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RAPID COMMUNICATION

Direct *ex vivo* analysis of dendritic cells in patients with hepatocellular carcinoma

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Abstract

AIM: To analyze the phenotype and function of dendritic cells (DC) from patients with hepatocellular carcinoma (HCC) in order to understand their role in this disease.

METHODS: Myeloid dendritic cells were enumerated in peripheral blood of HCC patients. CD80, CD83, CD86 and HLA-DR expression on naïve and stimulated myeloid dendritic cells from peripheral blood were analyzed. Myeloid dendritic cells were isolated from peripheral blood and their function was tested. Phagocytosis was analyzed using FITC-dextran beads, peptide specific stimulation, the capacity to stimulate allogeneic T cells and secretion of cytokines upon poly dI:dC was tested.

RESULTS: Myeloid dendritic cells were reduced in patients with HCC. No differences in CD80, CD83, CD86 and HLA-DR expression were found on naïve and stimulated myeloid dendritic cells from HCC patients and healthy controls. Normal phagocytosis or stimulation of peptide specific T cells was observed in contrast to an impaired allo-stimulatory capacity and a reduced IL-12 secretion.

CONCLUSION: Impaired IL-12 production of mDCs in patients could lead to an impaired stimulatory capacity of naïve T cells suggesting that IL-12 directed therapies may enhance tumor specific immune responses in HCC patients.

Key words: Myeloid DC; Hepatocellular carcinoma; CD80; CD83; CD86

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world with a poor prognosis and limited survival in the majority of the patients[1, 2]. A number of different studies suggest that immunotherapeutical approaches will be successful for the treatment of this disease[3, 4]. Lymphocytic infiltration of the tumors is indicative of a better survival after surgical resection of the tumors[5]. Adjuvant treatment with activated lymphocytes has also been shown to increase tumor free survival after removal of the tumor[6]. Others and we identified potential tumor antigens for HCC[7-9], which can be used for vaccination purposes including peptide pulsed dendritic cells (DCs).

DCs are the most potent professional antigen-presenting cells (APC) unique in their ability to efficiently prime both $CD4^+$ and $CD8^+$ cytotoxic T cell responses following encounter with antigen and maturation[10]. Upon antigen uptake, DCs undergo the process of maturation and migration and upregulate adhesion and costimulatory molecules and secrete cytokines[11-13].

Two main subsets of DCs namely myeloid (mDCs) and plasmacytoid DCs have been described. These subsets differ in their origin, expression of phenotypic markers, route of activation and immunological activity[14]. $CD11c^+$ mDCs originate from myeloid bone marrow precursors and produce large amounts of IL-12 upon stimulation, which in turn promotes cell-mediated immune responses that are crucial for the detection and elimination of malignant cells[14, 15]. $CD11c^-$ pDCs, however, are of lymphoid origin, express high levels of IL-3 receptor-α chain and secrete less IL-12. A number of different antibodies have recently been generated, which allow for detection and isolation of these DC subgroups from peripheral blood

and other tissues[16]. The development of new DC specific antibodies[16] allows for direct analysis of DC numbers in peripheral blood and minimizes isolation or culture procedures for the enrichment of DCs that may induce the selection of particular cell subsets and phenotypic or functional modifications of DCs present in the blood[17].

Several studies have suggested that impaired function of DCs might be an important factor in the escape of the tumor from the immune control in cancer patients[18]. At least two mechanisms by which tumors can alter DC maturation and DC function are currently known. The first one affects the ability of hematopoietic progenitor cells to differentiate into functional DCs during the early stages of their maturation[19], whereas the second one affects the *in vitro* maturation of DCs from $CD14^+$ monocytes[20]. An understanding of the mechanisms of defects in DC function in different cancers will shed light on the role of DCs in tumor escape mechanisms[21]. Moreover, correction of DC dysfunction might be one possible option to enhance tumor-specific immune responses in HCC, which can be either used alone or in combination with immunotherapy to treat HCC.

This study investigates the frequency and function of circulating mDCs from patients with HCC directly *ex vivo*. mDCs were analyzed in detail for expression of costimulatory molecules and MHC class II surface expression directly *ex vivo* and after poly dI:dC stimulation. Most importantly, we have directly isolated mDCs from blood of HCC patients to test their function in four different functional assays: stimulation of allo-responses, stimulation of peptide-specific responses, phagocytosis and secretion of IL-10, IL-12 and TNF-α. We could clearly demonstrate that mDCs from patients with HCC displayed a normal phenotype, but were impaired in IL-12 secretion possibly leading to a decreased allostimulatory capacity observed in our study.

MATERIALS AND METHODS

Human subjects

Blood samples were collected from patients in the Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Germany. Written consent was obtained from all patients before blood and tumor sampling and the Ethics Committee of Hannover Medical School approved the study protocol. HCC was diagnosed according to the diagnostic guidelines of the European Association for the Study of the Liver (EASL)[22]. Serological tests (i.e., HCV RNA, hepatitis B surface antigen) were performed as previously described[7]. Liver cirrhosis and tumor staging were done according to Child-Pugh[23] and CLIP[24].

Quantification and phenotyping of mDCs

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood as previously described[7] and stained using the following antibodies: anti-CD1c-PE, anti-CD14-PE-Cy5, anti-CD19-PE-Cy5, anti-HLA-DR, anti-CD80-FITC, anti-CD83-FITC and anti-CD86-FITC (Pharmingen) or the corresponding isotype controls and analyzed on a fluorescence-activated cell sorter (FACS Calibur, Becton-Dickinson, Heidelberg, Germany). When indicated PBMCs were incubated for 4 h in the presence of poly dI:dC (Amersham Pharmacia). A total of 500 000 to 1×10^6 events were acquired for each sample on an FACS Calibur. 7-amino-actinomycin D (7-AAD) was used to exclude dead cells from analysis. Four-color analysis was performed using Cell Quest Software (Becton Dickinson, Heidelberg, Germany). Absolute numbers of DCs per ml of blood were calculated using the following equation: (% of gated DCs-% of isotype control) × absolute number of leukocytes.

Intracellular cytokine analysis

Intracellular FACS analysis was performed to analyze cytokine secretion by mDCs. PBMCs were isolated from peripheral blood and stimulated with poly dI:dC (Amersham Biosciences) for 48 h. Golgi-Stop (Pharmingen) was added during the last 6 h of stimulation. PBMCs were stained with a panel of antibodies to detect mDCs as described above, fixed, permeabilised and labeled with FITC-labeled antibodies against TNF-α, IL-10 and IL-12 and corresponding isotype controls (Caltag Laboratories). The evaluation of cytokine production was based on the percentage of cytokine expressing $CD1c^+$,$CD19^-$, $CD14^-$ cells.

Isolation of PBMC and CD1c+ DCs

PBMCs were isolated from heparinized blood by standard density gradient separation using Ficoll density gradient (Biochrom AG, Berlin, Germany). $CD1c^+$ cells were positively selected using a two-step immunomagnetic cell sorting procedure (MACS) following the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, $CD19^+$ cells were depleted from freshly isolated PBMCs followed by positive selection of $CD1c^+$ cells. Purity of $CD1c^+$ DC was controlled by flow cytometry and found to be > 85%.

Quantification of endocytosis by freshly isolated mDCs

Measurement of FITC-dextran uptake was performed as previously described[25]. In brief, freshly isolated mDCs were suspended in culture medium and FITC-dextran (MW 40 000) (Molecular Probes, Leiden, The Netherlands) was added at a final concentration of 1.0 mg/mL and incubated at 37 ℃ for 30 min. The DCs were washed four times with cold PBS containing 1% fetal calf serum and the incorporation of FITC-dextran was analyzed using FACScan. The incorporation of FITC-dextran at 4 ℃ was used as control. The data are shown as fluorescence intensities.

Mixed lymphocyte reactions using freshly isolated CD1c positive DCs

Allogeneic PBMCs (1×10^5) from a healthy donor were resuspended in complete RPMI medium (Gibco BRL, Eggenstein, Germany) supplemented with 2% human AB serum, plated with indicated numbers ($10\text{-}10^4$) of $CD1c^+$ DCs in 96 well plates (Greiner, Frickenhausen, Germany) and incubated at 37 ℃ in a humidified incubator contain-

ing 50 ml/L CO_2. Phytohaemagglutinin 1 μg/mL (Sigma Aldrich, Munich, Germany) was added to PBMC in separate wells as a positive control. After 5 d, 1 μCi/well [3H]-thymidine (Amersham Biosciences, Freiburg, Germany) was added and plates were harvested after 16 h and harvested onto filters using an automated cell harvester (Wallac, Turku, Finland). The amount of [^{3}H]-thymidine incorporated into DNA was measured by a beta-counter (Wallac, Turku, Finland), and the results were expressed as mean counts per minute (cpm). A stimulation index (SI) was calculated for each donor by dividing the mean cpm of the replicates of DC stimulated PBMC by that of the control cultures (PBMC incubated in medium in the absence of DCs).

Analysis of antigen-specific stimulatory potential using a peptide specific T cell line

mDCs (1×10^5) were incubated with the M1 peptide or an irrelevant peptide control for 1 hour. After 1 hour, cells were washed and incubated with 1×10^5 M1 specific T cells. In parallel, T cells were stimulated with T2 cells pulsed with the M1 peptide or a negative control. After 20 hours supernatants were harvested and tested for GM-CSF release by ELISA (Pharmingen).

Statistical analysis

Kruskall-Wallis' analysis of variance and Student's *t* test were used to assess differences between the different study cohorts. Differences with a *P* value of < 0.05 were considered statistically significant.

RESULTS

Detection and quantification of mDCs in peripheral blood

Peripheral blood from a total of 25 patients with HCC was analyzed. Patients' and control group clinical characteristics are shown in Tables 1 and 2. Using a CD1c specific antibody, mDCs were detected in peripheral mononuclear cells and the frequency was determined for every individual subject tested. As shown in Figure 1, the median frequency of mDCs was significantly ($P < 0.001$) decreased for HCC patients (1.5 ± 0.24 mDCs/μL), compared with 23 healthy controls (3.14 ± 0.41 mDCs/μL). In order to exclude that the observations made was only due to chronic liver disease but not due to the presence of HCC, we have also analyzed mDCs from different control groups such as patients with chronic HBV and HCV infection, non-viral liver cirrhosis as well as patients with metastasis from colorectal carcinoma. However, significantly lower mDC frequencies were found in blood from patients with HCC, when compared to patients with chronic HBV infection (2.03 ± 0.52 mDCs/μL), non-viral liver cirrhosis (1.95 ± 1.2 mDCs/μL) and colorectal liver metastasis (1.48 ± 0.61 mDCs/μL) (Figure 1A). In addition, direct comparison of HCC patients with and without chronic viral hepatitis revealed no significant differences as shown in Figure 1B.

Phenotypic analysis of circulating mDCs in peripheral blood before and after in-vitro stimulation

mDCs from patients with HCC and healthy controls were further analyzed for the expression of costimulatory molecules and MHC class II by FACS analysis directly *ex vivo* (Figure 2A). No significant differences were found for the expression of CD80 (HCC patients 0.9 ± 0.05 *vs* healthy 1.0 ± 0.04), CD83 (HCC 1.54 ± 0.38 *vs* healthy 2.2 ± 0.51), CD86 (HCC 36.5 ± 9.54 *vs* healthy 34.7 ± 9.57) and HLA-DR (HCC 83.2 ± 10.1 *vs* healthy 89.5 ± 15.2). Next, mDCs, which are known to express TLR3[26], were stimulated with poly dI: dC and expression of CD83, CD86 and MHC class II was analyzed. As shown in Figure 2B, poly dI:dC stimulation induced a strong up-regulation of CD83 on mDCs. However, there was no difference in CD83 up-regulation between mDCs from HCC patients and healthy controls (6.58 *vs* 8.09). Similar results were found when stimulated mDCs from HCC patients and healthy controls were analyzed for up-regulation of CD86 (HCC 3.53 *vs* healthy 4.09) and HLA-DR (HCC 1.88 *vs* healthy 1.8), indicating no difference in the ability of mDCs to mature after poly dI:dC stimulation.

Table 1 Clinical characteristics of HCC patients analyzed in Figure 1 A and B

Age (yr)	Cause	Sex	Child score	AFP (μg/L)	CLIP score	mDCs (cells/μL)
58	Ethanol	M	A	419	1	0.76
61	Ethanol	M	A	5	1	1.46
65	Ethanol	M	A	380	1	1.67
67	Unknown	M	B	5	2	2.35
55	Ethanol	M	B	3399	3	2.02
70	Ethanol	M	C	5829	4	0.16
76	HBV	M	B	8	2	0.22
67	HBV	M	C	126	3	0.99
49	HBV	M	A	3845	1	0.54
55	HBV	M	B	5	0	4.93
46	HCV	M	B	32	1	1.17
69	HCV	M	A	8803	2	2.09
64	HCV	F	B	2458	4	0.28
63	HCV	M	A	5	3	1.03
70	Ethanol	F	B	6	3	0.82
72	Unknown	M	A	64	2	3.95
63	Ethanol	M	A	9	0	2.28
56	HCV	M	B	10	1	1.40
8	Ethanol	M	A	155509	3	0.55
64	HCV	F	A	6	3	2.04
67	None	M	n/a	18	2	1.37
55	HCV	M	B	33	1	1.07
71	HBV	M	A	10	2	0.71
61	Ethanol	M	B	455	3	2.63
70	HCV	F	A	3462	1	1.19

Table 2 Clinical characteristics of control groups with HCC in Figure 1 A

Group	Age (Mean, yr)	Liver cirrhosis
Healthy	64	None
HCV	54	None
HBV	45	None
Liver cirrhosis	46	Child A and B
Liver metastasis	64	None

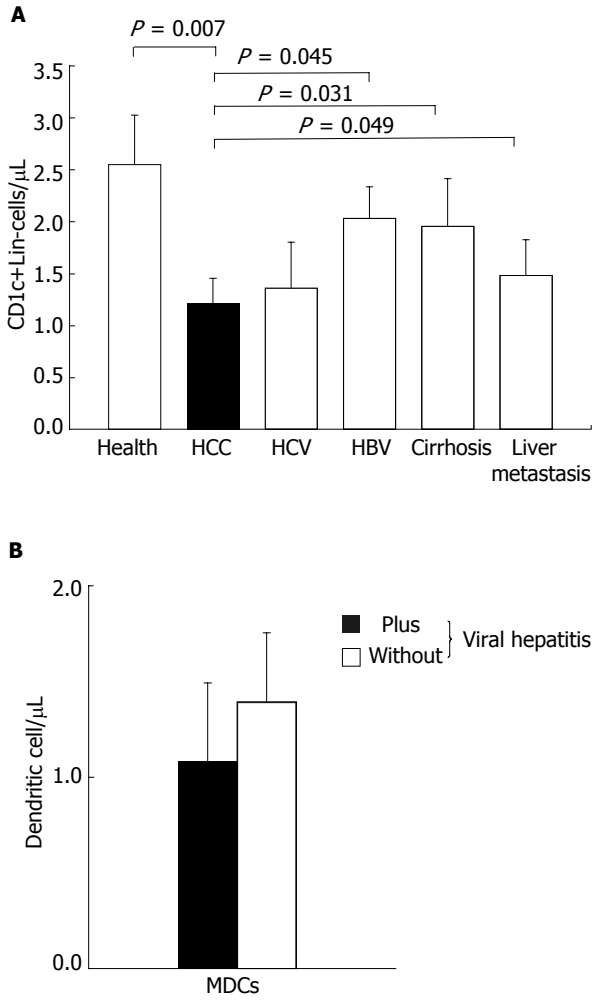

Figure 1 Quantification of dendritic cell subtypes. **A**: Analysis of different patient groups; **B**: Comparison of HCC patients with or without chronic viral hepatitis.

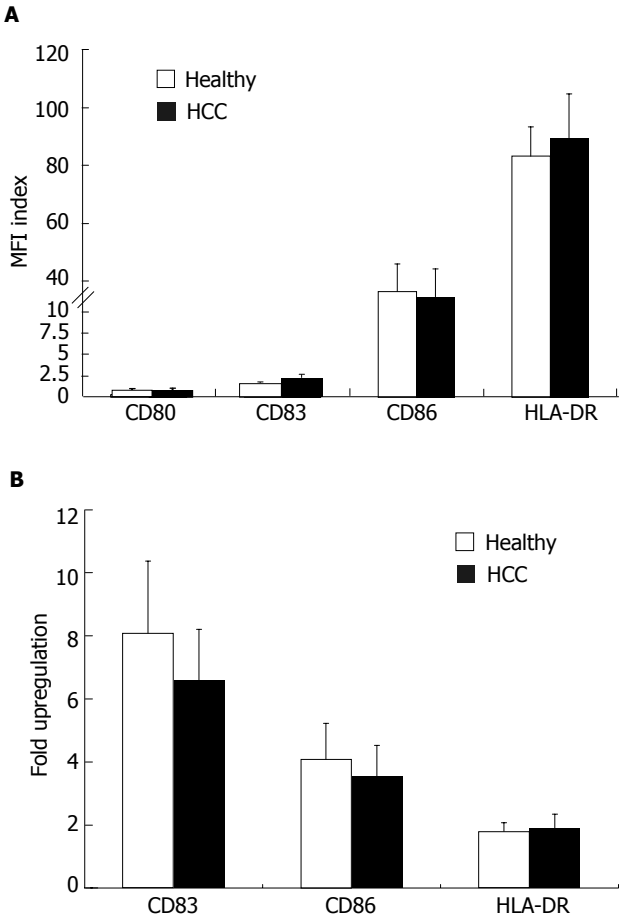

Figure 2 Phenotypic analysis of myeloid dendritic cells in peripheral blood.

FITC-dextran uptake by mDCs from HCC patients and healthy controls

Using CD19 depleted cells, we were able to isolate CD1c$^+$ mDCs from peripheral blood and analyze their function directly *ex vivo* and without *in vitro* stimulation or maturation. This procedure resulted in > 85% pure mDC populations (data not shown), avoided *in vitro* artifacts and therefore mimicked closely the *in vivo* situation. First, endocytosis ability of freshly isolated mDCs was measured using FITC dextran beads. FITC dextran uptake was compared between mDCs from HCC patients and healthy controls. As shown in a representative experiment (Figure 3A), phagocytosis could easily be followed over time with an increase of mean fluorescence intensity (MFI) from 6.3 to 14.6, 19.7 and 27.7 at 0, 7, 15 and 30 min respectively. Figure 3B shows the relative increase of MFI for the two groups after 7, 15 and 30 min. No significant difference was seen when endocytosis by mDCs from HCC patients and healthy donors was compared.

mDCs can stimulate antigen-specific T cells

Since mDCs from HCC patients and healthy controls could take up dextran beads equally well, we next analyzed the antigen presenting capacity of these cells. To assess the ability of mDCs to stimulate antigen-specific CD8$^+$ T cells, mDCs from HLA-A2 positive healthy donors and HCC patients were used to stimulate T cells specific for a common recall antigen, the Influenza matrix peptide M1[27]. mDCs were pulsed with M1 peptide and an irrelevant peptide and used to stimulate M1-specific CD8$^+$ T cells. T2 cells pulsed with the same peptides were used as control for every experiment in parallel. Cytokine secretion by M1 specific CD8$^+$ T cells was analyzed in the cell supernatants. Influenza M1 specific T cells secreted 306 pg/mL GM-CSF when mDCs from HLA-A2 positive donors were used (Figure 4) and slightly lower GM-CSF was released when M1 specific T cells were stimulated with mDCs from HCC patients (159 pg/mL). However, this difference was not significant, since the T2 stimulation (as a positive control) was also slightly different between the two experiments (Figure 4). As expected, no peptide specific GM-CSF secretion was found when mDCs were used from an HLA-A2 negative donor (data not shown).

The allo-stimulatory capacity of mDCs from HCC patients is impaired

To test the allostimulatory potential of mDCs from HCC patients, we studied their function in an allogeneic mixed

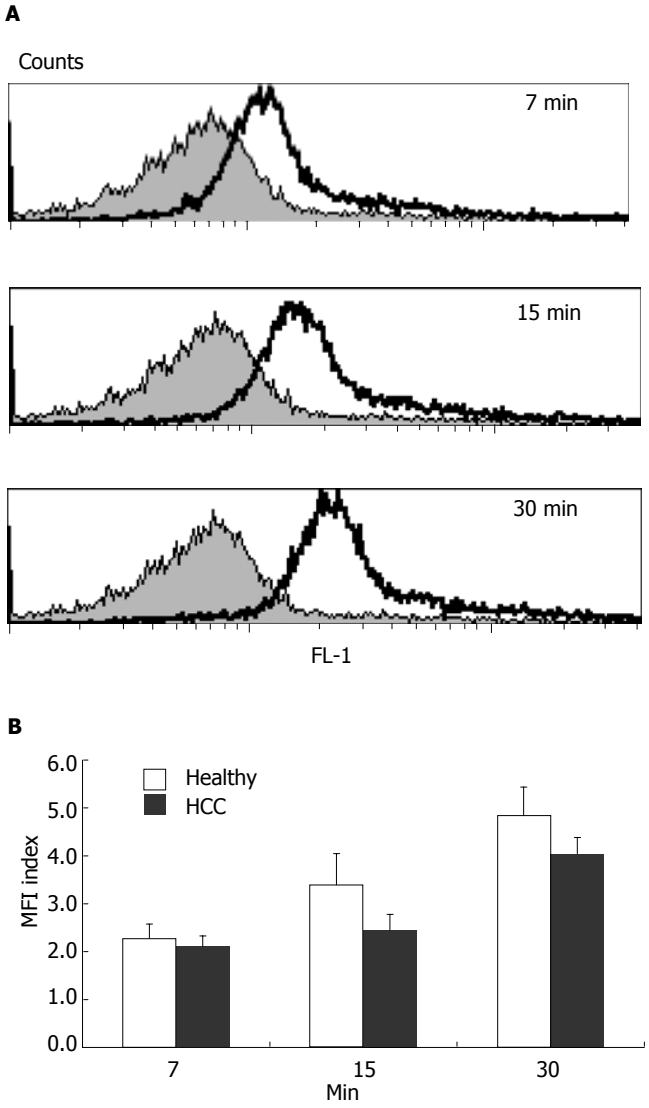

Figure 3 FITC-dextran uptake by mDCs.

lymphocyte reaction (MLR). mDCs were isolated from peripheral blood of HCC patients and healthy controls. PBMCs from a third healthy donor were used as responder cells. Figure 5 summarizes the average of eight HCC patients and six healthy donors. mDCs from HCC patients were clearly less efficient in inducing T cell proliferation than mDCs from healthy donors. For all the stimulator concentrations tested (10-10^4), a significant reduction in allostimulatory capacity of mDCs was seen ($P < 0.05$). Ten thousand mDCs from HCC patients stimulated allogeneic T cells almost 3-fold less than mDCs from healthy donors with a stimulation index of 13 *vs* 35. When 10 fold fewer mDCs were used, still a significant difference in allostimulatory function was seen. It has been shown that patients with chronic HCV infection have impaired function of circulating mDCs[28]. Therefore, we compared the allostimulatory potential of mDCs from patients with and without chronic HCV infection to mDCs from healthy controls. Our analysis clearly demonstrated that not only patients with chronic HCV infection and HCC but also those patients without chronic hepatitis have impaired circulating mDCs in peripheral blood (Figure 5B).

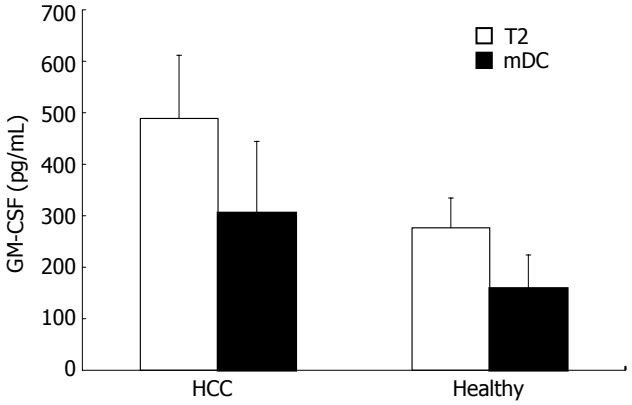

Figure 4 Peptide-specific stimulation by mDCs.

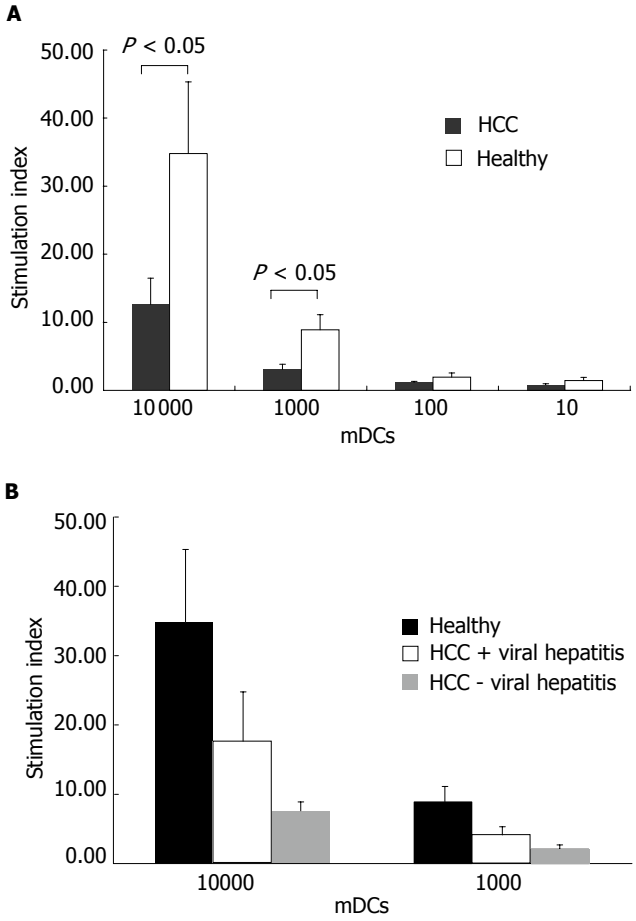

Figure 5 Impaired allogeneic MLR stimulation by mDCs from HCC patients. **A**: Stimulation index of mDCS from HCC patients and healthy controls; **B**: Comparison of stimulation index of mDCs derived from healthy controls and patients with or without chronic viral hepatitis.

Impaired cytokine production by mDC from HCC patients

We next analyzed the expression of cytokines by mDCs, since a perturbation of the cytokine network has been previously reported in patients with neoplasms including HCC[29-32]. Therefore, we analyzed the expression of IL-10, TNF-α and IL-12 by mDCs in HCC patients. PBMCs from healthy donors and HCC patients were stimulated for 48 h with poly dI: dC and analyzed for cytokine secretion by intracellular cytokine staining.

As shown in Figure 6, no significant differences for

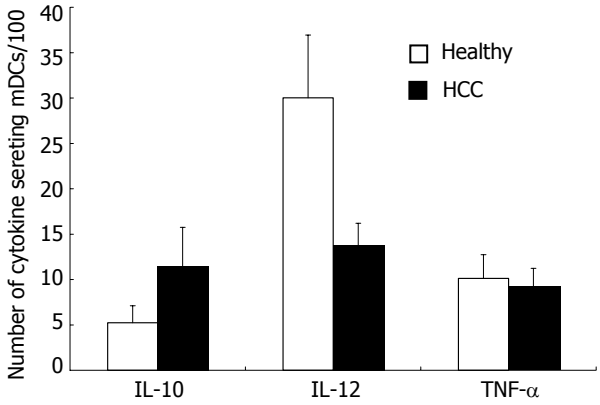

Figure 6 Impaired cytokine production by mDCs from HCC patients.

TNF-α and IL-10 production by mDCs were found. In contrast, the percentage of mDCs expressing IL-12 upon poly dI:dC stimulation was significantly lower in HCC patients (30.2) than in healthy controls (13.76, $P < 0.01$).

DISCUSSION

Until today treatment options for HCC are limited to surgery and local ablative therapy[33, 34]. Therefore, much emphasis has been put on understanding the development of HCC and investigating new therapeutic options, of which one is immunotherapy[3]. Previously, we have shown that more than 50% of HCC patients develop tumor specific immune responses against a well-defined tumor antigen[7]. However, in most patients analyzed, tumors progressed in spite of tumor specific humoral and cellular immune responses. Therefore, we are interested in investigating possible immunological mechanisms, which might lead to suppression of antigen-specific T cells in HCC patients. Recently, we have shown that $CD4^+CD25^+$ regulatory T cells are increased in both peripheral blood and tumor of HCC patients[35]. However, it is highly likely that a combination of regulatory T cells along with other factors is responsible for a defective anti-tumor immune response seen in HCC patients. Impaired function of professional antigen presenting cells has been suggested as a possible mechanism to promote tumor escape from immune control[21].

A number of different studies have analyzed the function of monocyte derived DCs in different liver diseases including HCC[25, 36, 37]. However, recent studies have shown that impaired function of monocyte derived DC might be the result of inefficient culture conditions rather than of patients' diseases[38]. Therefore we have performed our analysis on directly *ex vivo* isolated DCs avoiding *in vitro* manipulations. In order to verify that the findings made are not simply due to underlying liver diseases such as chronic viral hepatitis or liver cirrhosis, which can be found in the majority of patients with HCC[2], different control groups such as patients with HBV and HCV infection, liver cirrhosis and liver metastasis from colorectal cancer were included in the study for comparison. In addition, HCC patients with and without chronic viral hepatitis were compared with each other. Consistent with published data by others, we have observed a decreased number in circulating mDCs[31], which is an unlikely reason for impaired T cell responses seen in patients with different type of tumors including HCC[21]. Therefore, the main focus of our study was to investigate the function of mDCs isolated from patients with HCC. Professional antigen presenting cells pick up antigens, process and present them to T cells and cause potent T cell stimulation by expression of appropriate cytokines and costimulatory molecules[10]. Therefore, we performed a detailed step-by-step functional analysis of *ex vivo* isolated non-manipulated mDCs from patients with HCC and compared them to mDCs from healthy controls.

Interestingly, most functional assays performed showed no differences between mDCs from HCC patients and healthy controls such as expression of costimulatory molecules, most cytokines analyzed, antigen uptake and peptide presentation. However, when mDCs from HCC patients or healthy controls were tested to stimulate allogeneic PBMC, mDCs from HCC patients were clearly less potent stimulators. Similar results have been observed for patients with other tumors[29] and non-malignant liver diseases[39]. In addition, the observations made were not due to underlying chronic viral hepatitis, since mDCs from HCC patients with or without chronic viral hepatitis were less potent stimulators. However, the exact mechanism for this observation has not been elucidated. Because no differences were found for the MHC class II, CD80, CD83 and CD86 expression on mDCs, we extended our analysis for other potential factors leading to reduced stimulatory capacity of DCs.

Cytokines secreted by DC have been shown to have an important regulatory role in activation of the immune response. Therefore, we analyzed the expression of three different cytokines; TNF-α, IL-10 and IL-12 by mDCs upon stimulation. Although, we could not detect differences in the number of TNF-α or IL-10 expressing mDCs, significant differences were found in the numbers of IL-12 producing mDCs between HCC patients and healthy donors. IL-12 has been shown to play an anti-tumor role *in vivo*[32, 40]. An imbalance in the cytokine expression pattern as shown by low levels of IL-12 and normal or higher levels of IL-10 has been seen in other cancer patients[32, 41]. Our data suggests that reduced IL-12 expression of mDCs from HCC patients could lead to an impaired T cell stimulatory effect, which could not only account for a reduced allogeneic T cell response but also further account for an impaired general immune response.

Although, we have not been able to demonstrate this in our study due to limitations in the amount of blood that could be obtained from HCC patients, one could postulate that exogenous IL-12 might reverse impairment of DC function seen in our study. Indeed a number of clinical studies have seen promising results treating HCC patient with thalidomide in selected cases[42, 43]. Moreover, it has been shown that thalidomide might lead to an enhanced IL-12 secretion by DC s *in vitro*[44].

In summary, direct evaluation of DCs from HCC patients without *in vitro* manipulation has allowed us to

clearly demonstrate a decrease in the frequency of mDCs in peripheral blood of HCC patients and more importantly a functional defect of these cells leading to impaired stimulation of allogeneic T cells. Decreased IL-12 production by mDCs might be a possible cause of this impairment, which suggests that IL-12 directed therapies could reverse this phenomenon and should be further investigated in the context of immunotherapeutic approaches to the treatment of HCC.

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