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Monocyte-derived dendritic cells from cirrhotic patients retain similar capacity for maturation/activation and antigen presentation as those from healthy subjects ☆

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ABSTRACT

Few studies have investigated the impact of liver cirrhosis on dendritic cell function. The purpose of this study was to compare the activation and antigen-presentation capacity of monocyte-derived dendritic cells (MoDC) from cirrhotic patients (CIR) relative to healthy donors (HD). MoDC from CIR and HD were matured, phenotyped, irradiated and pulsed with 15mer peptides for two hepatocellular carcinoma-related antigens, alphafetoprotein and glypican-3, then co-cultured with autologous T-cells. Expanded T-cells were evaluated by interferon-gamma ELISPOT and intracellular staining. 15 CIR and 7 HD were studied. While CD14+ monocytes from CIR displayed enhanced M2 polarization, under MoDC-polarizing conditions, we identified no significant difference between HD and CIR in maturation-induced upregulation of co-stimulation markers. Furthermore, no significant differences were observed between CIR and HD in subsequent expansion of tumor antigen-specific IFNγ+ T-cells. *Conclusion:* MoDCs isolated from cirrhotic individuals retain similar capacity for *in vitro* activation, maturation and antigen-presentation as those from healthy donors.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and third leading cause of cancer death worldwide [1] and is the leading cause of death among cirrhotic patients [2]. Despite growing incidence, therapeutic options remain limited and novel approaches to treat or prevent HCC are urgently required. HCC expresses several potential tumor-associated antigen targets for immune-based therapy or prevention, including alpha-fetoprotein (AFP), MAGE-1, NY-ESO-1 and glypican-3 (GPC3) among others [3,4]. Antigen-loaded and unloaded DC have been investigated therapeutically with excellent tolerance but mixed clinical impact in Phase I–II trials in humans [3–15]. However, there are some data that suggest that DC function in hepatocellular carcinoma may be impaired [16]. In previous work, our laboratory showed that tumor antigen-specific CD8+ T-cells generated using 15mer peptide stimulation in cirrhotic patients with HCC were dysfunctional [17]. Similar findings have subsequently been reproduced by other groups [18]. Thus, by the time HCC is diagnosed it may be too late to effectively harness DC to expand tumor-reactive T-cells for optimal therapeutic benefit.

In our previous work, we identified that CD8+ T-cells from cirrhotic patients who had not yet developed HCC appeared to harbor a small population of multifunctional tumor antigen-specific CD8+ T-cells [17]. We therefore hypothesized that in the presence of optimized antigen-presenting cells that we would be able to expand multifunctional tumor antigen-specific T-cells with potential tumor preventing capacity in cirrhotic individuals. To address this hypothesis, we first aimed to evaluate the capacity of MoDC from cirrhotic donors at risk for future HCC to be generated and matured *in vitro* relative to those from healthy subjects. Secondly we aimed to compare the ability of MoDC derived from cirrhotic donors to prime autologous recall responses to highly immunogenic as well as tumor-related antigens with those generated from

Abbreviations: CIR, cirrhotic group; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HD, healthy donor; PBMC, peripheral blood mononuclear cells; MoDC, monocyte-derived dendritic cell; AFP, alpha-fetoprotein; GPC3, glypican-3.

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MoDC from healthy individuals. We found that MoDC from cirrhotic patients retained identical capacity for activation using a 48 h DC maturation protocol and induced a similar expansion of recall antigen- as well as tumor antigen-specific T-cell responses *in vitro*. These data suggest that antigen-specific cell-based vaccinations in pre-tumor cirrhotic patients could have the potential to expand functional tumor-reactive precursors *in vivo* that might prevent or delay progression to hepatocellular carcinoma.

2. Materials and methods

2.1. Patients

Subjects and controls were recruited from the Gastroenterology Clinic at the Philadelphia Veterans Affairs Medical Center following informed consent on an institutional review board-approved protocol. Viral status was determined using clinically-obtained hepatitis C (HCV) antibody, HCV viral load, HBsAg, and HBV DNA testing using standard definitions of chronicity. Alcohol, hemochromatosis, and non-alcoholic fatty liver disease/non-alcoholic steatohepatitis diagnoses were obtained from clinical records. Cirrhotic patients were routinely screened by sonography every 6 months to exclude interim development of HCC; data from any cirrhotic subject who developed HCC within 12 months of enrollment were excluded.

2.2. Peptides

15mer overlapping peptide pools spanning AFP and glypican-3 protein sequences were synthesized (Genscript USA Inc, Piscataway NJ). CMV, EBV, and influenza (CEF) 9–10mer control peptides (Cellular Technology Ltd., Cleveland, OH) were used as positive controls for effector T-cell responses.

2.3. Cell isolation and preparation

100–150 ml of peripheral blood was obtained, from which 100–200 million peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Histopaque (Sigma, St. Louis MO) density gradient centrifugation. T cells were purified from 30 to 40 $\times$ 10^6 PBMC by negative selection using the MACS Pan T-cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of CD3+ T cells was >95% as determined by flow cytometry. T-cells were plated in 24 well plates in RPMI1640 with L-glutamine (Invitrogen) with 10% human AB serum (Sigma Inc., St. Louis, MO), 1.5% HEPES (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

2.4. Antibodies and flow cytometry

All data were acquired on FACSCanto (BD) and analyzed using FlowJo (Tree Star Inc., Ashland OR) using cutoffs based on isotype antibody staining. All antibodies were purchased from Becton Dickinson (BD: Becton Dickinson, Franklin Lakes, NJ) unless specifically indicated.

2.5. Monocyte-derived dendritic cell (MoDC) maturation

CD14+ monocytes were purified from 40 $\times$ 10^6 PBMC using a human CD14+ cell isolation kit (Miltenyi Biotec), activated and matured using a 48 h protocol to generate MoDC as previously described [19–21]. Briefly, CD14+ monocytes were cultured in 24-well plates in X-vivo15 medium supplemented with 800 IU/ml GM-CSF (BioLegend) and 1000 IU/ml IL-4 (BioLegend) for 24h. The cells were matured for another 24 h in X-vivo15 medium supplemented a maturation cocktail (TNFα 10 ng/ml (Cell Signaling Technology), IL-1β 10 ng/ml (Cell Signaling Technology), IL-6 10 ng/ml (Cell Signaling Technology), and PGE_2 1 μg/ml (Sigma), GM-CSF 1600 IU/ml and IL-4 1000 IU/ml). MoDCs ($CD11c^{hi}$) before and after maturation procedure were assessed using CD40 FITC, CD70 FITC, CD83 PE, CD137L PE, HLA-DR APC, OX40L APC, CD86 V450 and Live/Dead Aqua [22–27].

2.6. Ex vivo assessment of monocyte nitric oxide production and macrophage polarization

PBMCs were incubated with LPS 10 μg/mL (*Escherichia coli* lipopolysaccharide, Sigma) for 60 min with or without 10 min pre-incubation with the nitric oxide synthase inhibitor L-NAME 10 mM (Nω-nitro-L-arginine methyl ester, Sigma) at 37 °C, 5% CO_2. Cellular levels of nitric oxide (NO) were assessed an NO probe DAF-2DA 2 μM (4,5-diaminofluorescein diacetate, Calbiochem) by flow cytometry. Briefly, cells were incubated with DAF-2DA at 37 °C for 20 min and were subsequently surface stained for CD14 (PerCP) and HLA-DR (APC) on ice. For macrophage polarization, purified CD14+ monocytes were plated in RPMI 1640 with 10% human AB serum containing with 100 ng/mL M-CSF (macrophage colony-stimulating factor, Biolegend) for 7 days for M0 differentiation. After 7 days, M1 and M2 polarization was induced by either 100 ng/mL LPS (Sigma) plus 20 ng/mL IFNγ (PeproTech), or rhIL-4 20 ng/mL (BioLegend) for 2 additional days. Cultured macrophages were stained for CD86, HLA-DR, and CD206 for analysis by flow cytometry.

2.7. Antigen-specific T-cell expansion

At 48 h MoDCs were harvested, count, and phenotyped. MoDc were irradiated to 30 Gy prior to peptide loading. For peptide loading, 0.4 x10^6 irradiated MoDCs per well in a 24 well plate were incubated in X-vivo15 media in the presence of AFP or GPC3 or CEF peptide pool at 1 μg/ml per peptide for 2–4 h. Pulsed MoDC were then centrifuged, washed, and co-cultured with 2.0 $\times$ 10^6 autologous CD3+ T-cells at T:DC ratio of 5:1 in RPMI 1640 with 10% human AB serum supplemented with IL-15 (10 ng/ml) and IL-12 (25 ng/ml). IL-2 (50 U/ml), IL-15 (10 ng/ml) and IL-21 (25 ng/ml) were added every 2–3 days. T-cells were harvested and replated for restimulation with autologous irradiated, peptide-pulsed MoDC (day 13) and PBMC 1.0 $\times$ 10^6/well (day 20). On day 27, *in vitro* expanded T-cells were restimulated for 6 h with media (negative control), AFP, GPC3 or CEF peptide pools at 1 μg/ml per peptide, or PMA/ionomycin (positive control) in the presence of anti-CD107a PE and monensin, fixed and permeabilized using BD Cytoperm/Cytofix (BD), then stained intracellularly for IFNγ PE-Cy7 and TNFα APC (BD). Dead cells (Live/Dead Aqua+) were excluded from analysis. Background positive responses from unrestimulated conditions were subtracted from stimulated conditions.

2.8. IFNγ Elispot

Antigen-specific T-cell IFNγ responses were examined after *in vitro* expansion in cytokine Elispot assay as previously described [17]. 5 $\times$ 10^4 antigen-expanded T-cells/well were restimulated with each peptide pool (1 μg/ml) in triplicates with positive (PHA) and negative (media) controls $\times$ 24 h in IFNγ Elispot plates. 96-well Elispot plates were pre-coated with anti-IFNγ (5 μg/ml, Thermo Scientific) and detected by biotinylated anti-IFNγ (0.5 μg/ml, Thermo Scientific). Spot-forming units were counted using IFL044 Elispot reader (AID, Strassberg Germany) excluding assays with high background (>10 SFU per well) or no response to PHA. Conditions with average greater than 500 SFU/10^6 cells were considered positive.