Circulating and intratumoral macrophages in patients with hepatocellular carcinoma: correlation with therapeutic approach

Pippa Newell, M.D.\textsuperscript{a,b,*}, Ben Cottam, B.S.\textsuperscript{b}, Talicia Savage, B.S.\textsuperscript{b}, Chet Hammill, M.S., M.D.\textsuperscript{a}, Ron Wolf, M.D.\textsuperscript{a}, Carlo Bifulco, M.D.\textsuperscript{b,c}, Hong D. Xiao, M.D.\textsuperscript{c}, Todd Crocenzi, M.D.\textsuperscript{b,d}, Paul Hansen, M.D.\textsuperscript{a}, Marka Crittenden, M.D., Ph.D.\textsuperscript{b,e}, Michael Gough, Ph.D.\textsuperscript{b}

\textsuperscript{a}Hepatobiliary Surgery, The Oregon Clinic, 4805 NE Glisan Street, Suite 6N50, Portland, OR, USA;\textsuperscript{b}Earle A. Chiles Research Institute, Providence Cancer Center, Portland, OR, USA;\textsuperscript{c}Department of Pathology, Providence Cancer Center, Portland, OR, USA;\textsuperscript{d}Division of Hematology and Oncology, Providence Cancer Center, Portland, OR, USA;\textsuperscript{e}Radiation Oncology, The Oregon Clinic, Portland, OR, USA

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Abstract


METHODS: Peripheral blood myeloid populations were quantified in 39 patients with hepatocellular carcinoma treated with surgical or endoluminal therapy. Macrophages were quantified in tissue when available.

RESULTS: There was a similar expansion of myeloid populations after operative procedures compared with endoluminal treatments. Immunostaining for CD68 revealed no significant differences in the number of macrophages within benign versus malignant tumors and when tumors were compared with nontumor liver. Cytotoxic CD8\textsuperscript{+} T cells were rare within tumors compared with the surrounding liver (P < .0001). Progression-free survival was reduced in patients with preoperative peripheral blood monocyte expansion (P < .05).

CONCLUSIONS: These data provide preliminary evidence of poor prognostic significance of elevated peripheral blood monocyte counts. We propose that the inflammatory environment of hepatocellular carcinoma may represent a consistent feature to both predict and alter the course of disease.

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer-related death in the world. HCC is a unique target for immune therapy in that most tumors arise within a background of chronic inflammation or cirrhosis. Although the association between chronic inflammation and HCC carcinogenesis is well documented, the biological mechanism underlying the relationship between the immune system and tumor treatment remains unclear. We believe the success of cancer therapy may be contingent on the underlying inflammatory biology of both the tumor and stroma.

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* Corresponding author. Tel.: +1-503-215-6685; fax: +1-503-215-6841.

E-mail address: philippa.newell@providence.org

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The liver presents an ideal environment in which to study this phenomenon because of the baseline level of inflammation present in the cirrhotic liver adjacent to the tumors. Molecular studies have shown that the gene signature of a surrounding cirrhotic liver, not tumor tissue itself, is prognostic of survival in patients with HCC.1 Hoechst et al2 isolated a subset of myeloid-derived suppressor cells that are significantly increased in peripheral blood and tumors of HCC patients. These cells express CD14, have high arginase activity, and are unable to stimulate an allogenic T-cell response. Radiofrequency ablation (RFA) of HCC has been shown to induce a functional transient activation of myeloid dendritic cells,3 and tumor protein lysates extracted before and immediately after RFA treatments have augmented T-cell responses.4 However, tumor-associated Kupffer cells suppress CD8+ T cells via the B7H1/PD-1 axis,5 and our previous work showed that tumor macrophages can limit the efficacy of both immunotherapy and conventional cancer therapy.6,7 We have only a limited understanding of the effect of liver-directed tumor treatment on the population of circulating cells, and, more importantly, on the ability of the immune system to detect and mount a response to an existing tumor in patients with HCC.

Methods

Patient recruitment

Patients were screened for inclusion in the study by the Clinical Trials Division of the Providence Cancer Center, Portland, OR. Patients with a diagnosis of HCC based on imaging or biopsy were eligible, including those with prior treatments. Patients were treated with resection, radiofrequency ablation (RFA), transarterial chemoembolization with drug-eluting beads (TACE/DEB) (Biocompatibles, Surrey, UK), or transarterial radioembolization (Yttrium-90) (Sir-spheres, Sirtex, Woburn, MA). The study excluded patients with other malignancies. Patients with benign cirrhosis but no HCC, and with normal livers were enrolled as controls (Fig. 1). The study was approved by the local institutional review board, and all patients gave written informed consent. Clinical data are summarized in Table 1.

Whole blood fluorescence-activated cell sorting (FACS) analysis

Blood samples were drawn before and 3 weeks after the procedure. Fifty microliters of whole blood was stained in BD Trucount Tubes (BD Biosciences, San Jose, CA) with the following antibodies: CD8-AF700 (eBioscience, San Diego, CA), CD15-FITC (eBioscience), CD4-eFluor 450 (eBioscience), CD14-PECy7 (BD Pharmingen, San Jose, CA), CD3-eFluor 780 (eBioscience), CD45-PETR (Beckman Coulter, Brea, CA), CD25-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), and human leukocyte antigen-DR (HLADR) APC (BD Pharmingen). Further macrophage phenotype analysis was performed using anti-CD33-APF700, anti-CD11b-e605, and anti-CD11c-PECy7 (BD Pharmingen). Blood cells were incubated with antibodies for 15 minutes at room temperature followed by red blood cell lysis using BD FACS lysing solution, and absolute cell counts were calculated using an LSRII flow cytometer (BD Bioscience) and BD FACSDivia software (BD Bioscience, San Jose, CA). Human leukocyte antigen DR mean fluorescence intensity (HLADR MFI) was measured in SSC<sup>+</sup> CD14<sup>+</sup> cells using BD FACSDivia software.

Cell isolation from tissue samples

Fresh tissue was minced and digested in 10 mL phosphate-buffered saline (Lonza Biologics, Hopkinton, MA) containing 33.3 mg collagenase (Sigma, Saint Louis, MO), 25 kunitz units DNase (Sigma), 8.3 mg hyaluronidase (Sigma), and 400 µL 25% human albumin for 45 minutes at room temperature with agitation. Cells were filtered through 70-µm nylon mesh, spun down, and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza) to be stored overnight at 4 °C.

Flow cytometry analysis of tissue samples

Cells, 1 × 10<sup>6</sup>, were stained with Fixable Viability Dye e450 (Invitrogen), CD8 a700, CD3 e780, CD15 FITC, CD4 e450 (all eBioscience), CD25 PE (Miltenyi Biotec), HLADR APC (BD), CD56 a700, CD16 a700, CD14 PE-Cy7 (BD Pharmingen), and CD45-PETXR (Coulter). Cell populations were then analyzed using the LSRII flow cytometer. For cell sorting, single-cell suspensions of tumor and nontumor were stained with a Fixable Viability Dye, CD14, and CD15 and sorted by FACS Aria.
(BD Biosciences). CD14+ and CD15+ cell morphology was verified by cytopsin (Shandon Cytospin 3, Block Scientific, Bohemia, NY).

Immunohistochemistry

Slides with 3-μm sections were deparaffinized and then rehydrated in graded alcohols. After antigen retrieval in citrate buffer and brief submersion in H2O2, slides were blocked in 4% horse/goat serum, 1% bovine serum albumin (BSA), and 0.3% Triton X-100 (Sigma, St. Louis, MO), followed by incubation in primary antibody overnight. Sections were washed and then incubated in secondary antibody antimouse or antirabbit ImmPRESS reagent (Vector Labs, Burlingham, CA) or fluorescent conjugated antimouse Alexa Fluor 568 (Life Technologies, Grand Island, NY) or antirabbit Alexa Fluor 488 (Invitrogen, Life Technologies, Grand Island, NY). ImmPRESS reagents were visualized with 3,3'-diaminobenzidine (DAB) or Very Intense Purple (VIP) substrates (Vector Labs). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) or hematoxylin. Antibodies used include anti-CD68 (Thermo, ThermoFisher, Walthman, MA), anti-CD3 (Spring Bioscience, Pleasanton, CA), anti-macrophage colony stimulating factor (MCSF) (Thermo, Abcam), anti-CD8 (Epitomics, Abcam), and anti-CD163 (Epitomics, Burlingham, CA). Dual immunofluorescence staining was performed with mouse anti-CD68 and rabbit anti-CD163 primary antibodies.

Image analysis

Whole slide images were obtained using the SCN 400 slide scanner (Leica Microsystems, Wetzler, Germany). Images were selected from whole slide image sets using the Digital Image Hub (Leica Microsystems). Pixels in random images were analyzed using NIH Image (National Institutes of Health, Bethesda, MD) and relevant plugins. Immunofluorescence images were generated using a Zeiss Axiovert 200 microscope (Carl Zeiss Microscopy, Gottingen, Germany) with an attached Nuance FX multispectral imaging system (Perkin Elmer, Waltham, MA).

Statistical methods

Kaplan-Meier plots were used to assess the association between peripheral blood monocyte counts and survival. The mean changes in cell populations were analyzed using a 1-sample paired t test. Variables such as Barcelona Clinic Liver Cancer (BCLC) stage, tumor size, satellitosis, and Child-Pugh Score were correlated with cell populations using either the Spearman rank test or the unpaired t test. High monocyte counts were calculated based on the mean

| Table 1 | Clinical characteristics of study patients |
|---|---|---|---|---|---|
| Variable (No.) | HCC Group | Control Group |
| | Resection (8) | Lap RFA (13) | Transarterial chemoembolization with drug-eluting beads (TACE/DEB) (14) | Yttrium-90 (4) | Liver disease (22) | Normal (12) |
| Median age | 64 | 65 | 60 | 67 | 55 | 50 |
| Male sex, n (%) | 6 (75) | 8 (67) | 11 (79) | 4 (100) | 14 (64) | 5 (23) |
| Etiology, n (%) | | | | | | |
| Hepatitis C | 3 (38) | 10 (83) | 11 (79) | 3 (75) | 14 (64) | 0 (0) |
| Hepatitis B | 2 (25) | 1 (8) | 2 (14) | 0 (0) | 0 (0) | 0 (0) |
| Alcohol | 2 (25) | 0 (0) | 3 (21) | 0 (0) | 5 (23) | 0 (0) |
| Other | 3 (38) | 1 (8) | 1 (7) | 1 (25) | 4 (18) | 0 (0) |
| Child-Pugh Class | | | | | | |
| A, n (%) | 8 (100) | 11 (92) | 9 (64) | 4 (100) | 17 (77) | 4 (100) |
| B, n (%) | 0 (0) | 1 (8) | 4 (29) | 0 (0) | 3 (14) | |
| C, n (%) | 0 (0) | 0 (0) | 1 (7) | 0 (0) | 2 (9) | |
| Tumor diameter, cm | | | | | | |
| Median | 5.1 | 2.1 | 3.4 | 2.3 | NA | 6 |
| Interquartile range | 6.2 | 2.4 | 3.1 | .85 | NA | .75 |
| Treated tumor no. | | | | | | |
| Median | 1 | 1 | 3 | 7.5 | NA | 1 |
| Interquartile range | .25 | 1 | 3 | 4 | NA | 0 |
| BCLC stage, n (%) | | | | | | |
| 0 | 1 (13) | 1 (8) | 0 (0) | 0 (0) | NA | NA |
| A | 5 (50) | 7 (58) | 2 (14) | 0 (0) | NA | NA |
| B | 2 (25) | 4 (33) | 10 (71) | 3 (75) | |
| C | 1 (13) | 0 (0) | 2 (14) | 1 (25) | |
| Median follow-up (mo) | 7.0 | 10.4 | 3.3 | 7.8 | NA | NA |
value of monocyte counts in control patients with liver disease but no HCC. Changes in myeloid populations were defined as high or low based on the mean change observed after operative treatment for benign disease. The correlation between T-cell subsets in peripheral blood and tissue was assessed with Spearman rho.

**Results**

**Patients**

The majority of patients treated had well-compensated liver disease. Median survival was not reached in any of the treatment groups; the median follow-up was 7 months (range 0 to 19 months). Clinical characteristics are summarized in Table 1.

**Myeloid cell populations in peripheral whole blood**

Populations of granulocytes and monocytes were identified within peripheral whole blood in all patients (Fig. 2A, i and ii). The cell phenotype was verified using cytofospin (Fig. 2B). Although there was a trend toward expansion in HCC patients, preoperative monocyte populations did not significantly differ in patients with normal livers...
(371.1 ± 40.99), patients with liver disease (388.3 ± 51.03), and patients with HCC (480.6 ± 41.71, \( P = .23 \) vs normal) (Fig. 2C, i). When compared with patients who underwent resection for benign liver lesions (control), there was not a consistent expansion of monocytes after either liver resection or laparoscopic RFA for HCC (operative) or endoluminal procedures (external) (Fig. 2C, ii).

Further analysis of SSCintCD14+ cells showed these to be CD11c+ CD11b−, and the majority were HLADR+ (data not shown). CD33 staining was not informative. There was significantly lower HLADR mean fluorescence intensity (MFI) in patients with HCC compared with normal controls \((P < .0001)\) and with patients with liver disease \((P < .0001)\) (Fig. 2D). There was also a lower HLADR MFI in patients with liver disease compared with normal controls \((P = .005)\). There was no difference in HLADR MFI in patients with hepatitis versus cirrhosis or in BCLC stage among HCC patients (data not shown).

Preoperative granulocyte counts in patients did not differ in patients with normal livers \((1,423 ± 345.0)\) versus those with liver disease \((2,315 ± 501.0)\) and HCC \((2,562 ± 184.9, P < .05 \) vs normal) (Fig. 2E, i). There was a trend toward greater granulocyte expansion in patients who underwent surgical intervention for HCC (operative group); there was also a trend toward expansion when operated patients were compared with those who underwent percutaneous intervention for HCC (external) (Fig. 2E, ii). Peripheral T lymphocyte CD3+CD8− subset populations did not vary between normal control patients and those with either liver disease or HCC (Fig. 2F).

**Myeloid cell populations detected by flow cytometry in tumor and nontumor tissue digests**

Discrete myeloid populations within digested tumor and nontumor tissue were sorted on the basis of side scatter and antibody staining (SSCintCD15+ for granulocytes and SSCintCD14+ for macrophages, data not shown). Both populations were gated on live cells as defined by fixable dye antibody staining. Of total live cells counted, macrophages accounted for an average of 3.1% in tumor tissue and 2.6% in nontumor tissue (\( P = \) not significant). Granulocytes accounted for average .1% of cells within tumor tissue and .25% within nontumor tissue (\( P = .09 \)). Sorted cells showed characteristic phenotypes when analyzed by cytospin.

**Immune cell populations detected by immunohistochemistry in fixed tumor and nontumor tissue**

Macrophages, including Kupffer cells, within tumor and adjacent nontumor liver were identified with CD68 immunostaining (Fig. 3A, i–ii). Morphology of the macrophages identified with CD68 staining was consistent with a mix of activated and nonactivated phenotypes.

![Image](image-url)
CD8+ T cells in tumor or nontumor tissue with any T-cell subsets in peripheral blood (data not shown).

Although there was no correlation between myeloid numbers in the peripheral blood and those in the tumor, the reduced T-cell numbers in the tumor could be explained by tumor macrophages exhibiting an inhibitory M2 phenotype. Although CD163 was initially proposed as a potential marker for M2 anti-inflammatory phenotype macrophages, we found that cells staining positively for CD163 did not overlap consistently with CD68 cells when analyzed by immunofluorescence (data not shown). For this reason, CD163 immunostaining was abandoned. Future studies will test additional markers of macrophage differentiation to examine this potential mechanism of T cell suppression.

Recurrence and progression in patients with peripheral myeloid expansion

Median survival was not reached in any of the 4 treatment groups; there were only 4 deaths total in all 4 groups during the median 7-month follow-up period. Monocyte expansion preprocedure was significantly associated with a poor progression-free survival: patients with a high preprocedural peripheral blood monocyte count had a median time to progression of 181 days versus 432 days (P < .05) (Fig. 4A, i). Preprocedural peripheral blood granulocyte counts did not seem to be associated with progression-free survival (Fig. 4A, ii).

Although no significant differences were observed in myeloid expansion after operative versus transarterial treatments for HCC, there was a more rapid time to progression in patients whose peripheral blood monocyte counts increased after any procedure (186 vs 372 days [P = not significant], Fig. 4B, i). Again, there was no correlation between change in granulocyte count and progression-free survival (Fig. 4B, ii).

Even with limited follow-up interval, progression-free survival of the HCC patients did vary predictably by BCLC stage (Fig. 4C, i). Notably, however, when analysis was limited to patients with intermediate stage (BCLC B) disease, there was still a trend toward poorer progression-free survival in patients with high preoperative peripheral blood monocyte counts (Fig. 4C, ii).

Conclusions

Macrophages are the dominant cell type that convert acute inflammation into resolution and wound healing through an ordered cascade of regulatory cytokines, including transforming growth factor (TGF)-β. Histological analysis of distinct cancer subtypes for the degree of macrophage

![Figure 4](image-url)
infiltrate has shown a significant association between high levels of macrophages and poor prognosis\textsuperscript{10,11}; the expansion of myeloid cells has been shown to correlate with cancer stage in patients with solid tumors.\textsuperscript{12} We hypothesize that a wound-healing phenotype in tumors causes mobilization of peripheral blood monocytes, which are polarized into an anti-inflammatory macrophage (ie, inflammatory resolution) phenotype within the tumor. Our results show that regardless of BCLC stage and treatment strategy, peripheral blood monocyte counts are a significant predictor of progression-free survival.

In this small cohort of patients, we did not observe a difference in myeloid expansion between treatments that induce hypoxia, such as chemoembolization, versus those that cause rapid cell death, such as RFA. Instead, we found that patients who developed exaggerated myeloid expansion after any intervention tended to have an earlier time to progression.

Future experiments are needed to characterize phenotype and function of intratumoral macrophages and to assess whether these affect the density and function of tumor-infiltrating lymphocytes. We observed a relative dearth of tumor-infiltrating cytotoxic CD8$^+$ lymphocytes within tumors; however, CD8$^+$ cells will need to be quantified in benign tumors and normal livers because the percentage of CD8$^+$ cells within nontumor tissue in HCC patients is likely elevated secondary to hepatitis. Nevertheless, because peripheral blood CD8$^+$ cell numbers did not change between patient groups, the presence of HCC did not cause an increase or decrease in systemic CD8$^+$ T cells.

In summary, as cancers progress, an array of noncancer cells are recruited into the tumor environment and directly influence the behavior of the tumor. The goal of this project was to link the inflammatory phenotype of peripheral blood myeloid cells in liver cancer patients with outcome and to evaluate whether treatment of the tumors resulted in alterations of this phenotype. We showed for the first time that the expansion of monocyte populations in peripheral blood is associated with significantly more rapid time to progression regardless of the type of treatment.

References