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Regulatory T Cell Infiltration Predicts Outcome Following Resection of Colorectal Cancer Liver Metastases

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Abstract

Background—Tumor-infiltrating lymphocyte (TIL) counts in colorectal cancer liver metastases (CRCLM) predict survival following resection. While CD4 and CD8 T cells have been correlated with outcome following CRCLM resection, the role of regulatory T cells (Treg) is not well defined.

Methods—TIL in 188 patients who underwent CRCLM resection between 1998 and 2000 were analyzed by immunohistochemistry using tissue microarrays. Correlation between TIL composition and outcome was determined while controlling for established prognostic factors. Total T cells (CD3), helper T cells (CD4), cytotoxic T cells (CD8), and Treg (FoxP3) were analyzed.

Results—Median follow-up time was 40 months for all patients and 95 months for survivors. Overall survival (OS) at 5 and 10 years was 40 and 25 %, respectively. The CD4 T cell count correlated with OS ($p = .02$) and recurrencefree survival ($p = .04$). A high number of CD8 T cells relative to total T cells (CD8:CD3 ratio) predicted longer OS times ($p = .05$). Analysis of Treg revealed that high FoxP3:CD4 ($p = .03$) and FoxP3:CD8 ($p = .05$) ratios were independent predictors of shorter OS. Patients with a high clinical risk score (CRS) were more likely to have a high number of intratumoral Treg, and patients ≥65 years old had a less robust CRCLM T cell infiltration.

Conclusions—A high number of Treg relative to CD4 or CD8 T cells predicted poor outcome, suggesting an immunosuppressive role for FoxP3 + TIL. The intratumoral immune response was an independent predictor of outcome in patients with colorectal liver metastases.

The immune response to neoplastic cells has been shown to predict survival in several human malignancies, including primary colorectal cancer.[1–6] Our interest in the immune response to colorectal cancer liver metastases (CRCLM) is prompted by data indicating that the vast majority of patients recur despite curative resection and optimal adjuvant therapy.[7,8] We speculate that the immunosuppressive nature of liver immune cells[9–14] contributes to the propensity of colorectal cancer to metastasize to the liver and recur following resection. Tumor-infiltrating lymphocyte (TIL) counts were shown to predict outcome in a selected group of short- and long-term survivors following resection of CRCLM.[15] More recently, our group reported that a high number of regulatory T cells (Treg) predicted decreased survival in patients with neuroendocrine tumor liver metastases.[16] A better understanding of Treg in CRCLM may allow for the identification of patients who fail to mount an effective

immune response and facilitate development of immunologic interventions designed to enhance intrahepatic immunity.

Our goals for this study were to determine the potential biologic importance of Treg in CRCLM patients and confirm that CD4 and CD8 T cell counts correlate with outcome. We characterized TIL from 188 patients who underwent liver resection. Tissue microarrays (TMA) were stained for CD3, CD4, CD8, and FoxP3 to quantify total T cells, helper T cells, cytotoxic T cells, and Treg, respectively. TIL subset counts and immune cell ratios were correlated with outcome. Several specific immunologic factors, including the Treg tumor infiltrate, were found to be independent predictors of outcome following CRCLM resection. Clinical correlates of TIL densities, including age and the clinical risk score (CRS), were demonstrated as well. These data support the biologic importance of the host immune response to intrahepatic metastases, including the intratumoral Treg infiltrate.

METHODS

Patients

A prospectively maintained hepatobiliary database was used with approval of the Institutional Review Board and in accordance with Health Insurance Portability and Accountability Act regulations. Of 293 consecutive patients who underwent resection of CRCLM at Memorial Sloan-Kettering Cancer Center (New York, NY) from September 1998 to 2000, we identified 188 who had sufficient tissue for TMA construction and analysis. Guidelines for resectability were medical fitness for major laparotomy and a resection encompassing all intrahepatic disease with an adequate remnant liver for recovery. Routine preoperative evaluation included chest X-ray, abdominal/pelvic computed tomography, and colonoscopy.

Immunohistochemistry and Cell Count Analysis

TMA were constructed following pathologic review and diagnostic confirmation as previously described.[15] Tissue blocks with minimal necrosis and fibrosis were selected to enable accurate acquisition of intratumoral lymphocyte counts, comparable among specimens. In brief, tumor tissue cores measuring 0.6 mm in diameter were made in triplicate from paraffin blocks and processed using the ATA-27 automated arrayer (Beecher Instruments, Sun Prairie, WI). TMA blocks were cut to 5-μm sections and deparaffinized, rehydrated in graded alcohol, and processed.[17] Antibodies were used to recognize CD3 (F7.2.38, Dako, Carpinteria, CA), CD8 (c8/144B, Dako), CD4 (polyclonal goat, R&D Systems, Minneapolis, MN), and FoxP3 (236A/E7, Abcam Inc., Cambridge, MA). Biotinylated secondary immunoglobulins were added (Vector Laboratories, Inc., Burlingame, CA) followed by avidinbiotin peroxidase complexes (1:25; Vector Laboratories, Inc.). Diaminobenzidine was used as the chromogen, and hematoxylin was used as the nuclear counterstain. TMA slides were analyzed with the Aperio ScanScope XT and Aperio IHC analysis algorithm (Aperio, Vista, CA). Our automated counting method was previously validated.[15] We excluded cases with fewer than 2 analyzable cores, which occurred in one instance each for CD3 and CD4 staining.

Statistical Analysis

The Kaplan–Meier method was used to estimate overall survival (OS) and recurrence-free survival (RFS). Cox regression multivariate models were used to identify independent prognostic factors. Separate multivariate models were constructed for each cell marker or cell marker ratio given the coexpression of the markers by single cell types and hence the potential for statistical interaction. The multivariate models for immunologic factors included the CRS and extrahepatic disease (EHD).[7] Unpaired *t* tests and chi-square tests

were used to compare TIL frequencies among subgroups. Optimal cutoff points for cell numbers were selected using the maximally selected chi-square method (maxχ^2, R version 2.7, www.r-project.org).[18] Use of the maxχ^2 method enables selection of an outcome-dependent cutoff point that may be most clinically useful and avoids arbitrary cutoff points such as the median or a percentile-based value. A p value of ≤.05 was considered statistically significant (SPSS, version 15.0; Chicago, IL).

RESULTS

Patient Characteristics and Follow-Up

The average age was 63 years (range, 23–86 years) for 188 patients who underwent CRCLM resection (Table 1). The median follow-up time for all patients was 40 and 95 months for survivors (range, 0–133 months). A minority of patients had EHD (17 %), 66 % had evidence of primary tumor nodal metastases, and 26 % had a clinical risk score (CRS) ≥3. Chemotherapy was administered to 89 % of patients, with 25 % of patients receiving systemic therapy prior to resection and 26 % undergoing hepatic artery infusion. Systemic therapy regimens included irinotecan or oxaliplatin in 54 and 7 % of all patients, while 22 % received 5-fluorouracil based treatment without inclusion of a platinum agent or topoisomerase inhibitor. To determine whether TIL numbers and ratios correlated with outcome following liver resection, we stained TMA for CD3, CD4, CD8, and FoxP3. We found that 20, 23, and 19 % of patients had high levels of CD3+, CD4+, and CD8+ TIL counts, respectively. A high level of FoxP3+ TIL, which are putative Treg, was documented in 14 % of those treated in this series (Fig. 1a).

TIL Subset Numbers Correlate with Survival and Recurrence Following CRCLM Resection

OS at 5 and 10 years was 40 and 25 % for the entire group. We analyzed nonimmunologic factors previously shown to be of prognostic importance in patients undergoing CRCLM resection.[7,19] Significant predictors of survival included the CRS and EHD (Table 2). The presence of EHD and a CRS ≥3 were significant predictors of poor recurrence-free survival (Table 2). Other correlates of OS and RFS included primary tumor lymph node metastases, tumor size, and CEA level (not shown).

Analysis of CD4 and CD8 staining demonstrated that OS was significantly greater among patients with a high level of TIL expressing either marker (Fig. 1b). The 5-year OS among those with a high level of CD4+ TIL was 55 % compared with 35 % for patients with a low CD4+ TIL count (p = .004). A significant 5-year survival advantage was also found for patients with a high number of CD8+ TIL (59 vs 35 %, p = .04). When multivariate analyses were performed, a high CD4+ TIL count proved to be a significant independent predictor of OS [odds ratio (OR) =1.8, p = .02], while a high CD8+ TIL count approached statistical significance (OR = 1.5, p = .08). Superior RFS at 5 years was associated with a high CD4+ TIL count on univariate (Fig. 1c) and multivariate analysis (58 vs 39 %, p = .04).

CD4 and CD8 T cell Ratios and Outcome Following Resection of CRCLM

As previously reported, immune cell ratios may be more informative than individual TIL subtype counts.[15] High CD4:CD8 and CD4:CD3 ratios were significant predictors of favorable OS and RFS on univariate analyses (Fig. 2a; Table 2). Although the absolute number of CD8 T cells was not an independent predictor of outcome, the CD8:CD3 ratio was a significant independent predictor of OS (OR = 1.6, p = .05). The median OS time for patients with a high CD8:CD3 ratio was 84 months, compared with 42 months for those with a low ratio.

Infiltration of CRCLM by Regulatory T Cells Predicts Poor Outcome Following Resection

FoxP3 is a marker for Treg, which can suppress the ability of CD4 and CD8 liver T cells to mount an effective immune response. The absolute number of FoxP3+ cells did not predict OS or RFS (Fig. 1b, c), although patients with a high FoxP3+ TIL count had a 5-year OS rate of only 31 % (Table 2). We also analyzed ratios of FoxP3+ cells to other TIL types, as the presence of Treg relative to CD4 and CD8 T cells has been well documented to influence their suppressive effects.[20] High FoxP3:CD4 and FoxP3:CD8 ratios were significant independent predictors of shorter OS (Fig. 2b; Table 2). At 5 years following CRCLM resection, 51 % of those with a low FoxP3:CD4 ratio were alive compared with 34 % for those with a high ratio (OR = 1.6, $p = .03$). Similarly, 5-year survival was significantly greater in those with a low FoxP3:CD8 ratio (46 %) when compared with those with relatively more FoxP3+ cells (35 %, HR = 1.5, $p = .05$).

Predicting the Extent of CRCLM T Cell Infiltration

To assess potential predictors of T cell counts within tumors, we compared CRCLM TIL frequencies among patient subsets defined by well-accepted clinical predictors of outcome (Fig. 3). Patients with a high CRS were more likely to have high FoxP3+ TIL counts (Fig. 3a). Since the majority of our patients (89 %) received some form of perioperative chemotherapy, we were interested in determining whether chemotherapy before resection impacted TIL frequency. A trend toward a higher T cell count was noted among patients who received neoadjuvant chemotherapy (30 %) compared with those who did not receive systemic therapy prior to resection (18 %, $p = .08$, not shown). However, when individuals who received irinotecan or oxaliplatin were analyzed separately, we found that administration of these drugs prior to liver resection correlated with a greater percentage of patients with high levels of favorable TIL counts. Among individuals who were given irinotecan or oxaliplatin before liver resection, 43, 34, and 36 % had high CD3+, CD4+, and CD8+ TIL counts, compared with 13, 15, and 18 % for those who did not receive these agents preoperatively ($p \leq .01$ for each comparison, data not shown). When the mean CD3+ TIL counts were compared among subgroups, older patients (≥65 years) had significantly lower levels of T cell infiltration (Fig. 3c, $p = .01$).

DISCUSSION

We have demonstrated an association between the degree of tumor T cell infiltration and outcome following resection of CRCLM. After controlling for EHD and the CRS, individual T cell counts and subset ratios were significant predictors of survival and recurrence following CRCLM resection. High ratios of CD4 and CD8 T cells relative to the total infiltrate were significant predictors of favorable outcomes following CRCLM resection. Patients with high FoxP3:CD4 and FoxP3:CD8 ratios had shorter survival times. The balance between Treg and conventional CD4 or CD8 T cells may be a determinant of a patient's capacity to mount an effective immune response to intrahepatic metastases.

In our prior study of highly selected 2- and 10-year survivors, we noted that patients with a more robust CD8 T cell response were more likely to survive long-term following CRCLM resection.[15] Another group recently substantiated our initial report of the prognostic importance of CRCLM TIL.[21] Presently, we confirmed that CRCLM CD8 T cell infiltration correlates with survival time following resection in a larger, more recent series of patients, consistent with what has been reported for primary colorectal cancer.[3,4,22] These findings fit well with the role cytotoxic CD8 T cells play as direct mediators of tumor killing and the enhanced activation status of CRCLM TIL compared with T cells in nontumor liver.[23]

CD4 T cells are essential for the generation of effective antitumor immune responses, as they provide the necessary help for cytotoxic CD8 T cells.[24] In the present study, we found that a high CD4 count was an independent predictor of survival and recurrence. We previously reported that CD4 T cells correlated with a decreased chance of longterm survival.[15] The patient groups from the present and prior studies are difficult to compare and differ in ways that have the potential to influence the nature of the immune response. As noted earlier, our previous work was confined to 2- and 10-year survivors and thus a limited spectrum of disease biology. The present series includes a more recent group of patients who were exposed to more modern cytotoxic therapy regimens. The differences in chemotherapeutic agents may have influenced the impact of the CD4+ infiltrate among the two studies. Oxaliplatin and irinotecan have been demonstrated to have significant effects on CD4 T cells and our patients who received these agents prior to hepatic resection had significantly higher levels of CD4+ TIL counts.[25–27] In this series, 25 % of patients received systemic therapy preoperatively. Only 19 % of subjects in our previous report were recipients of prehepatectomy systemic treatment, and none received oxaliplatin- or irinotecan-based regimens. When considering the functional plasticity of CD4 T cells, it is not surprising that the prognostic implications of CD4+ TIL vary among patient groups treated in different eras with alternative regimens.

Treg are a small subset of CD4 T cells and are identified by their expression of FoxP3, a transcription factor critical to their differentiation and suppressive function.[28] FoxP3+ cell counts have been associated with a higher likelihood of disease progression in several malignancies.[2,16,29,30] Although patients with a high number of FoxP3+ cells had lower OS and RFS rates, the results were not significant. The functional importance of Treg lies in their ability to interact with and suppress helper CD4 and cytotoxic CD8 T cells.[20,31,32] As such, we analyzed ratios of Treg to CD4 and CD8 T cells. High FoxP3:CD4 and FoxP3:CD8 ratios were independent predictors of shorter survival following CRCLM resection (Fig. 2b; Table 2). These findings suggest that a high number of Treg in relation to helper and cytotoxic T cells leads to a more suppressive tumor microenvironment and hence decreased survival.

In several instances, TIL ratios proved to be better predictors of outcome than individual cell counts, indicating the importance of immune cell interactions and networks within the liver.[32] The CD8:CD3, FoxP3:CD4, and FoxP3:CD8 ratios were independent predictors of outcome, whereas the individual CD8 and Foxp3 counts were not. Immune cell ratios may provide more insight into the functional impact of a subset of TIL, as the ultimate clinical impact of a TIL subset will depend on the ability of the cells to influence the immunologic response as a whole. In addition, TIL ratios allowed for the identification of a greater number of patients with favorable outcomes. TIL ratios may be more biologically relevant and of greater use in clinical decision making.

To enhance the potential utility of TIL counts and ratios, the identification of correlates of an effective immune response to CRCLM is of interest. We found that patients with poor prognoses, as defined by high CRS, were more likely to have a high level of suppressive Treg. The presence of a high number of Treg may have contributed to poor outcomes in those with a high CRS, although we cannot infer causation based on our data. In addition, patients over the age of 65 years were less likely to have a high level of CRCLM T cell infiltration and age-related declines in immunity have been described.[33] Confirmation of these correlations and identification of other surrogates of immune responses to CRCLM will increase the potential clinical applicability of our findings.

Our data must be interpreted in the context of study design limitations. Use of TMA to study TIL introduces the potential for sampling error. To minimize the influence of sampling

error, 3 tissue cores were included for each patient and we calculated the mean cell counts. Our tissue cores included tumor only, without normal liver or the tumor-liver interface. A prior report indicated that the total number TIL is of greater prognostic importance than separate analyses of intratumoral T cells and those at the margin.[3] In addition, we recognize that FoxP3 is not a perfect marker for Treg in humans.[34,35] Finally, treatment-related variables such as liver-directed therapy following resection or systemic regimens prior to referral for treatment of CRCLM may have impacted the outcomes of certain patients. While our data suggest a favorable T cell response may prolong survival following CRCLM resection, we cannot be certain that antitumor immunity was responsible for improved OS or if both simply reflect unidentified surrogates of tumor biology.

The present study demonstrates that T cell subset and ratio analyses provide independently significant prognostic information following resection of CRCLM. TIL analysis may be useful for refining prognoses following CRCLM resection. More importantly, our findings suggest that immunomodulatory therapy designed to enhance the endogenous antitumor response may be a valuable approach. Further work is needed to determine if agents that block T cell regulatory checkpoints, such as the PD-1 and CTLA-4 pathways, will enhance intrahepatic immune responses to CRCLM.[36–38] Future studies should focus on the immunologic network within the liver, in addition to individual cell type and their specific phenotypic and functional properties.

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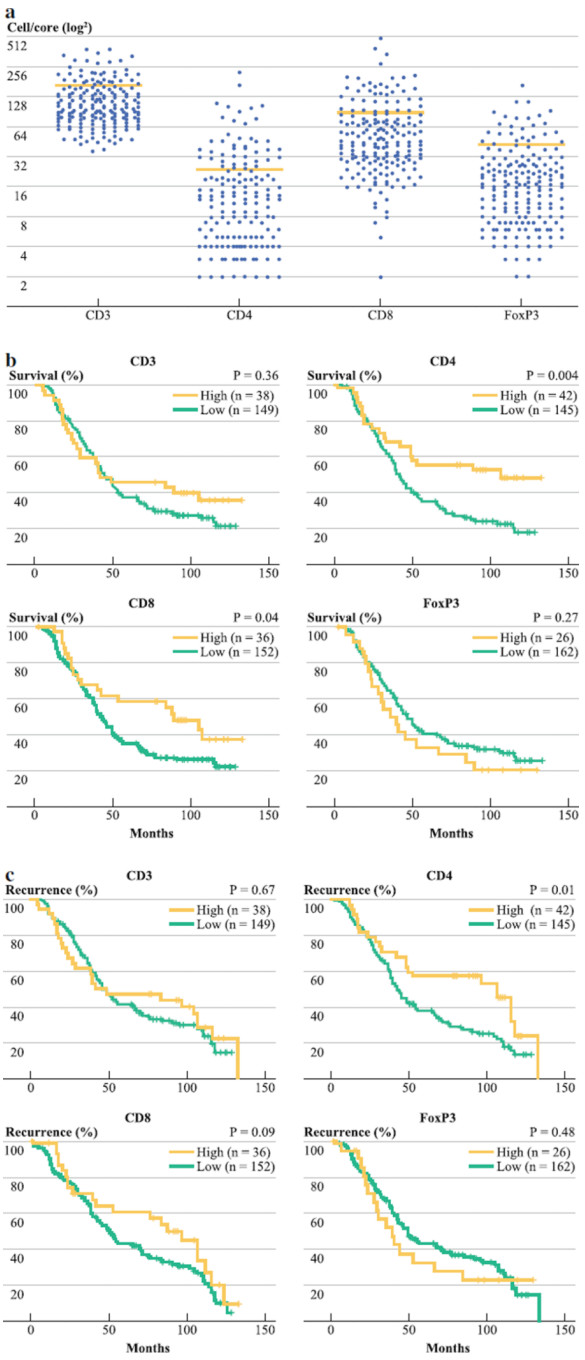

FIG. 1.

Tissue microarray samples from resected CRCLM were stained for various T cell markers. **a** The distributions of TIL counts for each marker are shown. The *bars* indicate the cutoff points (cells/tissue core) for each cell marker (CD3 = 174, CD4 = 26, CD8 = 95, FoxP3 = 44). Overall (**b**) and recurrence-free (**c**) survival were determined by the Kaplan–Meier method, and we used the log-rank test to compare groups

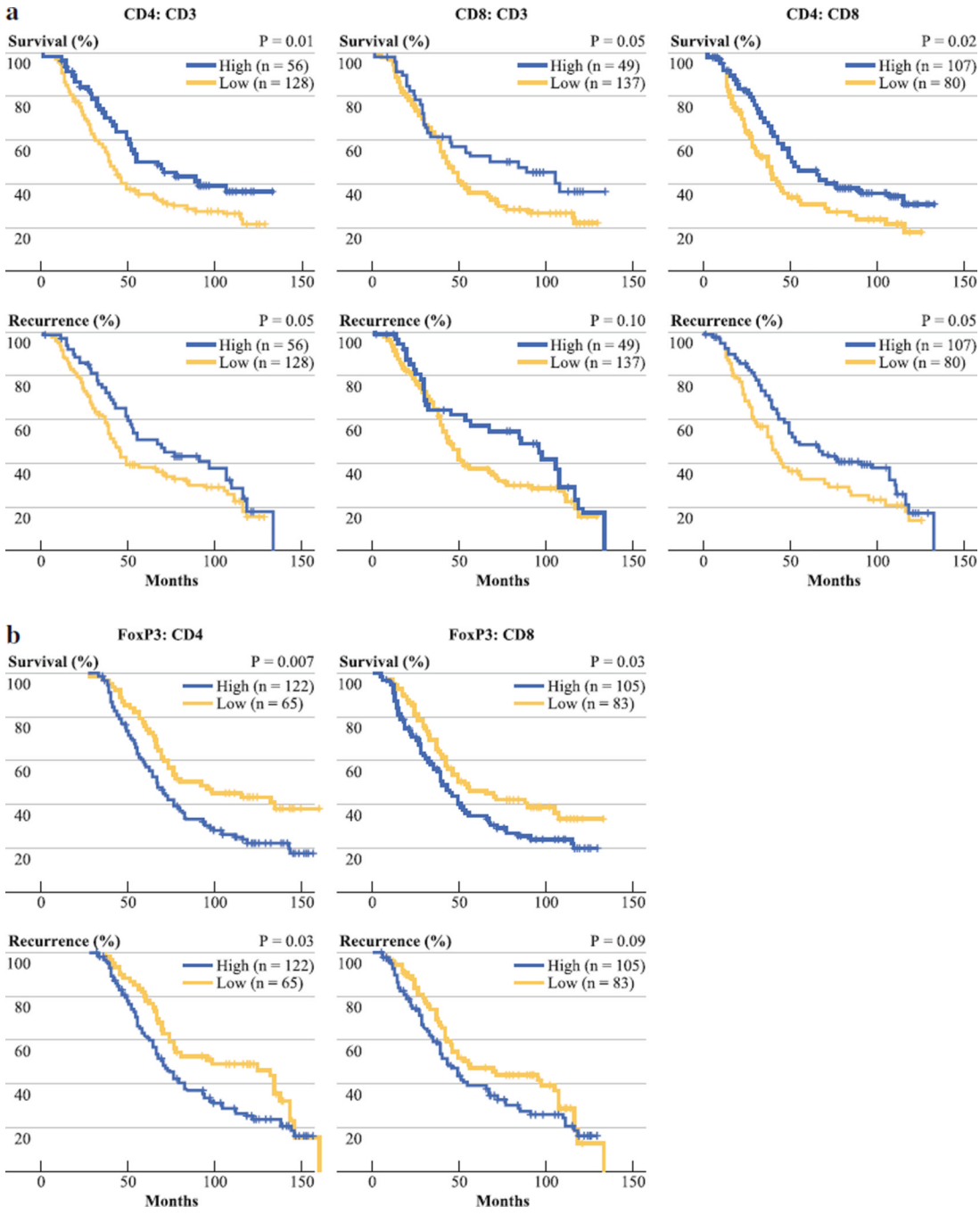

FIG. 2.

Tissue microarray samples from resected CRCLM were stained for various T cell markers and cell ratios were calculated. We analyzed various ratios based on CD3, CD4, and CD8 staining of TIL (**a**). In addition, we examined ratios of FoxP3+ TIL to CD4 and CD8 T cells (**b**). Overall and recurrence-free survival were determined by the Kaplan–Meier method and we used the log-rank test to compare groups

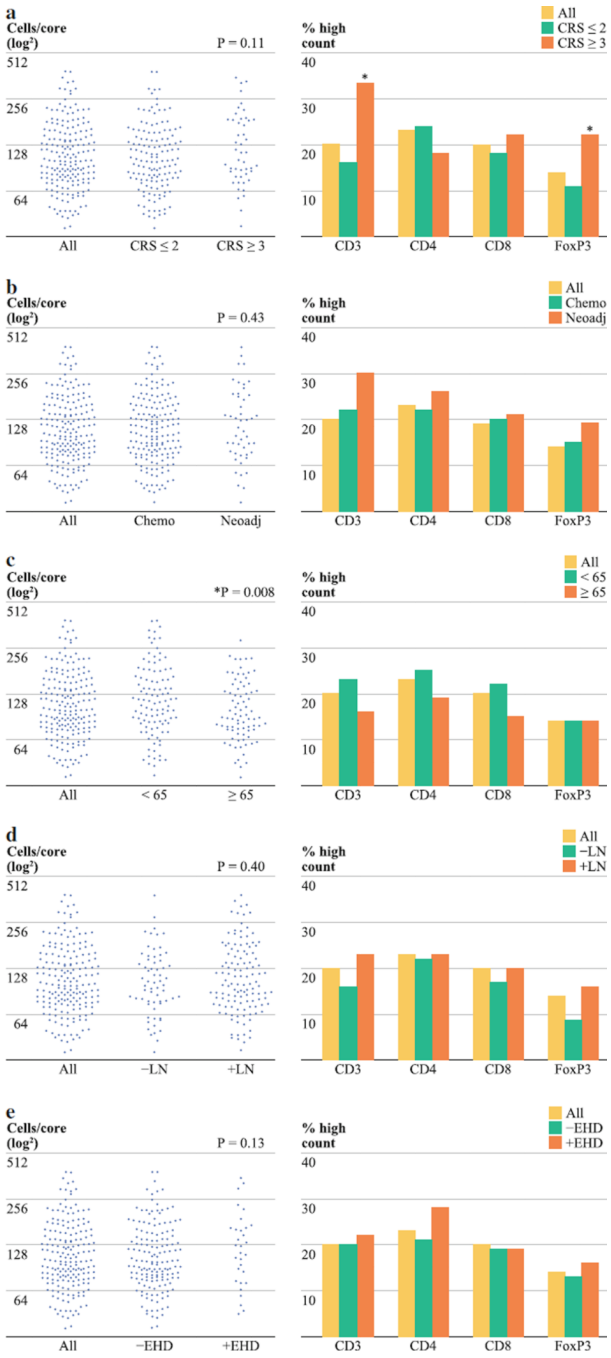

FIG. 3.

The frequencies of CD3+ TIL counts and proportion with high TIL subset counts is shown for subgroups defined by clinical risk score (**a**), systemic therapy (**b**), age (**c**), primary tumor node status (**d**), and presence of extrahepatic disease (**e**). Neoadjuvant therapy refers to treatment given prior to hepatic resection. The distribution plots on the left reveal the CD3+ TIL counts for all patients, and the *p* values represent comparisons of the mean cell numbers among subgroups. The *bar graphs* on the *right* demonstrate the proportion of patients within each subgroup who had high TIL counts for each marker. The cutoff points (cells/ tissue core) for each cell marker are as follows: CD3 = 174, CD4 = 26, CD8 = 95, FoxP3 = 44

TABLE 1

Patient characteristics

Median age, years (range)	**63 (23–86)**	
	N	**%**
Male sex	102	54
Extrahepatic disease	32	17
DFI <12	86	46
Tumor>5 cm	62	33
Multiple tumors	88	47
Node + primary	124	66
Synchronous disease	15	8
CEA >200	26	14
CRS ≥3	49	26
Any chemotherapy	168	89
5-FU based regimen	42	22
Oxaliplatin based regimen	13	7
Irinotecan-based regimen	102	54
Posthepatectomy chemotherapy	166	88
Prehepatectomy chemotherapy	47	25
Regional chemotherapy	48	26
CD3 High	38	20
CD4 High	42	23
CD8 High	36	19
CD4:CD3 High	56	30
CD4:CD8 High	107	57
CD8:CD3 High	49	26
FoxP3 High	26	14
FoxP3:CD4 High	122	65
FoxP3:CD8 High	105	56

TABLE 2

Analysis of the association of tumor-infiltrating lymphocyte numbers and other clinicopathologic variables with overall survival

Factor	5-year OS (%)	Median OS (months)	UV *P* value	MV *P* value	OR (95 % CI)	5-year RFS (%)	Median RFS (months)	UV *P* value	MV *P* value	OR (95 % CI)
CD3+										
High	46	42	.360	–	–	48	49	.67	–	–
Low	38	45				42	49			
CD4+										
High	55	107	.004	.02 **	1.8 (1.1–2.9)	58	107	.01	.04	1.6 (1.0–2.6)
Low	35	40				39	43			
CD8+										
High	59	89	.04	.08	1.5 (0.9–2.5)	59	97	.09	–	–
Low	35	43				39	46			
CD4+:CD3+										
High	53	68	.01	.07	1.5 (1.0–2.2)	55	71	.05	.23	1.3 (0.9–1.9)
Low	35	39				39	43			
CD8+:CD3+										
High	53	84	.05	.05	1.6 (1.0–2.4)	60	95	.10	–	–
Low	35	42				38	45			
CD4+:CD8+										
High	46	52	.02	.11	1.3 (0.9–1.9)	50	55	.05	.23	1.3 (0.9–1.8)
Low	31	37				34	40			
FoxP3+										
High	31	35	.27	–	–	34	39	.48	–	–
Low	41	46				46	49			
FoxP3+:CD4+										
High	34	39	.007	.03	1.6 (1.1–2.3)	38	43	.03	.09	1.4 (0.9–2.0)
Low	51	65				54	71			
FoxP3+:CD8+										
High	35	40	.03	.05	1.5 (1.0–2.1)	39	43	.09	–	–
Low	46	53				49	56			
Clinical risk score										

Factor	5-year OS (%)	Median OS (months)	UV *P* value	MV *P* value	OR (95 % CI)	5-year RFS (%)	Median RFS (months)	UV *P* value	MV *P* value	OR (95 % CI)
1–2	46	52	<.001	<.001**	2.1 (1.5–3.1)	51	66	<.001	<.001	2.2 (1.5–3.3)
>3	21	27				22	29			
Extrahepatic disease										
No	44	49	<.001	.002	2.0 (1.3–3.1)	48	55	.003	.01	1.8 (1.1–3.0)
Yes	15	24				21	37			

UV univariate analysis, *MV* multivariate analysis (separate MV models constructed for each cell marker or cell ratio, which also included CRS and extrahepatic disease)

** Statistically significant when analyzed as a continuous variable