Functional impairment of circulating FcεRI^{+} monocytes and myeloid dendritic cells in hepatocellular carcinoma and cholangiocarcinoma patients

Running title: **Circulating mDCs in primary liver malignancies**

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Abstract

Background: Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) represent the most common primary liver malignancies whose outcome is influenced by the immune response.

Methods: In this study, we have functionally characterized, by flow cytometry, circulating myeloid dendritic cells (mDCs) and FcεRI$^+$ monocytes in a group of healthy individuals (n=10) and in a group of patients with HCC (n=19) and CCA (n=8), at the time point of the surgical resection (T0) and once the patient had recovered from surgery (T1). Moreover, we proceeded to a more in depth phenotypic characterization of the FcεRI$^+$ monocyte subpopulation.

Results: A significant decrease in the frequency of TNFα producing FcεRI$^+$ monocytes and mDCs in HCC and CCA patients when compared to the group of healthy individuals was observed, and a close association between FcεRI$^+$ monocytes and mDCs dysfunction was identified. In addition, the phenotypic characteristics of FcεRI$^+$ monocytes from healthy individuals strongly suggest that this population drives to mDCs, which matches with the fact that both populations are functionally affected.

Conclusions: The frequency and the function of circulating mDCs and FcεRI$^+$ monocytes are affected in both HCC and CCA patients and FcεRI$^+$ monocytes could represent those fated to become mDCs.

Background

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the most common primary liver malignancies presenting high morbidity and mortality rates (1), associated with poor prognosis, generally due to its late presentation (2). It has been reported that dendritic cells (DCs) are critical for the induction of an antitumor immune response (3). However, *in vitro* propagated DCs from HCC patients display a decreased ability to produce IL-12 (4), HCC tumour-derived alpha-fetoprotein impairs both the differentiation and T cell stimulatory activity of human DCs (5). In the same line, tumour immune evasion is associated with the defective DC function in cancer patients as a result of decreased numbers of competent DCs and accumulation of immature cells (6,7).

High affinity receptor FcεRI has a role in mediating inflammatory signalling and enhancing T cell immunity (8). This receptor is homogeneously and constitutively expressed by the majority of human DC subsets (9), and it is expressed in a subset of monocytes, characterized as a $CD2^+$ population (10), that rapidly obtains DC-like features in culture (11,12).

In this study, we have characterized circulating mDCs and $FcεRI^+$ monocytes in peripheral blood (PB) from patients with HCC, CCA and in a group of healthy individuals, identifying a functional impairment both in mDCs and in $FcεRI^+$ monocytes.

Methods

Participants

A total of 19 patients with HCC (3 women and 16 men; average age: 62.0 ± 14.8 years) and 8 patients with CCA (5 women and 3 men; average age: 61.0 ± 14.7 years) were included in this study. Regarding the TNM classification, 1 CCA and 2 HCC patients were in stage I, 4 CCA and 14 HCC patients were in stage II, 1 HCC was in stage IIIA, and 3 CCA patients and 1 HCC patient were in stage IV. A group of 10 healthy individuals was included in the study as a control group (7 women and 3 men; average age: 51.6 ± 5.6 years). PB samples were collected at the time point of the surgical resection, just before the beginning of the surgical intervention (T0), and once the patient was completely recovered from the surgery (generally one month after the surgery) (T1). No patients took medication prior to surgery nor at T1. Nevertheless, 6 HCC patients underwent liver transplantation and took tacrolimus just after the surgery. Tacrolimus target T lymphocytes (13), but do not affect monocyte function (14) and has no influence in the maturation of DCs (15).

Phenotypic characterization of peripheral blood FcεRI^{+} monocytes and mDCs

For the identification of FcεRI^{+} monocytes and mDCs, 250µL of PB were stained with CD45-V500-C (clone 2D1, Becton Dickinson Biosciences (BD), San Jose, CA, USA), anti-IgE-PE (clone BE5, EXBIO Praha, Vestec, Czech Republic), HLA-DR-V450 (clone L243, BD), CD16-PE-Cy7 (clone 3G8, BD), CD33-APC (clone P67.6, BD) and CD14-APC-H7 (clone MϕP9, BD). Furthermore, for the characterization of the populations above mentioned, samples were stained with CD1c-BV421 (clone F10/21A3, BD),

HLA-DR- PerCP-Cy5.5 (clone L243, BD), CD35-FITC (clone E11, BD), CD11c-PerCP-Cy5.5 (clone B-ly6, BD), CD305-PE (clone DX26, BD), CD11b- APC (clone D12, BD), CD33- PerCP-Cy5.5 (clone P67.6, BD), CD2-FITC (clone RPA-2.10, BD), anti-IgE-FITC (clone BE5, EXBIO Praha) and CD13-PE (clone WM15, BD), using a lyse and wash procedure, as previously described (16). An isotype control was performed for CD2-FITC and CD1c-BV421 (Supporting Information).

TNFα expression in peripheral blood FcεRI$^+$ monocytes and mDCs after in vitro stimulation

LPS (100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA), IFNγ (100 U/ml, Promega, Madison, USA) and Brefeldin-A (10 µg/mL, Sigma-Aldrich) were added to 500 µL of PB sample diluted 1:1 (v/v) in RPMI 1640 complete culture medium (Invitrogen, Life Technologies, Carlsbad, CA, USA). All samples were then incubated in a 5% CO_2 humid atmosphere, at 37°C, for 6 hours. For all samples, a tube was included without any stimulating agent in order to evaluate the basal production of TNFα.

Each cultured sample was aliquoted (300µL) into one tube and stained with CD16-PE-Cy7 (clone 3G8, BD), anti-IgE- FITC (clone BE5, EXBIO Praha), HLA-DR-V450 (clone L243, BD), CD45-V500-C (clone 2D1, BD), CD14- APC-H7, (clone MϕP9, BD) and CD33- APC, (clone P67.6, BD) for 15 minutes in the dark at RT. For intracellular staining, Fix&Perm (GAS002, Life Technologies, Frederick, USA) reagent was used in parallel with TNFα- PE (clone MAb11, BD).

Flow cytometry data acquisition and analysis

Data acquisition was performed in a FACSCanto™ II flow cytometer (BD) and analyzed with Infinicyt™ 1.8 software (Cytognos SL, Salamanca, Spain).

Statistical analysis

To determine the statistical significance of the differences observed between groups, the non-parametric Mann-Whitney test was performed using the Statistical Package for Social Sciences software (SPSS, version 20, IBM, Armonk, NY, USA). Statistically significant differences were considered when $p < 0.05$. The Spearman rank test was used to evaluate the correlation among variables.

Results

Phenotypic characterization of peripheral blood FcεRI$^+$ monocytes and mDCs

The strategy employed for the identification and characterization of FcεRI$^+$ monocytes and mDCs is represented in figure 1A: in the first gate, the cell population positive for IgE bound to its receptor was selected; within this cell population, those events positive for both HLA-DR and CD33 were further selected. FcεRI$^+$ HLA-DR$^+$ CD33$^+$ cells include both mDCs (corresponding to CD16$^-$ CD14$^-$ events) and FcεRI$^+$ monocytes (CD14$^+$ events).

Within FcεRI$^+$ monocytes, two subpopulations were distinguished based on the expression levels of IgE bound to FcεRI$^+$ receptor and the expression levels of CD14:

CD14^{high} FcεRI^{+} monocytes (CD14 mean fluorescence intensity (MFI) = 5633) and **CD14^{dim} FcεRI^{+} monocytes** (CD14 MFI = 2749). **CD14^{high} FcεRI^{+} monocytes** are phenotypically characterized as CD14^{high}, HLA-DR ^{high}, CD33 ^{high}, negative for CD16, with similar SSC properties and CD45 expression than mDCs, and lower expression of IgE bound to high affinity FcεRI receptor compared to mDCs and CD14^{dim} FcεRI^{+} monocytes (figure 1B). Lower expression levels of CD1c and CD2, similar expression levels of CD13 and CD11c, and higher expression of CD35, CD305 and CD11b were exhibited by this population, in comparison to CD14^{dim} FcεRI^{+} monocytes and mDCs (figure 1B). CD35 expression appears in the later stages of monocytic maturation (17,18). CD35 is differentially expressed between monocyte subsets and mDCs, being highly expressed in classical monocytes, intermediately expressed in CD14^{dim} FcεRI^{+} monocytes, presenting lower expression in mDCs. The expression of CD305 (LAIR-1) on monocytes and monocyte-derived DCs is distinctly and reversibly controlled by myeloid cytokines and it has been associated with *in vitro* differentiation of CD14^{+} monocytes to DCs (19–21). Our results demonstrate that CD305 is highly expressed in FcεRI^{+} CD14^{high} monocytes, less expressed in CD14^{dim} FcεRI^{+} monocytes, being mDCs the population that exhibits the lowest expression.

CD14^{dim} FcεRI^{+} monocytes are phenotypically characterized as CD14^{dim}, HLA-DR ^{high}, CD33 ^{high}, negative for CD16, with similar SSC properties and similar expression of CD45 and IgE bound to high affinity FcεRI receptor than mDCs (figure 1B). When compared to mDCs and CD14^{high} FcεRI^{+} monocytes, CD14^{dim} FcεRI^{+} monocytes exhibit an intermediate expression of CD1c and CD2, similar levels of CD13 and CD11c, and decreasing expression of CD35, CD305 and CD11b (figure 1B). **mDCs** were

phenotypically identified as negative for CD14 and CD16, presenting a characteristic FSC/SSC light dispersion properties, together with high expression of HLA-DR, CD33 and IgE bound to high affinity receptor FcεRI (figure 1B). mDCs display higher expression levels of CD1c and CD2 than FcεRI^{+} monocyte populations, similar expression levels of CD13 and CD11c, decreasing expression of CD305 and CD35, and are negative for CD11b (figure 1B). The detailed phenotypic characterization of these three populations allows the observation of a clear differentiation path from CD14^{high} FcεRI^{+} monocytes to CD14^{dim} FcεRI^{+} monocytes and, finally, to mDCs (figure 1B).

Frequency and absolute value of peripheral blood FcεRI^{+} monocytes and mDCs in CCA and HCC patients

CCA and HCC patients displayed a decrease in the frequency and absolute numbers of peripheral blood FcεRI^{+} monocytes and mDCs, both at T0 and T1, compared to the control group. The frequency of mDCs was significantly decreased in both groups of patients when compared to the healthy group, both at T0 and T1, and the absolute counts of mDCs was significantly decreased at T0 and T1 in CCA patients and at T0 in HCC patients. A non-significant decrease in FcεRI^{+} monocytes, both in frequency and absolute numbers, was verified in comparison to the healthy group (table 1).

Functional characterization of peripheral blood FcεRI^{+} monocytes and mDCs in CCA and HCC patients

Regarding TNFα production (figure 2), a statistically significant decrease in the frequency of TNFα producing FcεRI^{+} monocytes and TNFα producing mDCs, for both groups of patients at T0, was observed when compared to the control group. A frequency of 61.3% ± 41.0 and 70.7% ± 26.7 of TNFα producing FcεRI^{+} monocytes was found for CCA and HCC patients, respectively, while the healthy group presented a frequency of 97.1% ± 5.80 (table 1). Of note, the frequency of TNFα producing classical monocytes in the healthy group was 98.1% ± 1.7 (unpublished data). Interestingly, a partial recovery of TNFα producing FcεRI^{+} monocytes was observed at T1, especially in CCA patients, without reaching the values of the control group. On the contrary, we did not observe significant differences in the amount of TNFα expressed *per* cell (MFI) among the different groups (table 1). Noteworthy, the decrease identified in the frequency of TNFα producing cells affected evenly both cell subsets, as there is a positive correlation between the percentages of TNFα producing FcεRI^{+} monocytes and TNFα producing mDCs in the PB of cancer patients (figure 2C), with a correlation coefficient of 0.789 in the Spearman rank test, significant at the 0.01 level.

Discussion

DCs are considered the most effective antigen-presenting cells for activating naïve T cells (22) and the interaction among DCs and tumour cells may profoundly dictate the outcome of a neoplasm (23). In fact, DC infiltration in HCC lesions has been associated with a better prognosis in resected patients (23) and it has been reported

that the inhibition of DC maturation in HCC may be a critical feature of tumour escape (24).

Therefore, to contribute to a better understanding of the role of fully differentiated mDCs as well as FcεRI$^+$ monocytes, in CCA and HCC cancers, we have functionally characterized these populations in PB from patients with HCC, CCA and in a group of healthy individuals.

We have observed a decrease in the frequency and the absolute numbers of circulating FcεRI$^+$ monocytes and mDCs, both at T0 and T1, in CCA and HCC patients compared to the control group. Significant differences were found in the frequency and absolute numbers of mDCs, in CCA patients, at T0 and T1, and in HCC at T0. Moreover, regarding the functional characterization of these cells, despite the high variability observed, a significant decrease in the frequency of TNFα producing FcεRI$^+$ monocytes associated to a significant decrease in TNFα producing mDCs was observed in both groups of cancer patients, at T0, when compared to the control group. This significant decrease was maintained for FcεRI$^+$ monocytes at T1 in both groups of patients, and for mDCs at T1 in HCC patients. The positive correlation verified in TNFα production dysfunction (figure 2C) suggests that FcεRI$^+$ monocytes may represent those fated to become mDCs, what is further supported by our results on the phenotypic characterization of this cell population (figure 1B), particularly by the expression kinetics of CD14, HLA-DR, CD1c (BDCA-1), CD2, CD35, CD305 and CD11b, that display a phenotypic continuum from circulating FcεRI$^+$ monocytes to circulating mDCs. However, in differentiation experiments it was reported that both culture-derived and primary CD1c$^+$ pre–conventional DCs purified

from peripheral blood acquire CD14 expression in culture (25). In addition, Meyerson et al. reported that CD1c myeloid DCs (mDCs) were noted to have partial expression of CD14 (26). Therefore, the classification of $CD14^{dim}$ $FcεRI^{+}$ $CD1c^{+}$ cells into mDCs or monocytes subsets is controversial and still under debate. Further studies in transcriptomics or/and at single cell level would be needed to clarify this classification. Nevertheless, it seems, from the phenotypic point a view, that there is a differentiation pathway from $CD14^{high}$ $FcεRI^{+}$ cells to $CD14^{dim}$ $FcεRI^{+}$ cells and finally to mDCs.

In conclusion, a functional defect in circulating mDCs and $FcεRI^{+}$ monocytes from HCC and CCA patients was identified. In addition, we have thoroughly characterized the phenotype of $FcεRI^{+}$ monocytes in healthy individuals, and our data strongly suggest that $FcεRI^{+}$ monocytes could differentiate into mDCs.

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Tables

Table 1. Frequency among total leukocytes (%) and absolute numbers ([], number of cells/µL) of peripheral blood FcεRI$^+$ monocytes and myeloid dendritic cells (mDCs) in cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) patients, at the time of surgery (T0) and once the patients were recovered from surgery (T1), and in healthy individuals (HG); frequency (%) of tumour necrosis factor (TNF)-α producing cells and amount of TNFα expressed per cell, measured as mean fluorescence intensity (MFI), within each cell population.

	Cholangiocarcinoma (N = 8)		**Hepatocellular carcinoma (N = 19)**		**HG (N = 10)**
	Mean ±SD T0	**Mean ±SD T1**	**Mean ±SD T0**	**Mean ±SD T1**	**Mean ±SD**
% FcεRI$^+$ monocytes	0.12 ± 0.23	0.13 ± 0.23	0.12 ± 0.22	0.11 ± 0.14	0.20 ± 0.21
FcεRI$^+$ monocytes [number of cells/µL]	4.80 ± 10.1	4.71 ± 8.30	10.25 ± 15.9	8.95 ± 11.3	14.2 ± 15.2
%TNFα producing FcεRI$^+$ monocytes	61.3 ± 41.0 [a]	84.7 ± 14.8 [a]	70.7 ± 26.7[a]	78.7 ± 15.1[a]	97.1 ± 5.80
MFI TNFα FcεRI$^+$ monocytes	11202 ± 9795	5580 ± 2204	6614 ± 7325	5293 ± 4087	5254 ± 3465
% mDCs	0.06 ± 0.06 [a]	0.07 ± 0.02 [a]	0.07 ± 0.05[a]	0.10 ± 0.07[a]	0.16 ± 0.06
mDCs [number of cells/µL]	3.24 ± 2.83[a]	3.40 ± 1.44 [a]	5.66 ± 5.07[a]	6.53 ± 5.80	9.69 ± 3.38
%TNFα producing mDCs	37.4 ± 30.8 [a]	48.5 ± 23.1	43.7 ± 26.7[a]	47.0 ± 23.7[a]	75.1 ± 8.10
MFI TNFα mDCs	6730 ± 7521	2529 ± 985	2902 ± 2215	2917 ± 2594	2127±1212

Independent-samples Mann-Whitney U test was performed with a significance level of 0.05 ($p<0.05$). [a] vs. CONTROL; [b] vs. CCA. The results are given by mean ± standard deviation.

Figures' legends

Figure 1. Phenotypic characteristics of peripheral blood FcεRI^{+} monocytes and myeloid dendritic cells (mDCs). **A)** Bivariate dot plot histograms illustrating the gating strategy for the identification of mDCs (up) and FcεRI^{+} monocyte subpopulations (down) from peripheral blood samples. **B)** Bivariate dot plot histograms illustrating the phenotypic characterization of the identified populations from peripheral blood samples. **CD14^{high} FcεRI^{+} monocytes** are represented as red events, **CD14^{dim} FcεRI^{+} monocytes** are represented as green events and **mDCs** are represented as blue events.

Figure 2. TNF-α production by mDCs and FcεRI^{+} monocytes. Bivariate dot plot histograms illustrating TNF-α production (indicated with dashed rectangle) by mDCs (left) and FcεRI^{+} monocytes (right) in basal condition (without stimulation agents) **(A)**, and after stimulation with LPS and IFN-γ **(B)**. Correlation between the frequency of TNFα producing mDCs and TNFα producing FcεRI^{+} monocytes in hepatocellular carcinoma (purple squares) and cholangiocarcinoma patients (pink circles), confirmed by the Spearman test **(C)**.