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Tumor-Associated Macrophage Infiltration in Colorectal Cancer Liver Metastases Is Associated With Better Outcome

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Abstract

Background—Tumor-associated macrophages (TAMs) support growth in most human cancers, with the notable exception of colorectal adenocarcinoma, in which TAM infiltration of primary tumors is correlated with a better outcome. The importance of TAMs in colorectal liver metastases (CLM) is unknown.

Methods—Using a tissue microarray of CLM resected at their institution from 1998 to 2000, the authors quantified immune marker expression by immunohistochemistry (IHC) using Metamorph Image Analysis software. Findings showed that CD68, CD3, CD4, CD8, FoxP3, and MHC-I were correlated with overall survival (OS) and disease-free survival (DFS).

Results—Tumor cores from 158 patients were analyzed. The median follow-up period was 117 months for survivors ($n = 39$). The univariate analysis showed a significant positive association between DFS and CD4⁺ ($p = 0.025$) and CD68⁺ ($p = 0.007$). The findings showed a significant positive correlation of OS with CD4⁺ ($p = 0.042$), whereas the correlation with CD68⁺ was not significant ($p = 0.17$). Cutoffs were determined to dichotomize each marker for the highest log-rank statistic. Patients with CD4^{high} had a median OS of 115 months and DFS of 41 months ($p = 0.007$ compared with 40 and 16 months, respectively, for patients with CD4^{low} ($p = 0.022$)). Patients with CD68^{high} had a median OS of 50 months and a median DFS of 25 months ($p = 0.67$) compared with 43 and 15 months ($p = 0.028$). In the multivariate analysis of factors affecting DFS, high CD68 was associated with longer DFS (hazard ratio [HR], 0.63, 95 % confidence interval [CI], 0.43–0.94; $p = 0.02$), independently of clinicopathologic variables and CD4.

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CONFLICT OF INTEREST There are no conflicts of interest.

Conclusions—High TAM infiltration in resected CLM is associated with better outcome, independently of known clinicopathologic and immune predictors. This suggests that TAM depletion, which is being tested clinically in other cancers, may be detrimental in CLM.

Tumor-associated macrophages (TAMs) usually are the most common immune cells in the tumor microenvironment.¹ Whereas macrophages exhibit a diverse range of biologic properties depending on the cellular context, it is thought that TAMs usually are M2-like, or pro-tumoral. Through a variety of molecular mechanisms, M2-like TAMs are thought to drive neoangiogenesis, suppress the adaptive immune response, and promote tumor cell proliferation, invasion, and metastasis. Clinically, however, determining the actual biologic relevance of TAMs in human cancer has proved to be difficult due to heterogeneity in methods of detection and analysis² and the limitations of immunohistochemistry (IHC).³ As a result, many individual studies have indeterminate findings.

A recent meta-analysis summarized 55 studies in which CD68⁺ TAMs were correlated with survival in 8692 patients.⁴ Consistent with the pro-tumoral phenotype mostly observed *in vitro* and in mice¹, most human cancers showed association of high TAM density with worse survival.

Studies have associated high TAM density with worse overall survival (OS) in breast cancer (5 studies; 2311 patients), gastric cancer (5 studies, 447 patients), oral cancer (3 studies, 280 patients), ovarian cancer (2 studies, 156 patients), and bladder cancer (2 studies, 104 patients). Other studies have been inconclusive. For example, lung cancer had three positive, three negative, and three indeterminate studies. These differences could be explained by nuances such as the exact location of the macrophages within the tumor,² a factor not accounted for in the meta-analysis. Nevertheless, only in colorectal cancer (CRC) was high TAM density found to have an association with better survival (5 studies, 1149 patients).

The studies of TAMs in CRC analyzed by Zhang et al. included only primary tumors, not metastases.^{5–9} It currently is well known that the adaptive immune response, specifically CD8⁺ cytotoxic T cells and FoxP3⁺ regulatory T cells, is prognostic in primary CRC, independently of traditional clinicopathologic variables.^{10–15} Likewise, we have shown that the immune signature in resected colorectal liver metastases (CLM)^{16–18} is associated with outcome independently of the clinical risk score (CRS).¹⁹ High CD4⁺ count, high CD8⁺:CD3⁺ ratio, and low FoxP3⁺:CD8⁺ ratio were associated with higher OS, independently of CRS.¹⁶ High tumor MHC-I in conjunction with high CD3⁺ count also was independently correlated with longer survival.¹⁸ The role of TAMs in resected CLM is unknown, so we performed additional analysis of our tissue microarray.^{16–18} We stained for the macrophage marker CD68 to assess the correlation of TAM infiltration with OS and disease-free survival (DFS) in the context of other previously studied immune markers and the CRS.

METHODS

Patients

We previously constructed a tissue microarray (TMA) of CLM from 188 patients who underwent resection with curative intent at our institution from 1998 to 2000.¹⁶ Briefly, 293 consecutive resections were reviewed, and those cases with sufficient material underwent pathologic review, excluding specimens with extensive necrosis or fibrosis. We applied our previously developed¹⁹ and validated²⁰ CRS to this cohort to assess the risk of recurrence.¹⁸ Institutional review board approval was obtained, and research was performed in compliance with Health Insurance Portability and Accountability Act regulations. The survival and recurrence status of survivors was last updated in January 2016, and we identified five additional deaths from our previous analysis in April 2013.¹⁸

Immunohistochemistry and Quantification

There were three 0.6-mm cores per tumor. Tissue microarray blocks were cut into 5- μ m sections, de-paraffinized, rehydrated serially through alcohol, and then stained and quantified as before.^{16–18} Briefly, for our previous study,¹⁸ slides were incubated with primary antibodies recognizing CD3 (F7.2.38; Dako), CD4 (polyclonal goat; R&D Systems), CD8 (c8/144B; Dako), FoxP3 (236A/E7; Abcam Inc.), and MHC-I (HC-10; provided by Hidde L. Ploegh, Whitehead Institute, Cambridge, MA, USA). For this study, we also used CD68 (KP1; Dako). Species-appropriate biotinylated secondary immunoglobulins then were added (Vector Laboratories, Inc.) followed by avidin-biotin peroxidase complexes (Vector Laboratories, Inc.). Diaminobenzidine was used as the chromogen. Nuclei were counterstained with hematoxylin using an automated Ventana XT and the OmniMap DAB Detection System (Roche). Slides were scanned in high resolution on a MIRAX SCAN (Carl Zeiss) and quantified using Metamorph Image Analysis Software (Molecular Devices). Quantification of T cell markers (CD3, CD4, CD8, FoxP3) had been accomplished as the cell count per core (mean for multiple cores) and the ratio of positive signal (brown) to total signal (brown plus area hematoxylin blue) for MHC-I using thresholds set to avoid connective tissue, fat, and necrosis.¹⁸ We quantified CD68 much the same as a positive:total signal ratio and analyzed this in the context of the previously quantified markers. Measurements were performed blinded to clinical data. Patients were excluded from analysis if no analyzable cores were available. Automated counts and ratios were visually confirmed.

Statistical Analysis

Both OS and DFS were calculated from the time of hepatectomy using the Kaplan—Meier method. For OS, death from any cause was counted as an event, and DFS was defined by recurrence or death from any cause. Groups were compared using the log-rank test. Median values are presented with their interquartile range (IQR).

We examined the univariate relationship of each immune parameter to OS and DFS on a continuous level using the Cox proportional hazards model. For immune markers showing significant association with survival in the univariate analysis, the optimal cutoff was determined using the method of maximally selected rank statistics. After cutoffs were

chosen, p values were adjusted for multiple comparisons. Using the optimal cutoffs to dichotomize values as high or low, significant markers were applied to a multivariate analysis including different levels of CRS. To examine the correlation between individual markers, Spearman correlation was used. All p values lower than 0.05 were considered statistically significant.

RESULTS

Patient Characteristics and Follow-up Evaluation

Of 188 patients in the TMA, 158 (84 %) were included in the analysis. Of the 30 patients excluded, 20 (11 %) did not have any analyzable cores for any marker, 8 (4 %) had inadequate follow-up data, 1 (0.5 %) had undergone palliative resection, and 1 was a duplicate (0.5 %). The total number of patients with at least one analyzable core was 154 to 158, depending on the marker. For example, there were 156 analyzable cores for CD68. Among the remaining 22 patients without analyzable cores, 3 were excluded due to necrosis, 4 due to fibrosis, 7 because the cores contained only mucin, and the remainder because of missing or damaged cores.

The median age at the time of liver resection was 63 years, and 57 % of the patients were men. The patient clinicopathologic characteristics have been reported^{16–18} and are summarized in Supplemental Table 1. A total of 52 patients (34 %) had a high CRS (≥ 3; 52 of 153 who had CRS available), corresponding to a median OS of 27 months (IQR, 15–42 months) compared with 68 months (IQR, 35–186 months) for those with a low CRS (<3). The median follow-up period 42 months for all the patients and 117 months (range, 7–199 months) for the survivors.

At the last follow-up visit, 39 patients were alive (25 %), 35 (90 %) of whom were disease-free. Of the 119 deaths, 98 (83 %) were due to cancer. Of 108 patients (68 %) who experienced recurrences, 4 were disease-free (after secondary resection or treatment), 4 were alive with disease, 98 had died of cancer, and 2 had died of unknown causes.

The median OS was 45.3 months (IQR, 26–132 months) for all the patients, with predicted 5- and 10-year survival rates of 39 % and 25 %, respectively. The median DFS was 17 months (IQR, 7–59 months), with predicted 5- and 10-year DFS rates of 25 % and 19 %, respectively.

TAM Infiltrate Positively Correlates With Disease-Free Survival

The IHC for CD68 was used to quantify TAMs (Fig. 1). The median positive-to-total signal ratio for CD68 was 0.05 (IQR, 0.03–0.1). In our previous study,¹⁸ the median T cell counts per core were as follows: CD3 (109; IQR, 79–170), CD4 (9; IQR, 3–23), CD8 (52; IQR, 30–90), and FoxP3 (16; IQR, 7–27), and the median positive-to-total signal ratio for MHC-I was 0.5 (IQR, 0.28–0.71). Assessment of the correlation between CD68 and T cell markers showed only weak positive correlation of CD68 with CD3 ($r = 0.18$; $p = 0.02$, two-tail), CD4 ($r = 0.27$; $p = 0.001$), and CD8 ($r = 0.27$; $p = 0.001$), and no correlation with FoxP3 ($r = -0.01$; $p = 0.89$).

The univariate analysis to determine the relationship of OS and DFS with the immune markers (Table 1) showed a significant association between longer OS and high CD4 ($p = 0.04$), whereas the association with CD68 was not significant ($p = 0.17$). Longer DFS was significantly associated with high CD4 ($p = 0.025$) and high CD68 ($p = 0.007$).

Cutoffs were determined for OS and DFS for CD4 and CD68 to dichotomize each marker and give the highest log-rank statistic (Fig. 2). For CD4, patients above the cutoff of 26 cells per core had a median OS of 115 months (IQR, 33–N/A) and a median DFS of 41 months (IQR, 11–N/A) compared with 40 months (IQR, 23–77 months) and 16 months (IQR, 7–33 months), respectively, for the patients below the cutoff ($p = 0.007$ and 0.022 , respectively, adjusted for multiple comparisons; Fig. 2A). For CD68, the optimal cutoff signal ratios (positive to total) were 0.076 for OS and 0.0685 for DFS. For the patients above the cutoff, the median OS was 50 months (IQR 28–157 months), and the median DFS was 25 months (IQR, 10–156 months) compared with 43 months (IQR 23–76 months) and 15 months (IQR, 7–29 months), respectively, for the patients below the cutoff ($p = 0.67$ and 0.028 , respectively; Fig. 2B). In summary, high CD4 was associated with longer OS and DFS, and high CD68 was associated with longer DFS.

Given these findings, we sought to determine whether the differences in DFS for CD4 and CD68 were independent of well-established predictors of recurrence after resection of CLM.^{19,20} In the multivariate analysis of factors affecting DFS (Table 2), different levels of CRS were associated with survival, with a CRS of 5, 4, or 3 compared with a CRS of 0 having respective hazard ratios (HRs) of 6.7 ($p = 0.002$), 3.6 ($p = 0.011$), and 2.4 ($p = 0.052$). Notably, in this dataset, perioperative and regional infusion chemotherapies were not associated with longer survival, and the proportion of either treatment did not differ in the CD68 or CD4 dichotomized groups (data not shown). High staining for CD68 was associated with longer DFS (HR, 0.63; 95 % confidence interval [CI], 0.43–0.94; $p = 0.023$), independently of CRS. In this multivariate model, CD4 trended toward significance (HR, 0.61; CI, 0.37–1.01; $p = 0.053$). Although high CD4 trended toward a positive correlation, with a longer DFS in the multivariate analysis, only 16 % of the patients with a CRS of 3 or higher had high CD4 (Table 3). In contrast, high CD68 appeared to identify an immunologically distinct subset of high CRS CLMs having a better prognosis, with 36 % of the patients who had a CRS of 3 or higher showing high CD68.

DISCUSSION

It is now well established that the magnitude and composition of T cell infiltration in primary and metastatic CRC bear prognostic significance, independently of known clinicopathologic factors.^{10–18} Although the clinical significance of TAMs has been more elusive due to heterogeneity in methods of study and analysis, it appears that in contrast to the majority of human cancers,⁴ high TAM infiltration in primary CRC is associated with prolonged OS, as well as less lymph node and distant metastases.^{5–9} To our knowledge, this has not been evaluated to date for CRC in the metastatic setting. In this report, we show that in resected CLM using IHC for CD68, high levels of TAMs correlated with longer DFS in the multivariate analysis (HR, 0.63; 95 % CI, 0.43–0.94; $p = 0.023$), independently of high

CRS and high CD4, the only other immune marker in this analysis to have significant association with survival in the univariate analysis.

Although TAM infiltration in primary CRC may provide additional prognostic information about the risk of recurrence, in primary tumors it may not lead to a change in therapy because the rate of cure with surgical resection and modern adjuvant therapy is high.²¹ However, given the high failure rate after resection of CLM,¹⁹ it is important to know whether TAM depletion could be beneficial in the event of recurrence because several agents are in clinical trials for therapeutic depletion of TAMs.²² Our results suggest that TAM depletion in CLM may be detrimental. In contrast, in addition to retrospective analysis showing a high relative risk of death in breast cancers that contain high levels of TAMs,⁴ there is a preclinical rationale and clinical confirmatory studies²³ for depletion of TAMs in clinical trials.²⁴ Nevertheless, although our study may not support depletion, strategies to activate TAMs could still be pursued in CLM. In advanced pancreas cancer, a CD40 agonist recruited tumoricidal macrophages, leading to a radiologic response in 88 % of patients and a progression-free survival of 5.6 months compared with 2.3 months historically for patients treated with gemcitabine.²⁵

Patients with Lynch syndrome, in which germline mutations in mismatched repair (MMR) genes lead to microsatellite instability (MSI),²⁶ are known to have a high mutational load, high T cell infiltrate, an overall better outcome, and high response rates to checkpoint blockade compared with microsatellite-stable CRC.²⁷ Among our 39 survivors, we found that MMR/MSI status was known in only 1 patient, whereas the remaining 38 patients had not been tested because this was not common practice at the time. However, patients with MMR-deficient tumors rarely experience liver metastasis, as shown in a study of 190 resected CLM. In that study, only five patients (2.7 %) were MSI^{high}, and their prognosis was similar to that of MSI^{low} patients.²⁸ Additionally, although MSI^{high} tumors have high T cell infiltrates, whether this correlates with high TAM infiltrates is unknown. Overall, it is unlikely that MMR/MSI status would explain the better DFS seen in our CD68^{high} patients.

It remains unclear why TAMs appear to be anti-tumoral in CRC, in contrast to most other cancers. We have shown that it does not depend on site. It is uncertain whether TAMs in CLM are recruited from blood monocytes or derived from resident Kupffer cells. Substantial preclinical work has been done to elucidate the mechanism of TAM polarization.²⁹ Ultimately, we and others believe that assessment of polarization and biologic relevance of TAMs should be through evaluation of many markers (not just one or two) and through primary functional analysis either *in vivo* or *ex vivo*.^{3,30} Functional *ex vivo* study of human CRC macrophages in CRC will need to be performed, as we did for gastrointestinal stromal tumor.³

The location of TAMs within the tumor may play a key role in primary CRC, with TAMs at the periphery of the tumor expressing high co-stimulatory molecules CD80 and CD86, compared with the tumor stroma,³¹ and causing cancer cell apoptosis in a Fas ligand-dependent manner.³² Multiple studies show that high levels of TAMs at the invasive margin are associated with better outcome or clinicopathologic factors.^{6,9,33} In contrast, one study, in which TAMs were counted in the entire tumor including necrotic areas, showed an

association of higher TAMs and higher stage, but no survival analysis was reported.³⁴ Bacman et al.⁵ (included in the meta-analysis⁴) used a TMA platform similar to ours to evaluate primary CRC tumor parenchyma and showed correlation of higher TAM infiltration with higher tumor stage and lymph node metastasis, but no difference in OS. One theory to explain this difference maintains that peripherally located TAMs have less exposure to tumor-derived cytokines and are less hypoxic, both of which are believed to drive a pro-tumoral phenotype.¹

Our results are similar to those in studies of primary CRC evaluating TAM infiltration at the invasive margin. Other studies evaluating primary tumor parenchyma came to different conclusions.^{33,34} However, those studies used manual histology techniques, which are subject to numerous limitations,³⁵ and included necrotic and fibrotic areas, which we excluded. Regardless, analysis of a core from tumor parenchyma is simpler and could be applied to percutaneous core needle biopsies, which could drive targeted therapy with TAM-depleting or TAM-modulating agents. Full assessment of a tumor's invasive margin would require resection or incisional biopsy, which are impractical for patients who are not candidates for resection. Nevertheless, our findings need further validation.

Inherent to any TMA study is the possibility of selection bias. Our goal was to assess the association of TAM infiltration in viable tumor with survival. Therefore, we excluded necrotic and fibrotic tumors before creating the TMA, and among those in the TMA, we did not include in the analysis cores that were largely necrotic or fibrotic (~4 % of total). Thus, our results apply only to TAM levels within viable tumor, and cannot be applied to tumors that are mostly fibrotic or necrotic. In our experience with TMAs, this is an acceptable fraction of exclusions, although we cannot definitively rule out the possibility of selection bias. Likewise, the quantification techniques used had limitations, mostly related to sampling error. However, we believe automated quantification is better than manual histology techniques.³⁵ Manual review of an entire tumor specimen for quantification is impractical, especially for large and multiple tumors, and not feasible or appropriate for patients with metastatic unresectable CLM.

Overall, high infiltration of resected CLM with TAMs is associated with longer DFS, independently of other immune markers and CRS. Our findings suggest that therapeutic TAM depletion may in fact be detrimental in CLM. Future work will focus on understanding the mechanism of increased TAM infiltration by examining the differential intratumoral cytokine and chemokine environments among tumors. A number of agents are being tested in clinical trials that stimulate TAMs, such as anti-CD40. These agents could be applied to colorectal cancer liver metastases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Immunohistochemistry (IHC) for tumor-associated macrophages (TAMs). CD68⁺ TAMs were quantified as the ratio of positive signal (*brown*) to total signal (*brown* plus *hematoxylin blue*). Representative CD68 staining is shown with three replicates of 0.6- μ m cores exhibited for one patient with high staining (*top row*) and one patient with low staining (*bottom row*). Each data point represents the mean of replicates for that patient, and the median is indicated.

FIG. 2.

Overall survival (OS) and disease-free survival (DFS) stratified by CD4 and CD68. Kaplan-Meier curves are shown depicting OS and DFS stratified by CD4 (**A**) and CD68 (**B**). Cutoffs were determined using the method of maximally selected rank statistics. The final p values shown were adjusted for multiple comparisons. The number of patients at risk is listed for each time point.

TABLE 1

Univariate analysis of immune markers with survival

OS marker	HR	95 % Lower	95 % Upper	<i>p</i> Value
CD3	1.00	1.00	1.00	0.90
CD4	0.99	0.98	1.00	0.04
CD8	1.00	1.00	1.00	0.32
FoxP3	1.00	1.00	1.01	0.45
FoxP3:CD8	1.27	0.95	1.68	0.11
MHC-I	0.72	0.37	1.40	0.33
CD68	0.11	0.01	2.59	0.17
DFS				
CD3	1.00	1.00	1.00	0.81
CD4	0.99	0.98	1.00	0.025
CD8	1.00	1.00	1.00	0.46
FoxP3	1.00	0.99	1.01	0.82
FoxP3:CD8	1.27	0.92	1.77	0.15
MHC-I	0.66	0.34	1.26	0.21
CD68	0.01	0.00	0.27	0.007

OS, overall survival;

HR, hazard ratio;

DFS disease-free survival

TABLE 2

Multivariate analysis of immune markers and CRS with DFS

	HR	95 % Lower	95 % Upper	p Value
CD68 (>0.0685 vs 0.0685)	0.63	0.43	0.94	0.023
CD4 (>26 vs 26)	0.61	0.37	1.01	0.053
CRS 1 vs 0	0.94	0.38	2.28	0.88
CRS 2 vs 0	1.39	0.59	3.27	0.46
CRS 3 vs 0	2.39	0.99	5.78	0.052
CRS 4 vs 0	3.59	1.33	9.66	0.011
CRS 5 vs 0	6.73	1.96	23.05	0.002

CRS, clinical risk score; DFS, disease-free survival; HR, hazard ratio

TABLE 3

Overlap of CD68^{high} and CD4^{high} patients with different levels of clinical risk score (CRS)^a

Marker	<i>n</i>	CRS >3 (<i>n</i> = 50) (%)	CRS >4 (<i>n</i> = 18) (%)	CRS 5 (<i>n</i> = 5) (%)
CD68 ^{high}	63	36	22	0
CD4 ^{high}	31	16	11	20
CD68 ^{high} and CD4 ^{high}	19	6	0	0

^aThere were 148 patients for whom CRS, CD68, and CD4 were available. The percentages reflect the proportion of patients in the listed CRS group bearing high levels of the listed immune marker or markers.