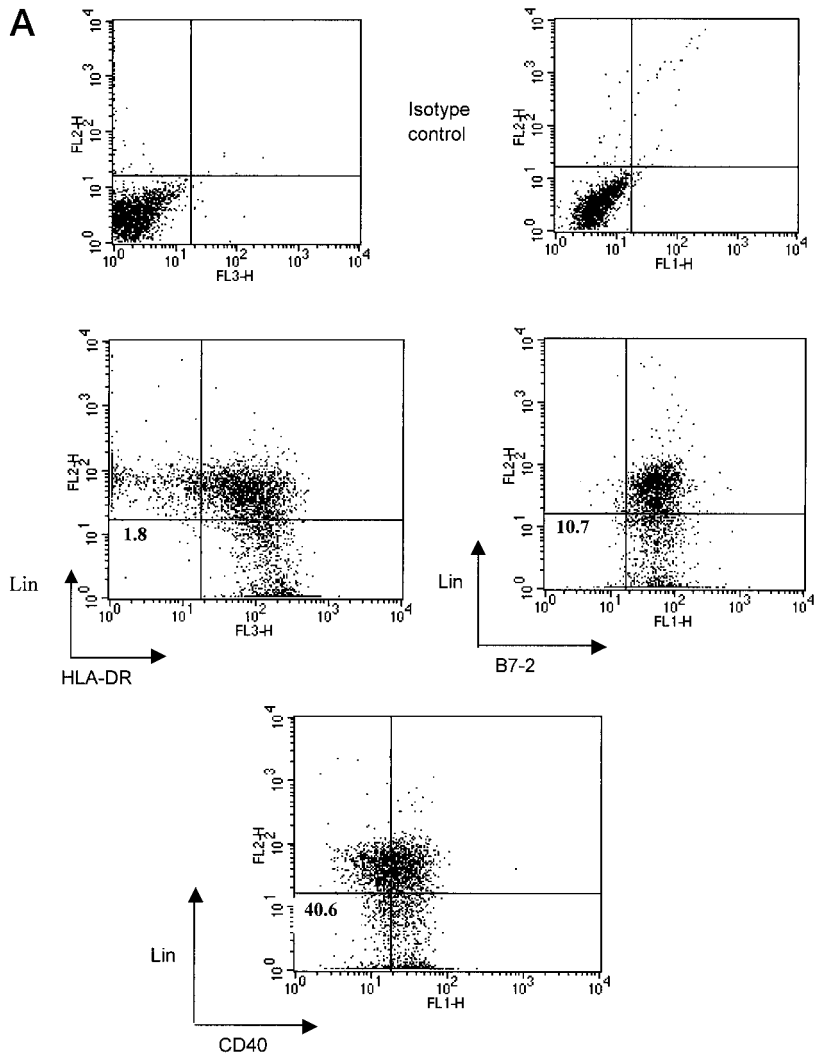

Fig. 3 Distribution of mature and immature DCs in a control donor and a cancer patient. PB cells were enriched for DCs as described in "Patients and Methods" and labeled with antibodies as indicated. Typical results of one healthy donor (*A*) and one patient with HNSCC (*B*) are shown. The percentage of HLA-DR$^-$, B7-2$^-$, and CD40$^-$ cells was calculated from Lin$^-$ cells.

The anatomical site of the tumor in patients with HNSCC has a significant impact on the clinical outcome of the disease (18). We investigated the relation between the presence of immature cells and the tumor location. Two groups of patients with stage 3–4 HNSCC without signs of distant metastases were compared: patients with tumors of the oral cavity (mostly tongue) and patients with tumors of the larynx. These patients did not significantly differ in their age (53.4 ± 5.7 and 57.5 ± 6.8, respectively) or in the presence of risk factors (tobacco and alcohol). We also could not determine statistically significant differences in the time between the first symptoms of the disease and the date of blood collection in these two groups of patients. However, the proportion of Lin$^-$ HLA-DR$^-$ and Lin$^-$ B7-2$^-$ was much higher in patients with tumor of oral cavity (the site associated with a worse prognosis) than in patients with laryngeal cancer (Fig. 5, *top panel*).

Surgical removal of the tumor had a great impact on the presence of immature cells in cancer patients. We compared the proportion of immature cells in five cancer patients. All patients had medically indicated surgical resections of their tumors. Blood was collected before and 3–4 weeks after the surgery but prior to adjuvant chemotherapy or radiation therapy. In all patients, removal of the tumor resulted in a dramatic reduction in the proportion of immature cells, although at the time of collection, these values did not quite reach control levels (Fig. 5, *bottom panel*).

We then asked whether the accumulation of immature cells was associated with the duration of the disease. Duration of the disease was calculated in weeks from the date of appearance of first symptoms of the disease and the date of blood collection. A statistically significant correlation between the presence of Lin$^-$ DR$^-$ cells and the duration of the disease was found for patients with breast cancer ($r = +0.81$, $P = 0.014$). However, no such correlation was seen for patients with HNSCC. Because of the relatively small numbers of patients with lung cancer, such an analysis was not performed in that group of patients. Thus, in all three types of cancer investigated here, the presence of immature cells was dramatically increased, and this increase was closely associated with the stage of disease, presence of tumor, and in some cases duration of disease.

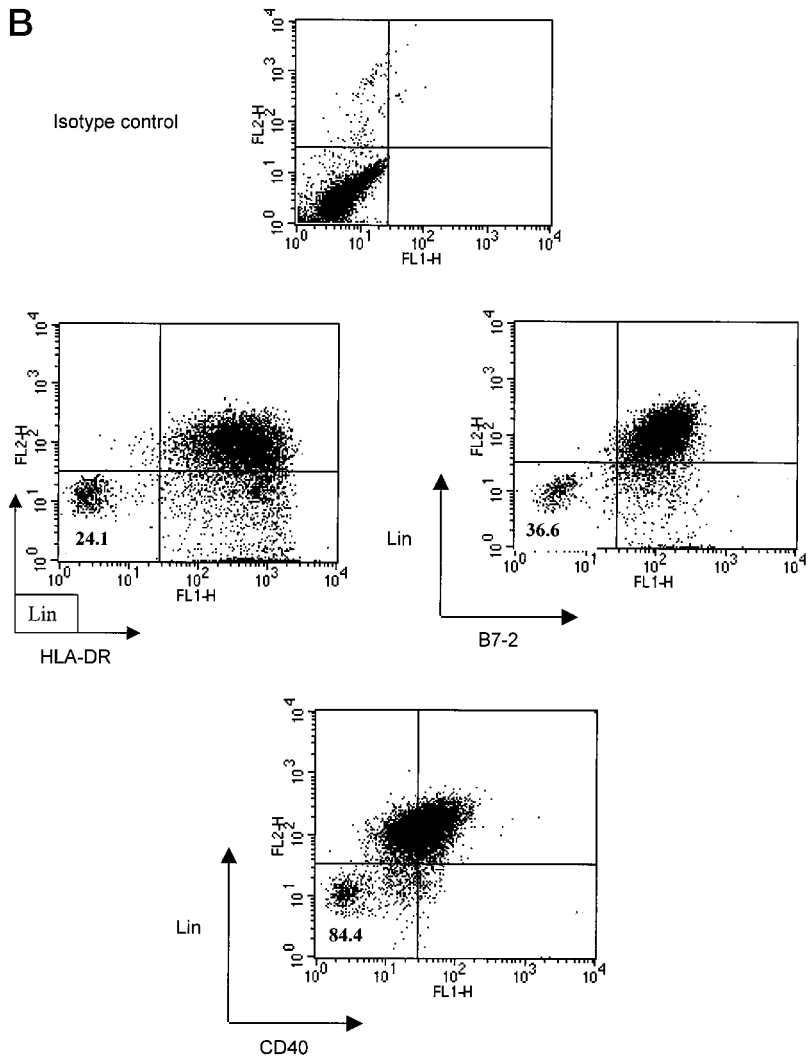

Fig. 3 Continued.

Association of the Increased Proportion of Immature Cells with Increased Concentrations of Growth Factors and Cytokines in the Plasma of Cancer Patients. Several factors have previously been implicated in defective DC maturation in cancer (15, 16, 19). Here, we studied the association between an elevated level of certain growth factors and cytokines in the circulation and the observed increased proportion of immature cells. Plasma samples were collected from 34 patients with advanced cancer, and the levels of M-CSF, GM-CSF, VEGF, IL-6, IL-10 and TGF-β were determined by ELISA (R&D Systems). Control levels of these cytokines were established using plasma from nine healthy volunteers. For each growth factor and cytokine, two groups of patients were compared: patients with the control levels of the factor (within 95% confidence intervals of control samples) and patients with increased levels of the factor. The proportion of immature cells was compared between these two groups. It is important to note that no statistically significant correlation between the levels of the factors was found (data not shown). A significant proportion of patients demonstrated increased concentrations of at least one of five factors (Table 3). We could not detect an increased level of IL-10 in any of the studied patients. The proportion of Lin^- $HLA\ DR^-$ and Lin^- $CD40^-$ cells was significantly lower in cancer patients with normal levels of VEGF in plasma than in those with an elevated VEGF concentration (Table 3). The same trend was evident in the presence of Lin^- $B7\text{-}2^-$ cells, but those differences did not reach statistical significance. No such association with any other factor was found (Table 3). Thus, these data suggest that VEGF might be involved in the generation of immature cells in cancer patients as suggested by animal and *in vitro* data.

The levels of immature cells were evaluated in metastatic lung cancer patients undergoing treatment with an antibody to VEGF. In this trial, six cycles of carboplatin and Taxol chemotherapy were given with anti-VEGF (15 mg/kg every 3 weeks). After six cycles, the chemotherapy was stopped, and the anti-VEGF was continued until disease progression. Blood was drawn for analysis before the start of treatment and then again after more than 3 months of antibody treatment alone. As can be seen, all three patients had improvement in the measured mark-

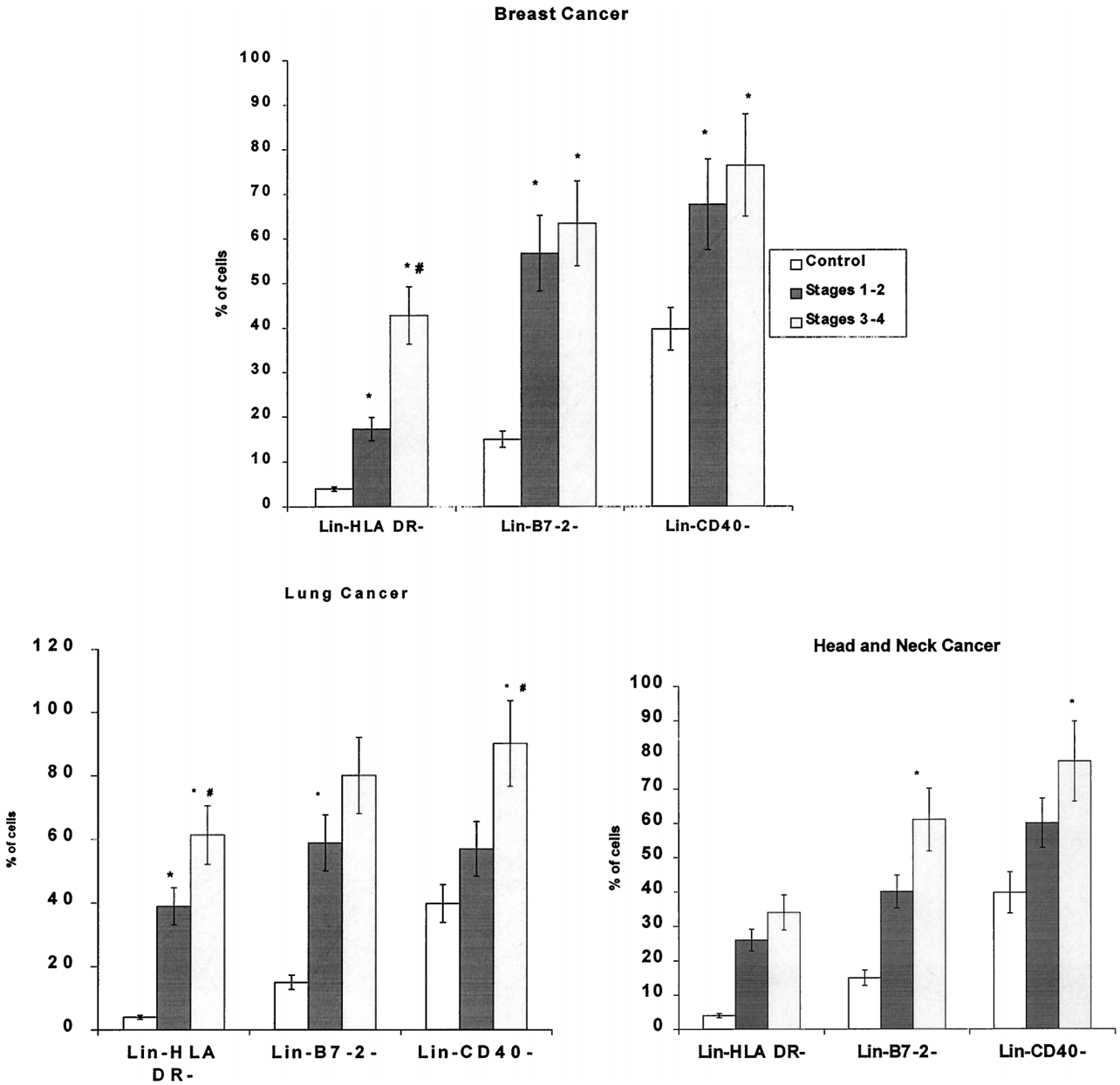

Fig. 4 Proportion of immature cells in patients with different types of cancer. DC fractions were isolated from PB mononuclear cells using sheep red cells and overnight incubation as described in "Patients and Methods." Cells were labeled with a mixture of PE-conjugated lineage-specific monoclonal antibodies and FITC-conjugated HLA-DR, anti-B7-2, or anti-CD40 antibodies as described in "Patients and Methods." The number of patients in each group is presented in Table 1. *, statistically significant differences from the control ($P < 0.05$); #, statistically significant differences between stages 1–2 and 3–4 ($P < 0.05$).

ers (Table 4). It should be noted that none of these patients had a significant tumor response, so the total amount of tumor present at both evaluations was identical.

DISCUSSION

DCs play a central role in the induction of antitumor immune responses. Adequate function of these cells is a crucial factor for effective antitumor control and the success of cancer immunotherapy. Significant evidence of inadequate function of these cells in tumor-bearing hosts has accumulated recently. Several groups have clearly demonstrated that populations of DCs isolated from PB, lymphoid tissues, and tumors of cancer patients and tumor-bearing mice contain cells with low levels of MHC class II molecules and undetectable levels of co-stimulatory molecules. These cells are impaired in their ability to stimulate T cells. At the same time, mature DCs remaining in peripheral lymphatics demonstrate normal levels of functional activity (5–9, 11, 12). Given the fact that functionally potent

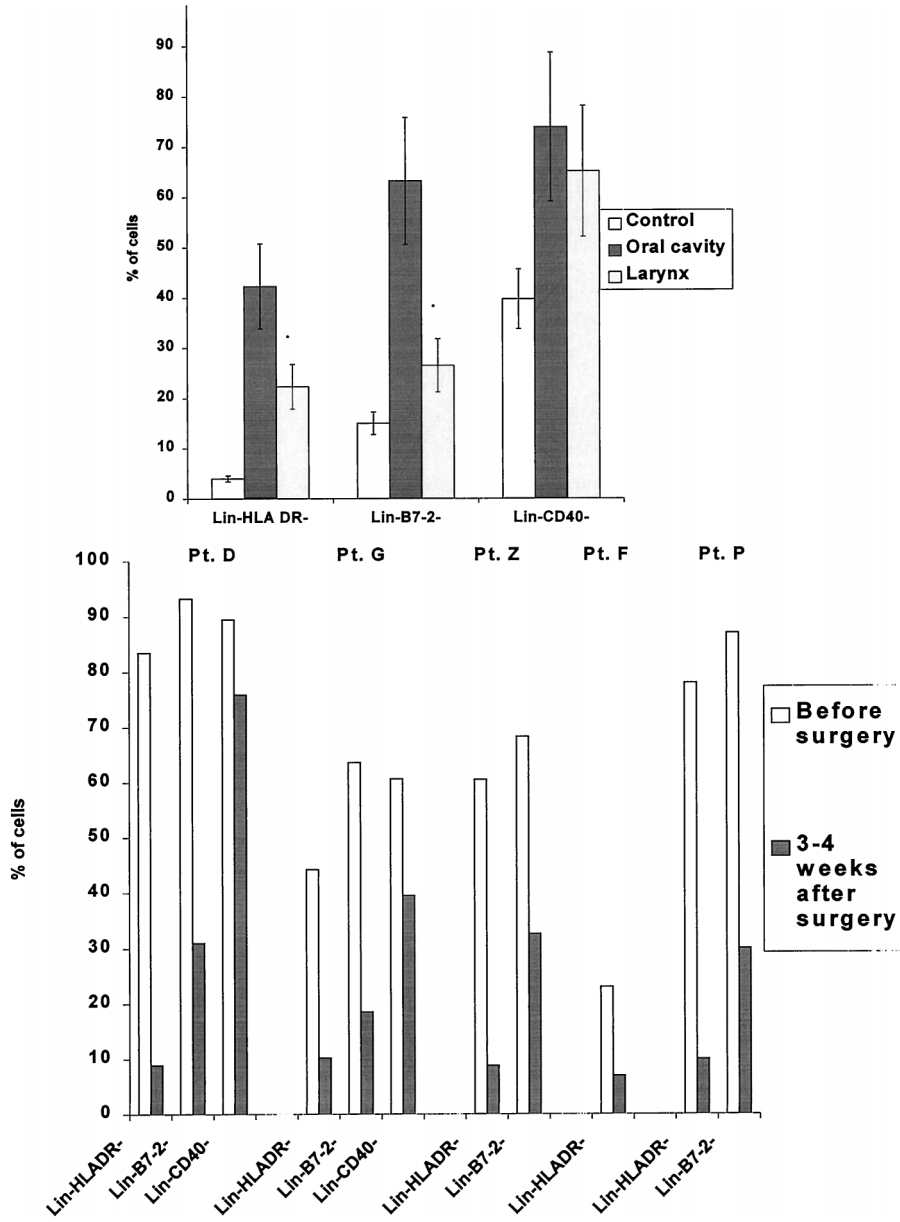

Fig. 5 Proportion of immature cells in PB is associated with tumor localization and tumor presence. *Top*, presence of immature cells in two groups of patients with stage 3–4 HNSCC. Seven patients with laryngeal cancer and 14 patients with oral cancer were compared. PB was collected, and cells were labeled as described in the legend to Fig. 4. *, statistically significant differences between the groups ($P < 0.05$). *Bottom*, five patients with different types of cancer were studied before and 3–4 weeks after surgical resection of their tumors but prior to the start of any adjuvant therapy.

DCs can be generated from progenitors isolated from patients even with advanced stages of cancer, as well as from mice with bulky tumors, we suggested that major events might occur during DC differentiation and maturation. In this study, we have tried for the first time to study defective DC features and function in a large cohort of human cancer patients and whether these DC defects are associated with clinical parameters and antitumor therapy.

The Defect in DC Function in Cancer Patients Is Systemic Rather Than Local. Two main mechanisms might be responsible for the observed DC deficiency. The first mechanism is that immature DCs may come in direct contact with tumor cells, and this contact could affect their maturation, including their ability to take up tumor antigen, migrate to regional LNs, or present antigen to T cells. If any of these mechanisms were prominent, one would expect more profound defects in the numbers and function of DCs isolated from draining LNs than in DCs isolated from PB. This might be especially evident in the earlier stages of cancer. In a recent study, sentinel LNs in breast cancer patients demonstrated a decreased density of paracortical DCs, a reduced frequency of double positive S100 and MHC class II cells and a predominance of immature DCs (20). Alternatively, tumors may exert their effects via tumor-derived factors able to systemically affect DC function or maturation in bone marrow and in other tissues. This would be manifested by equal dysfunction of PB and LN DCs. In our study of 18 patients with head and neck cancer, the function of both LN and PB DCs was equally impaired. These

Table 3 Association between production of immature cells and the presence of growth factors and cytokines in plasma from cancer patients

Growth factors and cytokines				
Name	Level in plasma	$Lin^-HLA\text{-}DR^-$	$Lin^-B7\text{-}2^-$	Lin^-CD40^-
GM-CSF	Normal (n = 14)	25.2 ± 6.1	45.1 ± 8.9	63.6 ± 7.0
	Elevated (n = 15)	34.8 ± 7.1	55.6 ± 10.8	65.7 ± 8.9
TGF-β	Normal (n = 18)	24.5 ± 5.4	42.3 ± 7.7	58.4 ± 6.3
	Elevated (n = 14)	32.8 ± 6.1	46.5 ± 8.2	70.9 ± 6.5
VEGF	Normal (n = 7)	13.4 ± 8.3	32.2 ± 11.7	39.5 ± 10.0
	Elevated (n = 23)	33.0 ± 4.5*	52.4 ± 6.3	72.2 ± 4.9*
IL-6	Normal (n = 15)	27.6 ± 5.9	42.6 ± 7.9	59.5 ± 6.9
	Elevated (n = 18)	28.1 ± 5.4	49.9 ± 7.7	67.9 ± 6.9
M-CSF	Normal (n = 20)	29.2 ± 5.0	50.6 ± 7.0	71.0 ± 6.1
	Elevated (n = 14)	25.0 ± 6.1	39.3 ± 8.0	55.6 ± 6.8

Table 4 Correction of immature cell numbers toward normal after chemotherapy and anti-VEGF treatment in the absence of tumor response. ND, not done. The dose of anti-VEGF is given in parentheses.

	Date	Response	%HLA-DR−	%CD86−	%CD40−
Patient 1 (15 mg/kg)					
Start chemo	7/8/98				
Last chemo	10/20/98	Stable			
Prestudy	7/7/98	Stable	77.6	34.1	69.0
During anti-VEGF	1/19/99	Slight progression	1.5	6.2	11.5
Patient 2 (15 mg/kg)					
Start chemo	7/28/98				
Last chemo	11/18/98	Stable			
Prestudy	7/16/98	Stable	74.0	ND	ND
During anti-VEGF	2/3/99	Stable	4.7	3.26	28.9
Patient 3 (7.5 mg/kg)					
Start chemo	8/6/98				
Last chemo	11/18/98	Stable			
Prestudy	7/30/98	Stable	65.7	87.2	99.4
During anti-VEGF	2/17/99	Stable	6.0	10.9	90.6

data indicate that defects in DCs is a systemic phenomenon rather than an effect confined to local LNs.

The Appearance of Immature Cells Co-purified with DCs Is Clinically and Functionally Relevant. As was reported earlier, the presence of tumor-derived factors inhibits DC differentiation from hematopoietic progenitors *in vitro*, resulting in the generation of immature cells (13, 15–17). Here, we asked whether this process takes place in cancer patients and whether it results in a significant decrease in the numbers of DCs. We used three markers closely associated with DCs: HLA-DR, B7-2, and CD40. The two latter markers reflect the level of functional maturation and activation of DCs. Using a combination of purification and labeling techniques, we calculated the proportion and absolute number of Lin^- $HLA\text{-}DR^+$ DCs in the PB of patients with head and neck cancer. Our results clearly demonstrate a significant decrease in the proportion and number of DCs in patients with early stage cancer and even more profound changes in patients with advanced disease. This decrease was caused by the appearance of cells lacking typical markers specific for the normal cell lineages. We have called them immature cells. The proportion of these immature cells was the same for patients with the three different types of cancer evaluated here (breast, head and neck, and lung). In all three types of cancer, this proportion increased with disease progression. The presence of tumor was critical in the generation of immature cells, because the proportion of these cells dropped dramatically 3–4 weeks after surgery. This is consistent with the hypothesis that the generation of these cells was caused by the production of soluble tumor-derived factors. Thus, it appears that removal of the source of these factors by surgical excision of the tumors restored the DC differentiation process and resulted in improved numbers of DCs in the circulation 3–4 weeks later. We tried to establish a correlation between the presence of immature cells and the duration of disease. A direct correlation was found for patients with breast cancer, but the small number of patients evaluated with lung cancer did not allow this analysis.

Patients with tumors of the oral cavity have a significantly higher rate of recurrence and a poorer prognosis than patients with similar stages of laryngeal cancer. The cause of these differences is not clear. It has been suggested that tumors of the oral cavity manifest later because of anatomical features of the site, and therefore, patients with oral cancer have a longer duration of the disease before diagnosis. We found a significantly higher proportion of immature cells for patients with oral cancer than in those with the same stage of laryngeal cancer. There was no difference in the duration of disease, age, or other factors between these two groups. These data suggest that there is a possibility that clinical differences between these two ana-

tomical sites of otherwise similar tumors may be associated with immunological differences.

Taken together, these data suggest that the appearance of immature cells in the PB of cancer patients is closely associated with decreased presence of DCs and is clinically relevant. It is possible that tumor-derived factors might affect the normal process of DC differentiation, which results in the decreased presence of mature cells. Our data demonstrate that these immature cells were also functionally relevant. Elimination of these cells using fluorescence-activated cell sorting completely restored the functional potency of the DC fraction *in vitro*.[5]

Tumor-derived Factors Involved in Generation of Immature Cells. At this time several factors have been implicated in defective DC differentiation. Using neutralizing antibodies, we demonstrated previously an important role for VEGF and possibly M-CSF in defective DC differentiation (15). Continuous *in vivo* VEGF infusions resulted in dramatic inhibition of DC production (21). VEGF, which is produced by a majority of tumors, plays an essential role in blood vessel formation, and an elevated level of VEGF in the plasma of cancer patients is closely associated with an adverse prognosis (22, 23). It has been recently reported that an elevated levels of VEGF in the vicinity of tumors is closely associated with decreased tumor infiltration by DCs in patients with gastric cancer (24). IL-10 is another factor implicated in the defective DC function. IL-10 has been shown to block the differentiation of monocytes into DCs (25) and to inhibit the function of epidermal Langerhans cells (26–28) and of monocyte- and $CD34^+$-derived DCs (19, 29). Two other factors, M-CSF and IL-6, have recently been reported to be involved in defective DC differentiation. Neutralizing anti-IL-6 and anti-M-CSF antibody abrogated the negative effect of supernatants from renal cell carcinomas on DC differentiation, and incubation of $CD34^+$ progenitor cells with these factors shifted cell differentiation from DCs to monocytes (16). It is important to note that all these studies were performed *in vitro*. To investigate the role of these and some other factors in the generation of immature cells in cancer patients, we measured plasma levels of six growth factors and cytokines, M-CSF, GM-CSF, IL-6, IL-10, TGF-β, and VEGF. Patients were divided into two groups, with normal or elevated levels of each factor. The proportion of immature cells was compared in these two groups. Only those patients with elevated levels of VEGF showed statistically significantly increased numbers of immature cells. Thus, these data support the hypothesis that VEGF plays an important role in abnormal DC differentiation. This is also supported by the data from patients treated with anti-VEGF antibody. However, the proportion of immature cells for patients with normal levels of VEGF was still higher than in controls, suggesting the involvement of additional factors.

In conclusion, for the first time, we have demonstrated a dramatic decrease in the presence of mature DCs in the blood and regional LNs of cancer patients. This was caused by the accumulation of immature myeloid cells at different stages of differentiation. The presence of these cells correlated directly with the stage of the cancer and, in some cases, with its duration. Their presence also decreased after surgical removal of the tumor. The detailed study of the nature and the functional role of these cells is currently under way in our laboratories. Identification of the molecular pathways involved in these effects may lead to therapeutic approaches for blocking their production or inducing their differentiation in cancer patients. This would result in a significant improvement of the function of endogenous DCs and therefore possibly improve the efficacy of immunotherapy and the clinical outcome of the disease.

[5] Manuscript in preparation.

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