Increased Levels of Interleukin-10 in Serum from Patients with Hepatocellular Carcinoma Correlate with Profound Numerical Deficiencies and Immature Phenotype of Circulating Dendritic Cell Subsets

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ABSTRACT

Increased levels of interleukin (IL)-10 have been described as a negative prognostic indicator for survival in patients with various types of cancer. IL-10 exerts tolerogenic and immunosuppressive effects on dendritic cells, which are crucial for the induction of an antitumor immune response. Blood dendritic cell antigen (BDCA)-2 and BDCA-4 are specifically expressed by CD123^{bright} CD11c^{-} plasmacytoid dendritic cells; whereas BDCA-1 and BDCA-3 define 2 distinct subsets of CD11c^{+} myeloid dendritic cells. In this study, the T-helper cell (Th)1/Th2 cytokine serum profile of 65 hepatocellular carcinoma patients was assessed. We found that serum levels of IL-10 were substantially increased in hepatocellular carcinoma patients as compared with controls. Peripheral blood mononuclear cells from healthy volunteers were exposed to recombinant human (rh)IL-10 *in vitro* to additionally characterize its impact on distinct blood dendritic cell subsets. A dramatic decrease of all myeloid dendritic cell (MDC) and plasmacytoid dendritic cell (PDC) subsets was detectable after 24 hours of continuous rhIL-10 exposure. Moreover, the expression of HLA-DR, CD80 and CD86, was significantly reduced on rhIL-10-treated dendritic cell subsets. Direct *ex vivo* flow cytometric analysis of various dendritic cell subpopulations in peripheral blood from hepatocellular carcinoma patients revealed an immature phenotype and a substantial reduction of circulating dendritic cells that was associated with increased IL-10 concentrations in serum and with tumor progression. These findings confirm a predominantly immunosuppressive role of IL-10 for circulating dendritic cells in patients with hepatocellular carcinoma and, thus, may indicate novel aspects of tumor immune evasion.

INTRODUCTION

Hepatocellular carcinoma represents the most common primary malignant tumor of the liver and is one of the major causes of death among patients with cirrhosis (1). Hepatocellular carcinoma ranks as the fifth most common cancer in the world and the third most frequent cause of cancer-related death. The incidence of hepatocellular carcinoma is steadily increasing in both Europe and the United States (1, 2). Increased serum levels of interleukin (IL)-10 have been associated with poor prognosis in patients with hepatocellular carcinoma (3, 4). IL-10 is a pleiotropic cytokine that differently affects various cellular components of the immune system (5). For instance, IL-10 is able to prevent dendritic cell maturation and differentiation by down-regulation of costimulatory molecules and major histocompatibility complex class II, thus inhibiting dendritic cell-mediated antigen priming of naïve T cells (6–9). Moreover, the induction of tolerance and promotion of regulatory T cells have been described for IL-10-treated dendritic cells (10). Although initial data mainly suggested an immunosuppressive role for IL-10, recent results showed that IL-10 may also have immunostimulatory properties on immune effector cells under certain conditions (5, 10). IL-10 down-regulates the expression of major histocompatibility complex class I, thus reducing tumor recognition by CTL and rendering at the same time tumor cells more susceptible to NK cell-mediated lysis (11, 12). Moreover, it has been shown that IL-10 administration before anticancer vaccination results in tumor progression, whereas IL-10 is able to inhibit tumor growth and actually enhances antitumor immunity by promotion of antitumor CTL when administered just after tumor-specific immunization (13–15). The conflicting experimental findings relative to the immunomodulatory effects of IL-10 may depend on the predominant type of cells involved in the immune response at the time of IL-10 exposure, the experimental conditions such as the timing of IL-10 administration, and the dosage (16). It has been previously shown that IL-10 inhibits interferon (IFN)-γ production of T lymphocytes by suppression of IL-12 synthesis in dendritic cells, thereby shifting the T-helper cell (Th) response toward a predominant Th2

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profile (16, 17). Initially, it has been thought that the regulation of Th1 and Th2 responses may depend on the particular dendritic cell subset (18, 19). It has been suggested that $CD11c^+$ myeloid dendritic cells (MDC) primarily induce Th1 differentiation; whereas plasmacytoid dendritic cells (PDC), which express the receptor for IL-3 (CD123), mainly promote a Th2 response (18). However, recent evidence suggests that during inflammation and viral infection, PDC are also able to drive a potent Th1 response, whereas MDC may be influenced by IL-4 and IL-10 to stimulate a Th2 response (20, 21). Therefore, the mechanisms by which dendritic cells control the Th1/Th2 differentiation *in vivo* appear more complex than previously presumed and may depend on various factors such as the maturational state, the cytokine microenvironment, and the type of pathogen to which dendritic cells are exposed (22, 23). It has become evident that tumor cells are inadequate at tumor antigen presentation and CTL activation and that specialized antigen-presenting cells such as dendritic cells are critical for induction of an antitumor immune response. Previous studies have shown that *in vitro* propagated dendritic cells from hepatocellular carcinoma patients have lower expression of major histocompatibility complex class II and impaired capacity to produce IL-12 with consecutive lower potential to stimulate allogeneic T cells (24, 25). However, culturing of dendritic cells for selective growth with certain cytokine combinations may manipulate and alter dendritic cells functionally and thus does not allow for quantitation of dendritic cell frequencies in peripheral blood. Methods for the detection and isolation of fresh dendritic cells in humans are commonly based on immunophenotypic criteria including the absence of a panel of leukocyte lineage (Lin)-specific antigens (CD3, CD14, CD16, CD19, CD20, and CD56) and the presence of HLA-DR (26, 27). These Lin^-DR^+ dendritic cells have originally been differentiated exclusively into a myeloid and plasmacytoid fraction (18, 27). However, novel markers of dendritic cells have been described recently that allow for additional characterization of distinct dendritic cell subsets in peripheral blood (28, 29): (*a*) Blood dendritic cell antigen (BDCA)-1 is expressed on a major subpopulation of $CD11c^{bright}CD123^{dim}$ MDC (MDC1); and (*b*) $BDCA\text{-}3^+$ cells represent a small subpopulation of $CD11c^+CD123^-$ MDC (MDC2); whereas expression of BDCA-2 and BDCA-4 is strictly confined to $CD123^{bright}CD11c^-$ PDC. BDCA-2, a novel type II C-type lectin, is a potent inhibitor of IFN-α/β induction in PDC (30). It expresses CD45RA and the pre-T–cell receptor and has been characterized as being down-regulated on PDC in *in vitro* cultures. In contrast, BDCA-4 expression is induced on culturing. MDC1, which are monocytoid in appearance, express myeloid markers (CD13 and CD33) as well as Fc receptors (CD32, CD64, and FcεRI), and spontaneously develop in mature dendritic cells on culturing even without addition of exogenous cytokines. Unlike BDCA-1, $BDCA\text{-}3^+$ cells lack the expression of CD2 and several Fc receptors such as CD32 and CD64. Previous studies in patients with breast cancer and squamous carcinoma of the head and neck indicated that freshly isolated dendritic cells, in particular MDC, were markedly reduced in number (31, 32). In another study, quantitative and functional alterations of MDC in patients with chronic myeloid leukemia have been described previously (33). However, a more subtle analysis of the frequency and the phenotype of distinct Lin^-DR^+ dendritic cells subsets, as done in our study, provides an opportunity to additionally characterize dendritic cells in the circulation of cancer patients. Most of the existing reports in the literature have been mainly focused on the effect of IL-10 on myeloid monocyte-derived dendritic cells. To our knowledge, this is the first study that directly investigates *in vitro* the influence of the immunoregulatory cytokine IL-10 on distinct dendritic cell populations, including the BDCA subsets, without the addition of exogenous dendritic cell growth factors. Additionally, we provide evidence that low proportion and impaired maturity of freshly isolated dendritic cell subsets from patients with hepatocellular carcinoma are correlated with increased levels of IL-10 in serum.

MATERIALS AND METHODS

Subjects. Peripheral blood was obtained from 65 patients with histomorphologically confirmed hepatocellular carcinoma and from 70 healthy volunteers after obtaining written informed consent. The demographics of the hepatocellular carcinoma patients are depicted in Table 1. Hospital records were reviewed, and tumor-node-metastasis stage of each patient was evaluated according to the International Union against Cancer Classification (6th edition, 2002). Mean age of the 37 male and 33 female healthy volunteers was 57 years (±21.56 SD).

Table 1 Clinicopathological characteristics of the patients with HCC

Characteristics	Patients ($N = 65$)
Sex	
Female	14
Male	51
Age (yr)	
Mean ± SD	60 ± 12.27
Range	26–78
Cirrhosis	54
HCV-induced	21
HBV-induced	10
Alcohol-induced	9
Cryptogenic	13
Autoimmune-induced	1
No cirrhosis	11
HCV infection	2
HBV infection	1
AFP (units/mL)*	
Median	25.9
Range	1–26,000
Tumor status	
T1	14
T2	20
T3	20
T4	11
Nodal status	
N0	48
N1	17
Distant metastasis	
M0	51
M1	14
Tumor differentiation	
Well	20
Moderate	32
Poor	13

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; AFP, α-fetoprotein.

* AFP, <10 IU/ml as reference value.

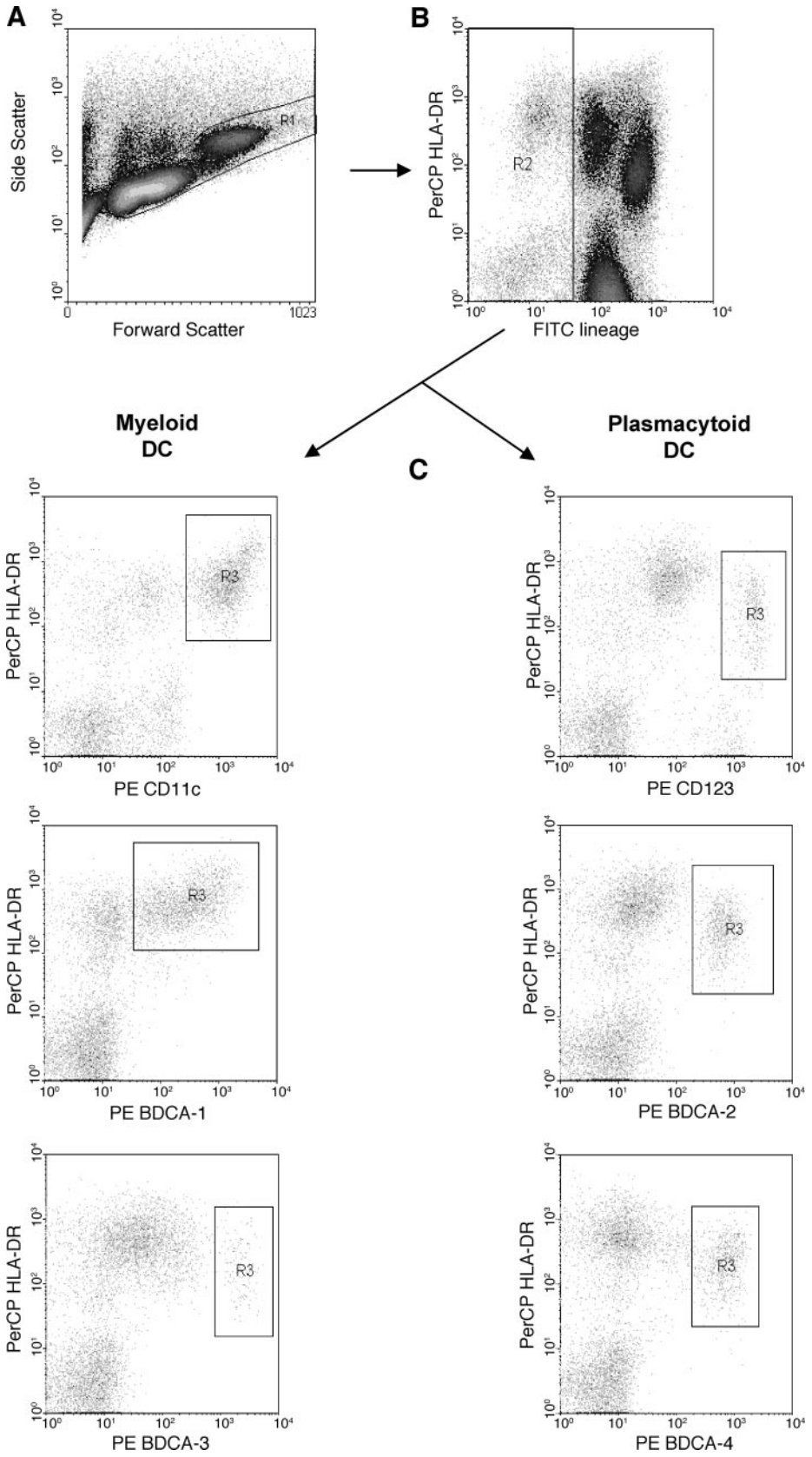

Fig. 1 Gating strategy used to identify circulating PDC and MDC subsets in peripheral blood. A representative experiment done with freshly isolated peripheral blood mononuclear cells from a healthy control is shown. *A*. Using light scatter properties, region *R1* was defined to include lymphocytes and monocytes and to exclude debris. *B*. Cells were gated on region 1 (*R1*). Region *R2* describes HLA-DR$^+$ and Lin$^-$ cells. *C*. By compound gating on *R1* and *R2*, Lin$^-$ cells that are HLA-DR$^+$ and either CD11c^{high} (MDC), CD123^{high} (PDC), BDCA-1$^+$, BDCA-2$^+$, BDCA-3$^+$, or BDCA-4$^+$ can be identified as a distinct dendritic cell population in gate *R3*. (*DC*, dendritic cells)

Th1/Th2 Cytokine Quantitation in Serum. Sera from 65 hepatocellular carcinoma patients and 60 controls were collected for cytokine quantitation. Cytokines were assessed by flow cytometry with the "Human Th1/Th2 Cytokine Cytometric Bead Array (CBA) Kit" (BD PharMingen, Heidelberg, Germany) as described previously (34). The assay sensitivities for IL-2, IL-4, IL-5, IL-10, tumor necrosis factor α, and IFN-γ are 6.6, 6.5, 2.8, 4.7, 4.3, and 15.6 pg/mL.

Exposure of Peripheral Blood Mononuclear Cells to Recombinant Human (rh)IL-10 *In vitro* over Different Time Periods. It has been shown previously that freshly isolated dendritic cells survive poorly in culture, whereas coincubation with peripheral blood mononuclear cells substantially increases dendritic cell viability (35). Thus, instead of directly isolating MDC and plasmacytoid dendritic cell subsets from blood, peripheral blood mononuclear cells from healthy volunteers ($N = 20$) have been purified with Ficoll-separation (Biochrom AG Seromed, Berlin, Germany) as described previously (34). Peripheral blood mononuclear cells were then cultured in X-VIVO 10 (Cambrex Bio Science, Verviers, Belgium), a serum-free medium, at 4×10^6 cells/mL in round-bottomed, 12-well culture plates without addition of exogenous growth factors, and their viability was assessed after 6, 12, 24, 48, and 96 hours. Peripheral blood mononuclear cells were then exposed to rhIL-10 *in vitro* to additionally characterize the role of IL-10 for various dendritic cell subsets in circulation. It has been shown previously that rhIL-10 is most effective when added to dendritic cells at early time points of culture (9). In preliminary experiments, we tested different concentrations of rhIL-10 to assess its effect on circulating dendritic cells. At 20 ng/mL, the activity of rhIL-10 reached a plateau and could not be augmented additionally. Therefore, we added rhIL-10 at a concentration of 20 ng/mL (R&D System, Wiesbaden-Norderstedt, Germany) to peripheral blood mononuclear cells immediately at onset of culture. Cultures from the same healthy volunteers with (*a*) non-rhIL–10-treated peripheral blood mononuclear cells plus IgG isotype and (*b*) peripheral blood mononuclear cells exposed to rhIL-10 and anti-IL–10 monoclonal antibody (mAb; Sigma-Aldrich, Taufkirchen, Germany) simultaneously served as controls. Preliminary dose-response experiments indicated that 10 μg/mL of anti-IL–10 mAb was sufficient to block the effect of rhIL-10. Cells were harvested after 6, 12, 24, 48, and 96 hours of continuous exposure to rhIL-10. Viability of cells harvested from *in vitro* cultures was determined by staining with FITC-conjugated Annexin V and propidium iodide (PI) with the Annexin V-FITC apoptosis detection kit (BD PharMingen).

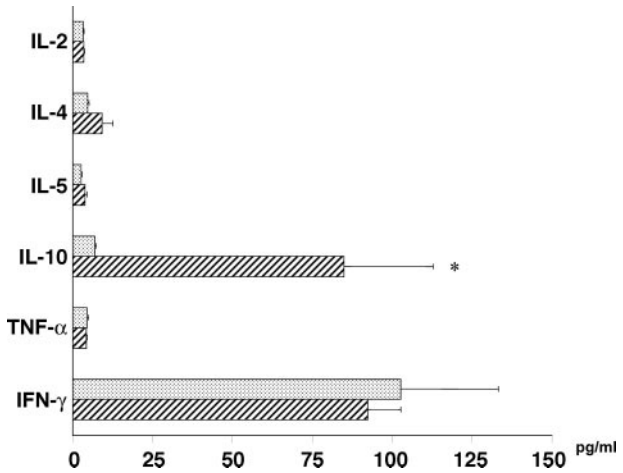

Fig. 2 Serum samples were assessed by FACS analysis for production of Th1 and Th2 cytokines. IL-10 levels were significantly higher in patients ($N = 65$) as compared with controls ($N = 60$). *, $P < 0.001$. There was a trend toward higher concentrations of IL-4 in patients. The results are expressed; *bars*, ±SEM. *P*s are indicated in case of statistical significance. (*TNF*, tumor necrosis factor; *HCC*, hepatocellular carcinoma). ▨ healthy controls; ▨ HCC patients.

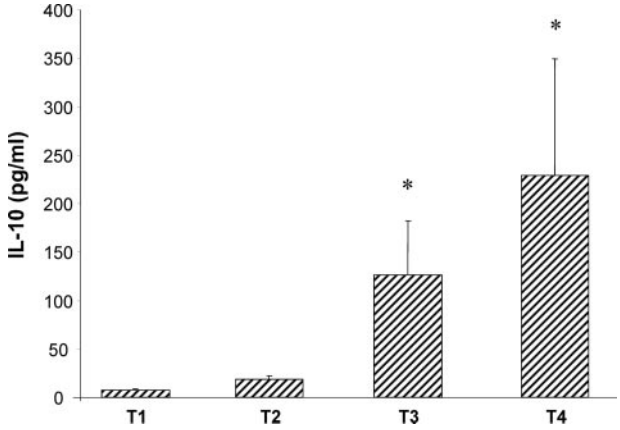

Fig. 3 IL-10 concentrations in serum from hepatocellular carcinoma patients ($N = 65$) are significantly higher at T3 and T4 categories as compared with T1. *, $P < 0.05$. Results are summarized; *bars*, ±SEM.

Flow Cytometric Analysis of Distinct Dendritic Cell Subsets. Peripheral blood mononuclear cells from hepatocellular carcinoma patients and controls were isolated from peripheral blood as described above. Freshly isolated peripheral blood mononuclear cells as well as harvested cells from *in vitro* cultures were analyzed by flow cytometry for quantitation and immunophenotyping of dendritic cell subsets. A cocktail of FITC-labeled mAb against CD3, CD14, CD16, CD19, CD20, and CD56 (Lineage Cocktail 1, BD PharMingen) was used to define Lin^- peripheral blood mononuclear cells (27) that were stained with anti-HLA-DR-peridinin chlorophyll protein (PerCP) and either anti-CD11c-phycoerythrin (PE), anti-CD123-PE (both from BD PharMingen), anti-BDCA-1-PE, anti-BDCA-2-PE, anti-BDCA-3-PE, or anti-BDCA-4-PE mAb (Miltenyi Biotec, Bergisch Gladbach, Germany). For dendritic cell immunophenotyping, four color staining has been done with (*a*) Lineage Cocktail 1, (*b*) anti-HLA-DR-PerCP, and (*c*) either biotinylated anti-CD80 or anti-CD86–allophycocyanin (APC), and (*d*) either anti-CD11c–PE or anti-CD123–PE mAb. Streptavidin-APC (BD PharMingen) was used for indirect immunofluorescent staining with biotinylated anti-CD80 mAb. Respective IgG isotype controls were run for each specimen. Cells were incubated with mAb for 30 minutes on ice and washed twice in PBS containing 0.1% (w/v) BSA and 0.1% (w/v) NaN_3 as described previously (26). After staining, cells were fixed with 2% (w/v) paraformaldehyde in PBS, and flow cytometry was done with a FACScalibur Flow Cytometer (BD PharMingen).

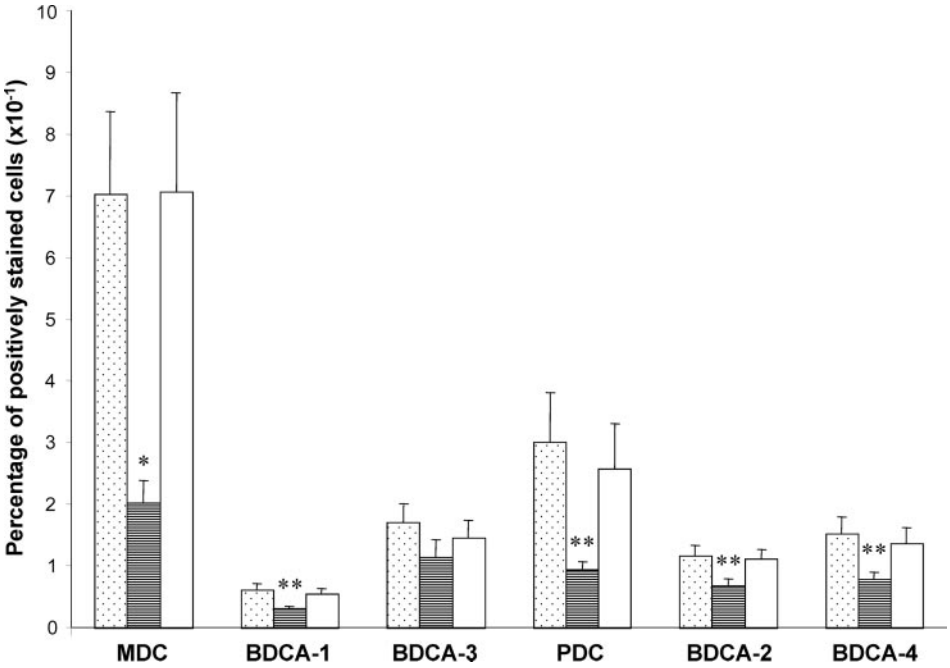

Fig. 4 Exposure of peripheral blood mononuclear cells from healthy volunteers ($N = 20$) to rhIL-10 in *in vitro* short-term (24 hours) cultures leads to depletion of MDC and PDC, including their distinct subsets. Non-rhIL–10-treated peripheral blood mononuclear cells plus IgG isotype and peripheral blood mononuclear cells exposed to rhIL-10 plus anti-IL–10 from the same healthy volunteers served as controls. The effect of rhIL-10 was inhibited by the simultaneous addition of blocking anti-IL–10 mAb to the cell cultures. *P*s are indicated in case of statistical significance. *, $P < 0.001$ in rhIL-10–treated MDC *versus* corresponding controls; **, $P < 0.05$ in rhIL-10–treated PDC, BDCA-1$^+$, BDCA-2$^+$, BDCA-4$^+$ dendritic cell subsets *versus* controls. *Bars*, ±SEM. ▨, control IgG; ▤, rhIL–10; □, rhIL–10 + anti–IL–10.

At least 200,000 events were acquired for each sample. The acquired data were analyzed with the WinMDI program (Version 2.8, Joe Trotter, Scripps Institute, La Jolla, CA). The gating strategy used for the discrimination of the distinct dendritic cell subsets is depicted in Fig. 1.

Statistical Methods. Mann-Whitney *U* test, Kruskal-Wallis rank ANOVA, and ANOVA were used to detect differences between groups. Spearman rank correlation and multinomial logistic regression analyses were done to evaluate effects of gender, age, tumor stage, tumor differentiation, etiology of cirrhosis, α-fetoprotein, alanine aminotransferase values, and Child-Pugh-Turcotte score on serum IL-10, on the proportions of Lin$^-$ peripheral blood mononuclear cells and on dendritic cell subsets. Results are expressed as mean ± SEM unless otherwise specified. For all of the analyses, $P < 0.05$ was defined as statistically significant. Statistical analysis was done with SPSS 12.0 (Chicago, IL) and JMP 5.0 (Cary, NC) statistical software.

RESULTS

Serum Concentration of IL-10 Is Substantially Increased in Hepatocellular Carcinoma Patients. Overall serum concentration of IL-10 was dramatically higher in patients as compared with that of controls ($P < 0.001$; Fig. 2). Furthermore, performing Spearman rank correlation analysis, we found a moderate positive correlation between increased IL-10 concentration and advanced T status (tumor status; T3 and T4) according to the tumor-node-metastasis classification ($r = 0.502$, $P < 0.001$). IL-10 levels were significantly higher in patients categorized T3 or T4 as compared with T1 patients ($P < 0.05$; Fig. 3). No correlations were found between IL-10 levels and demographics of patients such as age, gender, etiology of liver disease, presence or absence of cirrhosis, stage of cirrhosis, tumor differentiation, α-fetoprotein levels, and transaminases. There was a trend toward higher concentrations of IL-4 in patients; however, this difference did not achieve statistical significance. The concentrations of IFN-γ, tumor necrosis factor α, IL-2, and IL-5 in serum were comparable in both study groups (Fig. 2).

IL-10 Directly Suppresses Dendritic Cell Subsets *In vitro*. After short-term culture (24 hours), the percentage of intact viable cells (Annexin V$^-$PI$^-$) was >95% for untreated peripheral blood mononuclear cells and for those pre-exposed to rhIL-10 or rhIL-10 plus anti-IL–10 mAb (data not shown). Peripheral blood mononuclear cells harvested after 96 hours of culture revealed a substantial increase of cells that underwent apoptotic death or died as a result of a necrotic pathway (data not shown). This is consistent with results from recent studies showing that dendritic cells, when maintained in culture with peripheral blood mononuclear cells, are able to survive for ~3 days without the addition of exogenous cytokines (35). A dramatic decrease of MDC and PDC including their BDCA subsets was detectable as early as after 24 hours of continuous rhIL-10 exposure *in vitro* (Fig. 4). Prolongation of the rhIL-10 exposure time did not additionally affect the proportion of dendritic cell subsets (data not shown). The effect of rhIL-10 was inhibited by simultaneous addition of blocking anti-IL–10 mAb to the peripheral blood mononuclear cell in culture (Fig. 4). Under the influence of rhIL-10, a substantial decrease of the mean fluorescence intensity for HLA-DR on MDC, MDC1, PDC, and the BDCA-2$^+$ and BDCA-4$^+$ fractions was detectable (Table 2*A*). Furthermore, the expression of costimulatory molecules CD80/CD86 on MDC and PDC was substantially lower as compared with untreated and rhIL-10 plus anti-IL–10 mAb-treated controls (Table 2*B*).

Table 2 A. Expression of HLA-DR on various DC subsets from healthy volunteers after 24 h of exposure to rhIL-10 *in vitro*

		Control + IgG	rhIL-10	rhIL-10 + anti-IL–10 mAb
Antigen	DC subset	MFI (mean ± SEM)	MFI (mean ± SEM)	MFI (mean ± SEM)
HLA-DR	MDC	1040.7 ± 86.9	784.5 ± 55.8*	993.6 ± 84.2
	BDCA-1$^+$	2156.3 ± 313.4	1280.2 ± 157.0*	1978.9 ± 292.9
	BDCA-3$^+$	1212.2 ± 165.1	854.2 ± 81.5	1183.2 ± 165.2
	PDC	737.1 ± 103.7	482.7 ± 39.1*	686.7 ± 64.3
	BDCA-2$^+$	603.4 ± 72.1	429.7 ± 41.9*	714.9 ± 77.0
	BDCA-4$^+$	819.1 ± 81.5	577.9 ± 54.3*	924.2 ± 85.8

Abbreviations: DC, dendritic cells; MFI, mean fluorescence intensity; PDC, plasmacytoid dendritic cells.
* $P < 0.05$ as compared to both controls.

Table 2 B. Expression of costimulatory molecules on DC subsets from healthy volunteers after 24 h of exposure to rhIL-10 *in vitro*

		Control + IgG	rhIL-10	rhIL-10 + anti-IL-10 mAb
Antigen	DC subset	% positive cells (mean ± SEM)	% positive cells (mean ± SEM)	% positive cells (mean ± SEM)
CD80	MDC	41.2 ± 9.6	21.1 ± 1.4*	42.4 ± 8.6
	PDC	29.4 ± 1.8	21.8 ± 1.7*	29.5 ± 1.8
CD86	MDC	110.0 ± 7.3	52.4 ± 11.0*	98.6 ± 9.1
	PDC	113.0 ± 15.2	43.9 ± 8.0*	95.8 ± 14.9

Abbreviations: DC, dendritic cells; PDC, plasmacytoid dendritic cells.
* $P < 0.05$ as compared to both controls.

Lin$^-$ Cells in Freshly Isolated Peripheral Blood Mononuclear Cells from Hepatocellular Carcinoma Patients Are Comparable in Frequency to That of Healthy Controls but Exhibit Lower Major Histocompatibility Complex Class II Expression. The percentage of Lin$^-$ cells compared with the total number of cells analyzed was not significantly different in patients and healthy controls (Fig. 5). Mean values were 6.14 ± 0.58% for controls and 7.10 ± 0.64% for hepatocellular carcinoma patients. There were no significant associations between characteristics of patients such as disease, age, gender, presence or absence of cirrhosis, stage of cirrhosis, tumor differentiation, α-fetoprotein levels, or transaminases and the overall proportion of Lin$^-$ cells. However, the expression of HLA-DR on the Lin$^-$ fraction was significantly reduced in patients as compared with controls (mean fluorescence intensity 171.69 ± 12.27 *versus* 356.42 ± 46.71; $P < 0.001$), thus indicating the presence of a higher percentage of immature Lin$^-$ cells in the circulation of hepatocellular carcinoma patients.

Low Frequencies of MDC and PDC Subsets in Freshly Isolated Peripheral Blood Mononuclear Cells from Hepatocellular Carcinoma Patients Are Associated with Increased IL-10 Levels in Serum and Advanced T Status. The relative proportion of MDC was dramatically reduced in patients in comparison to controls (Fig. 6). Mean values were 0.09 ± 0.01% *versus* 0.34 ± 0.03%, respectively. Similarly, the percentage of MDC1 was significantly lower in the patient group (0.08 ± 0.01% *versus* 0.23 ± 0.04% in controls). The BDCA-3$^+$ subpopulation of MDC, MDC2, represents a small population in peripheral blood; the difference in the corresponding values, 0.031 ± 0.003% and 0.073 ± 0.01% in patients and controls, was statistically significant. Similarly, a reduced frequency of PDC (0.07 ± 0.01% *versus* 0.17 ± 0.03%) and the corresponding BDCA-2$^+$ (0.08 ± 0.01% *versus* 0.21 ± 0.03%) and BDCA-4$^+$ subsets (0.08 ± 0.01% *versus* 0.20 ± 0.03%) was noted in patients as compared with controls.

Furthermore, we found a close association between reduced percentages of circulating MDC and PDC including the BDCA-1$^+$ and BDCA-2$^+$ subsets and advanced T status (Table 3). Subdividing both study groups relative to their gender revealed maintenance of the statistically significant decrease of dendritic cell subsets in the patients as compared with the

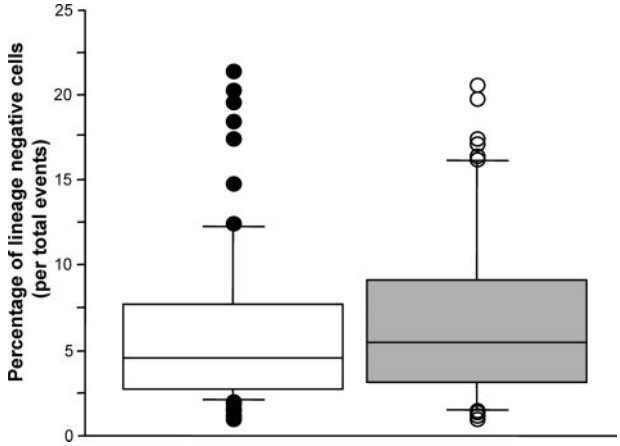

Fig. 5 Hepatocellular carcinoma patients ($N = 65$) and controls ($N = 70$) have comparable percentages of Lin$^-$ cells in freshly isolated peripheral blood mononuclear cells from peripheral blood. The *horizontal line* within each group represents the median. The *bottom* and *top edges* of the *box* correspond to the 25th and 75th percentiles. The whiskers extend up to a distance of 1.5 interquartile ranges. Values farther away are defined as potential outliers. *Bars*, ±SEM. (*HCC*, hepatocellular carcinoma). □, healthy controls; ■, HCC patients.

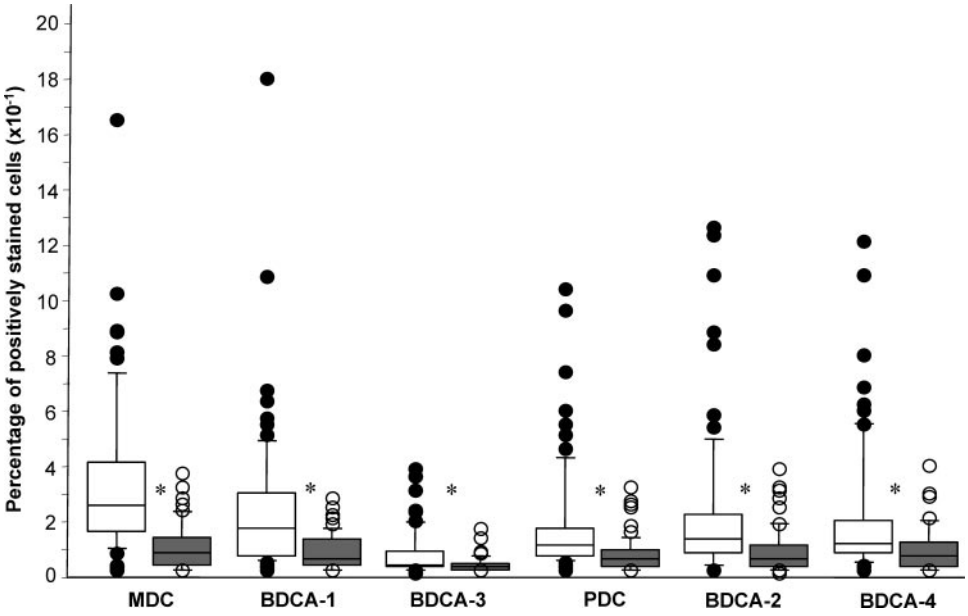

Fig. 6 Three color flow cytometry has been done to distinguish dendritic cell subsets in freshly isolated peripheral blood mononuclear cells of patients and controls. Analysis of the 2 study groups revealed significantly lower percentages of circulating MDC, including the BDCA-1$^+$ and BDCA-3$^+$ dendritic cell subsets, and of PDC, including BDCA-2$^+$ and BDCA-4$^+$ subsets in hepatocellular carcinoma patients ($N = 65$) as compared with normal controls ($N = 70$). *, $P < 0.001$ for all tested subpopulations. See the Fig. 5 legend for the description of the dot plots. *Bars*, ±SEM. (*PDC*, plasmacytoid dendritic cells; *HCC*, hepatocellular carcinoma). □, healthy controls; ■, HCC patients.

Table 3 Association between the T category and the proportion of MDC and PDC subsets in HCC patients ($N = 65$).* Low proportions of MDC and PDC including BDCA-1$^+$ and BDCA-2$^+$ subsets in HCC patients are associated with advanced T status

Cell type	Logistic regression coefficient	χ^2 value	*P*
MDC	−0.277	7.501	<0.05
BDCA-1$^+$	−0.274	7.610	<0.05
BDCA-3$^+$	−0.089	1.922	0.589
PDC	−0.36	9.466	<0.05
BDCA-2$^+$	−0.349	9.085	<0.05
BDCA-4$^+$	−0.004	0.904	0.825

Abbreviations: PDC, plasmacytoid dendritic cells; HCC, hepatocellular carcinoma.

* Evaluated by likelihood ratio χ^2 test.

controls; whereas no significant difference was detectable comparing dendritic cell frequency in males and females within one study group. There was a trend toward an even more pronounced decrease of MDC including the BDCA-3$^+$ subset in patients older than 60 years and a higher percentage of PDC in the older as compared with the younger hepatocellular carcinoma patients. Spearman rank correlation analysis for patients and healthy controls revealed an inverse correlation between number of all dendritic cell subsets and IL-10 concentration in serum (Table 4). Additionally, a markedly reduced MDC number was associated with lower tumor differentiation ($r = 0.304$, $P < 0.05$). No significant correlations were detectable between the percentages of dendritic cell subsets and the type of underlying liver disease, presence or absence of cirrhosis, Child-Pugh-Turcotte score, transaminases, or α-fetoprotein levels.

Circulating Dendritic Cells in Hepatocellular Carcinoma Patients Mainly Consist of Immature Nonactivated Cells. HLA-DR expression was significantly lower on all freshly isolated MDC fractions as well as on BDCA-2$^+$ and BDCA-4$^+$ PDC subsets from patients ($N = 65$) as compared with healthy volunteers ($N = 70$; Fig. 7*A*). The percentages of expression of costimulatory molecules were significantly lower on MDC and PDC from patients ($N = 65$) as compared with controls ($N = 21$; Fig. 7*B*). There was a weak inverse correlation between measured IL-10 levels in serum of patients and the expression of CD80 and CD86 on freshly isolated MDC ($r = -0.442$, $P < 0.001$ and $r = -0.265$, $P < 0.05$, respectively) and PDC ($r = -0.349$, $P < 0.005$ and $r = -0.350$, $P < 0.005$, respectively). In addition, patients with dramatically low CD80 expression on MDC tended to qualify for advanced T status ($r = -0.245$, $P < 0.05$).

DISCUSSION

We investigated the Th cytokine bias in the serum from hepatocellular carcinoma patients and found that IL-10 concentrations were substantially higher as compared with that of healthy controls. It has been suggested that a shift from a Th1 to a Th2 cytokine profile and elevated IL-10 concentrations in serum contribute to an impaired antitumor immune response in

Table 4 Spearman rank correlation between the percentage of freshly isolated DC subsets and IL-10 levels. Frequencies of circulating DC subsets are inversely correlated with measured IL-10 concentrations in serum

Cell type	r*	*P*
MDC	−0.369	<0.001
BDCA-1$^+$	−0.461	<0.001
BDCA-3$^+$	−0.291	<0.005
PDC	−0.358	<0.001
BDCA-2$^+$	−0.444	<0.001
BDCA-4$^+$	−0.387	<0.001

NOTE. $n = 65$ HCC patients plus 60 healthy controls.

Abbreviations: DC, dendritic cells; PDC, plasmacytoid dendritic cells; HCC, hepatocellular carcinoma.

* r, Spearman rank correlation coefficient.

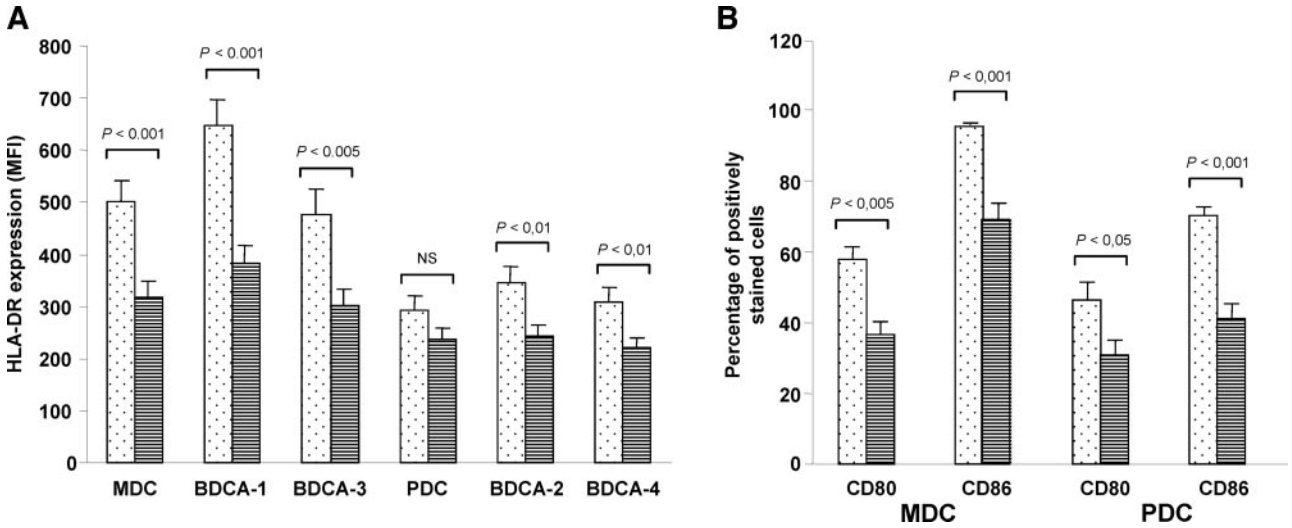

Fig. 7 *A*. HLA-DR expression, measured as mean fluorescence intensity, is significantly lower on freshly isolated distinct MDC fractions and on BDCA-2^+ and BDCA-4^+ dendritic cell subsets from patients ($N = 65$) as compared with controls ($N = 70$). *P*s are indicated in case of statistical significance. *B*. The percentages of CD80 and CD86 are significantly reduced on freshly isolated MDC and PDC from patients ($N = 65$) as compared with healthy volunteers ($N = 21$). (*NS*, not significant; *PDC*, plasmacytoid dendritic cells; *HCC*, hepatocellular carcinoma). ▨, healthy controls; ▤, HCC patients.

patients with hepatocellular carcinoma and other types of cancer (3, 36–39). In line with these previous reports, we found a positive correlation between substantially elevated levels of IL-10 in serum and advanced T category. Similarly to others, we did not observe substantially elevated IL-10 levels in nontumor patients with virally, autoimmune- or alcohol-induced cirrhotic or noncirrhotic liver disease (40, 41).[5] Taken together, these findings suggest that increased IL-10 concentrations found in hepatocellular carcinoma patients are directly related to the presence of the tumor itself. However, tumors have evolved numerous strategies to evade the immune response, and increased IL-10 levels may not necessarily be directly derived from the tumor (5, 42, 43). For instance, the release of prostaglandin E_2, tumor necrosis factor-α, or transforming growth factor β by tumor cells has been shown to induce host immune cells to enhanced production of IL-10 (44–46). IL-10 may have growth-factor or -cofactor activity for tumor cells (5, 47) and may confer tolerogenic properties to dendritic cells, resulting in tumor-specific T-cell anergy (8, 48, 49). Recently, functional deficiencies of dendritic cells, related at least partly to IL-10, have been described in animal tumor models (50, 51). However, most of the previous studies, particularly in the human system, have been conducted by using dendritic cells propagated *in vitro* under the influence of certain cytokine combinations. For instance, Rissoan *et al.* (18) observed that the Th2 cytokines IL-4 and IL-10 decreased the number of PDC and induced their apoptosis during a 6-day culture period. In our *in vitro* study, however, the lower proportion of isolated dendritic cell subsets after short-term exposure to IL-10 was not related to increased cell death (data not shown). Both MDC and plasmacytoid dendritic cells exhibited an immature phenotype, as defined by low expression of major histocompatibility complex class II, CD80, and CD86. Therefore, it might be conceivable that IL-10 inhibits the differentiation as well as the maturation of MDC and PDC subsets. Consistent with our findings *in vitro*, we noted an inverse correlation between IL-10 concentrations measured in serum and frequency of dendritic cell subsets in blood. In particular, in hepatocellular carcinoma patients we observed a dramatic decrease of all circulating MDC and PDC, including the BDCA subsets, with a more pronounced reduction of the myeloid fraction as expressed by a 3.6 *versus* a 2.5-fold decline of the MDC and PDC populations, respectively. In addition, low frequencies of circulating MDC and PDC including the BDCA-1^+ and BDCA-2^+ subsets in hepatocellular carcinoma patients were associated with advanced tumor status and low proportion of MDC correlated with low tumor differentiation. These results are consistent with those obtained from studies with breast cancer and squamous carcinoma of the head and neck patients in which lower numbers of PDC and MDC were observed in advanced tumor stage (31, 32). In our study, we found that increased systemic levels of IL-10 were negatively correlated with the expression of costimulatory molecules, in particular CD80 and CD86, on both circulating MDC and PDC. In addition, all circulating dendritic cell subsets exhibited low major histocompatibility complex class II expression. Thus, circulating dendritic cells in hepatocellular carcinoma patients consist mainly of immature, nonactivated cell subsets. This is concordant with a recent report from Ratta *et al.* (52), showing that peripheral blood dendritic cells of multiple myeloma patients have lower expression of HLA-DR, CD40, and CD80 antigens as compared with that of healthy controls. In contrast, a recent study in patients with breast cancer has shown that freshly isolated MDC, although markedly reduced in number and ability to stimulate antigen-specific, T-cell responses, ex-

[5] Unpublished observations.

hibit a more mature phenotype as compared with controls (31). These contrasting findings possibly reflect the distinct mechanisms by which the tumors interfere with the immune system of the host. In summary, our findings strongly suggest that in hepatocellular carcinoma patients, increased systemic levels of IL-10 may directly account for the alterations in the frequency and maturity of circulating dendritic cell subsets. Recently, it has been shown that the decrease of dendritic cells in peripheral blood from cancer patients is closely associated with the appearance of many cells lacking markers of mature lymphoid and myeloid cells capable of actively suppressing antigen-specific, T-cell responses (53, 54). We also noted in hepatocellular carcinoma patients the existence of immature Lin^- cells; however, similarly to observations in squamous carcinoma of the head and neck patients (32), in our study the overall frequency of Lin^- peripheral blood mononuclear cells in the circulation was comparable between patients and controls. Another explanation for the "loss" of peripheral blood dendritic cells may be because of altered dendritic cells homing behavior with increased recruitment to peripheral tissues and secondary lymphoid organs. In fact, IL-10 has been shown to modulate expression of chemokines and chemokine receptors implicated in the recruitment of dendritic cells, resulting in down-regulation of CCR7 and up-regulation of CCR5 (55). It has also been reported that dendritic cells exposed to IL-10 are more susceptible to lysis by autologous NK cells (56). However, the numerical deficiencies of circulating dendritic cells noted in hepatocellular carcinoma patients may negatively impact the induction of an antitumor immune response. As indicated by others, surgical removal of the tumor only results in partial improvement of the dendritic cells number in peripheral blood (32, 53). Additional adjuvant treatment modalities, therefore, may be warranted to overcome tumor-related dendritic cell suppression. Recently, it has been shown that administration of Flt3 ligand, a growth factor for hematopoietic progenitors, and/or granulocyte-macrophage colony stimulating factor leads to enhanced mobilization of dendritic cells into peripheral blood of patients with cancer (57–59).

In conclusion, our findings indicate novel aspects of tumor immune evasion that are associated with IL-10–mediated suppression of circulating dendritic cell subsets in hepatocellular carcinoma patients. Combined systemic administration of immunomodulating growth factors, as well as cytokines for enhancement of dendritic cells number and function directly *in vivo* may represent a promising adjuvant immunotherapeutic approach for patients with liver cancer.

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REFERENCES

1. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–17.

2. Szabo E, Paska C, Kaposi Novak P, Schaff Z, Kiss A. Similarities and differences in hepatitis B and C virus induced hepatocarcinogenesis. Pathol Oncol Res 2004;10:5–11.

3. Chau GY, Wu CW, Lui WY, et al. Serum interleukin-10 but not interleukin-6 is related to clinical outcome in patients with resectable hepatocellular carcinoma. Ann Surg 2000;231:552–8.

4. Hattori E, Okumoto K, Adachi T, et al. Possible contribution of circulating interleukin-10 (IL-10) to tumor immunity and prognosis in patients with unresectable hepatocellular carcinoma. Hepatol Res 2003; 27:309–14.

5. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19: 683–765.

6. Buelens C, Willems F, Delvaux A, et al. Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. Eur J Immunol 1995;25:2668–72.

7. Buelens C, Verhasselt V, De Groote D, et al. Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor. Eur J Immunol 1997;27:756–62.

8. McBride JM, Jung T, de Vries JE, Aversa G. Il-10 alters DC function via modulation of cell surface molecules resulting in impaired T-cell responses. Cell Immunol 2002;215:162–72.

9. Allavena P, Piemonti L, Longoni D, et al. IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. Eur J Immunol 1998;28:359–69.

10. Mocellin S, Panelli MC, Wang E, Nagorsen D, Marincola FM. The dual role of IL-10. Trends Immunol 2003;24:36–43.

11. Zheng LM, Ojcius DM, Garaud F, et al. Interleukin-10 inhibits tumor metastasis through an NK cell-dependent mechanism. J Exp Med 1996;184:579–84.

12. Kundu N, Fulton AM. Interleukin-10 inhibits tumor metastasis, downregulates MHC class I, and enhances NK lysis. Cell Immunol 1997;180:55–61.

13. Groux H, Cottrez F, Rouleau M, et al. A transgenic model to analyze the immunoregulatory role of IL-10 secreted by antigen-presenting cells. J Immunol 1999;162:1723–9.

14. Berman RM, Suzuki T, Tahara H, et al. Systemic administration of cellular IL-10 induces an effective, specific, and long-lived immune response against established tumors in mice. J Immunol 1996;157: 231–8.

15. Fujii S, Shimizu K, Shimizu T, Lotze MT. Interleukin-10 promotes the maintenance of antitumor CD8(+) T-cell effector function in situ. Blood 2001;98:2143–51.

16. Mocellin S, Marincola F, Rossi CR, Nitti D, Lise M. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. Cytokine Growth Factor Rev 2004;15:61–76.

17. Corinti S, Albanesi C, la Sala A, Pastore S, Girolomoni G. Regulatory activity of autocrine IL-10 on dendritic cell functions. J Immunol 2001;166:4312–18.

18. Rissoan MC, Soumelis V, Kadowaki N, et al. Reciprocal control of T helper cell and dendritic cell differentiation. Science (Wash DC) 1999;283:1183–6.

19. Pulendran B, Smith JL, Caspary G, et al. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. Proc Natl Acad Sci USA 1999;96:1036–41.

20. Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J Exp Med 2000;192:219–26.

21. Cella M, Facchetti F, Lanzavecchia A, Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive potent TH1 polarization. Nat Immunol 2000;1:305–10.

22. Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. Nat Immunol 2000;1:199–205.

23. Steinman RM. Some interfaces of dendritic cell biology. APMIS 2003;111:675–97.

24. Kakumu S, Ito S, Ishikawa T, et al. Decreased function of peripheral blood dendritic cells in patients with hepatocellular carcinoma. J Gastroenterol Hepatol 2000;15:431–6.

25. Ninomiya T, Akbar S, Masumoto T, Horiike N, Onji M. Dendritic cells with immature phenotype and defective function in the peripheral blood from patients with hepatocellular carcinoma. J Hepatol 1999;31:323–31.

26. Beckebaum S, Cicinnati VR, Dworacki G, et al. Reduction in the circulating pDC1/pDC2 ratio and impaired function of ex vivo generated DC1 in chronic hepatitis B infection. Clin Immunol (Orlando) 2002;104:138–56.

27. Willmann K, Dunne JF. A flow cytometric immune function assay for human peripheral blood dendritic cells. J Leukoc Biol 2000;67:536–44.

28. Dzionek A, Fuchs A, Schmidt P, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 2000;165:6037–46.

29. Dzionek A, Inagaki Y, Okawa K, et al. Plasmacytoid dendritic cells from specific surface markers to specific cellular functions. Hum Immunol 2002;63:1133–48.

30. Dzionek A, Sohma Y, Nagafune J, et al. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J Exp Med 2001;194:1823–34.

31. Della Bella S, Gennaro M, Vaccari M, et al. Altered maturation of peripheral blood dendritic cells in patients with breast cancer. Br J Cancer 2003;89:1463–72.

32. Hoffmann TK, Muller-Berghaus J, Ferris RL, et al. Alterations in the frequency of dendritic cell subsets in the circulation of patients with squamous cell carcinomas of the head and neck. Clin Cancer Res 2002;8:1787–93.

33. Mohty M, Isnardon D, Vey N, et al. Low blood dendritic cells in chronic myeloid leukaemia patients correlates with loss of CD34+/Cd38− primitive haematopoietic progenitors. Br J Haematol 2002;119:115–8.

34. Beckebaum S, Cicinnati VR, Zhang X, et al. HBV-induced defect of monocyte-derived dendritic cells leads to impaired Th1 response in vitro: Mechanisms for viral immune escape. Immunology 2003;109:487–95.

35. Ho CS, Munster D, Pyke CM, Hart DN, Lopez JA. Spontaneous generation and survival of blood dendritic cells in mononuclear cell culture without exogenous cytokines. Blood 2002;99:2897–904.

36. Lauerova L, Dusek L, Simickova M, et al. Malignant melanoma associates with Th1/Th2 imbalance that coincides with disease progression and immunotherapy response. Neoplasma (Bratisl) 2002;49:159–66.

37. Aoki Y, Tsuneki I, Sasaki M, et al. Analysis of TH1 and TH2 cells by intracellular cytokine detection with flow cytometry in patients with ovarian cancer. Gynecol Obstet Investig 2000;50:207–11.

38. Lee BN, Duvic M, Tang CK, et al. Dysregulated synthesis of intracellular type 1 and type 2 cytokines by T cells of patients with cutaneous T-cell lymphoma. Clin Diagn Lab Immunol 1999;6:79–84.

39. De Vita F, Orditura M, Galizia G, et al. Serum interleukin-10 is an independent prognostic factor in advanced solid tumors. Oncol Rep 2000;7:357–61.

40. Yumoto E, Higashi T, Nouso K, et al. Serum gamma-interferon-inducing factor (IL-18) and IL-10 levels in patients with acute hepatitis and fulminant hepatic failure. J Gastroenterol Hepatol 2002;17:285–94.

41. Krams SM, Cao S, Hayashi M, Villanueva JC, Martinez OM. Elevations in IFN-gamma, IL-5, and IL-10 in patients with the autoimmune disease primary biliary cirrhosis: association with autoantibodies and soluble CD30. Clin Immunol Immunopathol 1996;80:311–20.

42. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Adv Immunol 2000;74:181–273.

43. Pawelec G. Tumour escape: antitumour effectors too much of a good thing? Cancer Immunol Immunother 2004;53:262–74.

44. Loercher AE, Nash MA, Kavanagh JJ, Platsoucas CD, Freedman RS. Identification of an IL-10-producing HLA-DR-negative monocyte subset in the malignant ascites of patients with ovarian carcinoma that inhibits cytokine protein expression and proliferation of autologous T cells. J Immunol 1999;163:6251–60.

45. Menetrier-Caux C, Bain C, Favrot MC, Duc A, Blay JY. Renal cell carcinoma induces interleukin 10 and prostaglandin E2 production by monocytes. Br J Cancer 1999;79:119–30.

46. Maeda H, Shiraishi A. TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. J Immunol 1996;156:73–8.

47. Asadullah K, Sterry W, Volk HD. Interleukin-10 therapy-review of a new approach. Pharmacol Rev 2003;55:241–69.

48. Steinbrink K, Jonuleit H, Muller G, et al. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. Blood 1999;93:1634–42.

49. Gunzer M, Grabbe S. Dendritic cells in cancer immunotherapy. Crit Rev Immunol 2001;21:133–45.

50. Lee WC, Chiang YJ, Wang HC, et al. Functional impairment of dendritic cells caused by murine hepatocellular carcinoma. J Clin Immunol 2004;24:145–54.

51. Yang AS, Lattime EC. Tumor-induced interleukin 10 suppresses the ability of splenic dendritic cells to stimulate CD4 and CD8 T-cell responses. Cancer Res 2003;63:2150–7.

52. Ratta M, Fagnoni F, Curti A, et al. Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. Blood 2002;100:230–7.

53. Almand B, Resser JR, Lindman B, et al. Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res 2000;6:1755–66.

54. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol 2001;166:678–89.

55. Takayama T, Morelli AE, Onai N, et al. Mammalian and viral IL-10 enhance C-C chemokine receptor 5 but down-regulate C-C chemokine receptor 7 expression by myeloid dendritic cells: impact on chemotactic responses and in vivo homing ability. J Immunol 2001;166:7136–43.

56. Carbone E, Terrazzano G, Ruggiero G, et al. Recognition of autologous dendritic cells by human NK cells. Eur J Immunol 1999;29:4022–9.

57. Maraskovsky E, Daro E, Roux E, et al. In vivo generation of human dendritic cell subsets by Flt3 ligand. Blood 2000;96:878–84.

58. Morse MA, Nair S, Fernandez-Casal M, et al. Preoperative mobilization of circulating dendritic cells by Flt3 ligand administration to patients with metastatic colon cancer. J Clin Oncol 2000;18:3883–93.

59. Kiertscher SM, Gitlitz BJ, Figlin RA, Roth MD. Granulocyte/macrophage-colony stimulating factor and interleukin-4 expand and activate type-1 dendritic cells (DC1) when administered in vivo to cancer patients. Int J Cancer 2003;107:256–61.3

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