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BRIEF REPORT

Tumor-associated macrophages and risk of recurrence in stage III colorectal cancer

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Abstract

Tumor-associated macrophages (TAMs) have a unique favorable effect on the prognosis of colorectal cancer (CRC), although their association with stage-specific outcomes remains unclear. We assessed the densities of $CD68^+$ and $CD163^+$ TAMs at the invasive front of resected CRC stage III CRC from 236 patients, 165 of whom received post-surgical FOLFOX treatment, and their relationship with disease-free survival (DFS). Associations between macrophage mRNAs and clinical outcome were investigated *in silico* in 59 stage III CRC and FOLFOX-treated patients from The Cancer Genome Atlas (TCGA). Biological interactions of SW480 and HT29 cells and macrophages with FOLFOX were tested in co-culture models. Low TAM densities were associated with shorter DFS among patients receiving FOLFOX ($CD68^+$, $p = 0.0001$; $CD163^+$, $p = 0.0008$) but not among those who were untreated. By multivariate Cox analysis, only low TAM ($CD68^+$, $p = 0.001$; $CD163^+$, $p = 0.002$) and nodal status ($CD68^+$, $p = 0.009$; $CD163^+$, $p = 0.007$) maintained an independent predictive value. In the TCGA cohort, high *CD68* mRNA levels were associated with better outcome ($p = 0.02$). Macrophages enhanced FOLFOX cytotoxicity on CRC cells ($p < 0.01$), and drugs oriented macrophage polarization from M2- to M1-phenotype. Low TAM densities identify stage III CRC patients at higher risk of recurrence after adjuvant therapy, and macrophages can augment the chemo-sensitivity of micro-metastases.

Keywords: colorectal cancer; tumor-associated macrophages; adjuvant therapy; cancer immunology; macrophage polarization

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No conflicts of interest were declared.

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Introduction

The standard of care for patients with stage III colorectal cancer (CRC) includes postsurgical adjuvant chemotherapy, which is aimed at eradicating clinically occult micro-metastasis. Although the addition of oxaliplatin to 5-fluoropyrimidines has ameliorated the disease-free survival (DFS) [1], about 30% of treated patients still suffer recurrences within 5 years [2]. Tumor pathological features (pT4 and/or N2) identify stage III CRCs at higher risk of recurrence, so that a reduced duration of the standard 6-month FOLFOX (folinic acid [leucovorin, FOL], fluorouracil [5-FU, F], and oxaliplatin [Eloxatin, OX]) therapy has been proposed for patients at low risk of recurrence (i.e. pT1-pT3 and/or N1) [3]. However, the lack of predictive biomarkers limits our capacity to select patients who really benefit from current adjuvant treatments. Molecular markers, such as microsatellite and *KRAS/BRAF* mutational status, define CRC subsets with variable prognosis, but their predictive role remains controversial [4,5]. Similarly, high densities of tumor-infiltrating T-cells (TILs) characterize CRC with better prognosis but seem to be predictive only in patients at low risk of recurrence [6]. Even circulating tumor DNA, a promising marker of minimal residual disease, was recently reported to predict less than 50% of post-chemotherapy recurrences from stage III CRC [7].

Tumor-associated macrophages (TAMs) are generally associated with poor prognosis of solid tumors [8] but have a unique favorable prognostic impact in CRC [9], especially if peri-tumoral and M1-like polarized cells are considered [10]. We previously reported the association between $CD68^+$ TAM densities and stage III CRC outcome as highly significant but limited to subjects treated with 5-fluorouracil-based adjuvant therapy [11]. This study was designed to confirm the interaction of TAMs with standard FOLFOX adjuvant therapy in determining the clinical outcome of stage III CRC patients.

Materials and methods

Tumor tissues were obtained from 236 patients consecutively resected for stage III CRC at the Humanitas Research Hospital, between January 2014 and December 2017. One hundred and sixty-five patients received postoperative FOLFOX adjuvant therapy, whereas 71 patients with co-morbidities, poor performance status, or reduced life expectancy had no medical treatment. Clinical data were collected in a prospectively maintained database for a mean follow-up of 4.79 ± 0.16 years (mean ± SEM). The study was limited to patients giving their informed consent and conducted by protocols approved by the local Ethics Committee (approval no. 1052/2013). Serial sections of tumor specimens were stained by immunohistochemistry with anti-CD68 (clone KP-1, Dako, Santa Clara, CA, USA) and anti-CD163 (clone 10D6, Leica Biosystems, Wetzlar, Germany) antibodies. Digital images from whole tissue slides were taken along the tumor invasive front (CCD camera XC50, Olympus, Tokyo, Japan) and computer-analyzed (Image-Pro Premier 9.2 software, Media Cybernetics, https://www.mediacy.com/). The percent immuno-reactive area was taken as the measure of TAM density (supplementary material, Figure S1). Cox proportional regression models and Kaplan–Meier curves were applied to assess the predictive value of TAM densities.

As a validation set, an *in silico* cohort of 59 stage III CRC patients treated with FOLFOX adjuvant therapy was extracted from The Cancer Genome Atlas for Colorectal Adenocarcinoma (TCGA-COAD) and explored for interactions between macrophage-related mRNA expression and DFS. The raw data from the Genomic Data Commons (GDC, https://portal.gdc.cancer.gov) [12] were analyzed using R/Bioconductor, normalized to fragments per kilobase of transcript per million of mapped reads (FPKM), and mRNA signature matrix was estimated by CIBERSORT computational approach [13]. A classification and regression tree (CART) analysis was applied to test the predictive effect of macrophage mRNA levels on recurrence. The decision tree model exploited the Orange Data Mining tool (3.25.0 version, https://orangedatamining.com/) with pruning to avoid overfitting.

In vitro, macrophages were co-cultured with SW480 and HT29 CRC cells and exposed to FOLFOX agents. The effect of co-culturing on the cytotoxic effect of drugs and on macrophage M1/M2 polarization was tested (see Supplementary materials and methods).

Statistical analysis was performed using STATA13.1 (https://www.stata.com/). Two-sided P values of <0.05 were considered statistically significant.

Results

TAM densities by CRC pathological and molecular features are reported in supplementary material, Table S1. As expected [10], TAMs were more

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abundant in TIL-rich cancers ($p < 0.01$). $CD163^+$ cells were denser in MSI ($p = 0.01$) and *BRAF*-mutated ($p = 0.001$) tumors, but less so in *KRAS*-mutated CRC ($p = 0.03$).

Increasing densities of TAMs, but not of TILs, were significantly ($p < 0.0001$) associated with a lower risk of postsurgical CRC recurrence and an interaction between TAMs and FOLFOX adjuvant therapy was detected ($CD68^+$ cells, $p < 0.0001$; $CD163^+$ cells, $p = 0.001$) (supplementary material, Table S2). Accordingly, increasing densities of both $CD68^+$ and $CD163^+$ TAMs were progressively associated with a lower risk of recurrence in FOLFOX-treated patients ($p < 0.0001$) but not those who were untreated (supplementary material, Table S3).

By using median values as cut-offs, low densities of both $CD68^+$ and $CD163^+$ TAMs predicted CRC recurrence by univariate ($CD68^+$, HR 3.89, 95% CI [1.85–8.15], $p < 0.0001$; $CD163^+$, HR 3.19, 95% CI [1.56–6.51], $p = 0.001$) and by multivariate ($CD68^+$, HR 3.62, 95% CI [1.71–7.67], $p = 0.001$; $CD163^+$, HR 3.15, 95% CI [1.53–6.47], $p = 0.002$) analyses (Table 1). Tumor nodal status was the only additional independent predictive variable ($p < 0.01$). By Kaplan–Meier analysis, low TAMs were significantly associated with poorer DFS in the institutional cohort of adjuvant-treated patients ($CD68^+$ cells, $p = 0.0001$; $CD163^+$ cells, $p = 0.0008$) (Figure 1). The DFS of FOLFOX-treated patients was significantly better than that of untreated subjects only for high-TAM tumors ($CD68^+$ cells, $p = 0.002$; $CD163^+$ cells, $p = 0.007$) (supplementary material, Figure S2). Consistently, CART analysis of the TCGA cohort showed that higher expression of *CD68* mRNA was associated with no recurrence of FOLFOX-treated patients ($p = 0.02$) (Figure 2 and supplementary material, Figure S4). When SW480 and HT29 colon cancer cells were co-cultured with macrophages, the cytotoxic effect of FOLFOX agents on colon cancer cells increased significantly over time (Figure 3A and supplementary material, Figure S3).

In addition, macrophages co-cultured with cancer cells and exposed to FOLFOX had higher expression of M1-like surface markers (HLA-DR and CD86) and lower expression of M2-like markers (CD14 and CD163) (Figure 3B).

Table 1. Predictors of recurrence in patients resected for stage III CRC and treated with FOLFOX

		No. of cases	Recurrences	Univariate		Multivariate by $CD68^+$ TAMs		Multivariate by $CD163^+$ TAMs	
			n (%)	HR* (95% CI)	*p*	HR* (95% CI)	*p*	HR* (95% CI)	*p*
All cases		165	41 (24.8)						
$CD68^+$ TAM†	High	79	9 (11.4)	1.00 Ref		1.00 Ref		Not included	
	Low	86	32 (37.2)	3.89 (1.85–8.15)	< 0.0001	3.62 (1.71–7.67)	0.001		
$CD163^+$ TAM†	High	79	10 (12.7)	1.00 Ref		Not included		1.00 Ref	
	Low	86	31 (36.1)	3.19 (1.56–6.51)	0.001			3.15 (1.53–6.47)	0.002
Tumor nodal status	pN1	90	15 (16.7)	1.00 Ref		1.00 Ref		1.00 Ref	
	pN2	75	26 (34.7)	2.48 (1.31–4.69)	0.005	2.36 (1.24–4.49)	0.009	2.44 (1.28–4.64)	0.007
Local invasion	pT3	133	29 (21.8)	1.00 Ref		1.00 Ref		1.00 Ref	
	pT4	32	12 (37.5)	2.12 (1.08–4.15)	0.03	1.69 (0.84–3.40)	0.14	1.90 (0.95–3.82)	0.07
Tumor differentiation‡	Well-to-moderate	116	26 (22.4)	1.00 Ref		1.00 Ref		1.00 Ref	
	Poor	47	15 (31.9)	1.69 (0.90–3.20)	0.10	1.45 (0.75–2.77)	0.27	1.56 (0.81–2.99)	0.18

*Hazard rates and corresponding 95% confidence intervals were estimated using Cox proportional hazards model. All demographic, pathological, and molecular variables (as listed in supplementary material, Table S1) were tested by univariate analysis. Only variables with $p \leq 0.10$ were included in the multivariate analysis.
†Assessed as percent immunoreactive area; 'high' and 'low' defined by median of percent immunoreactivity of TAMs in 236 patients (CD68, 2.78%; CD163, 0.93%).
‡Not available in two cases.

Discussion

Defining the prognostic interaction of TAMs with FOLFOX therapy, this study recognizes patients

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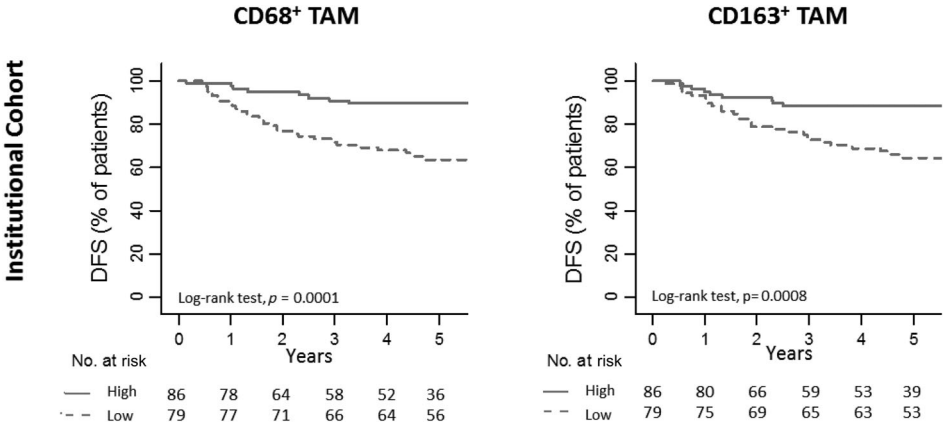

Figure 1. DFS by TAM densities in patients resected for stage III CRC and treated with FOLFOX. High versus low densities of both CD68+ and CD163+ TAM were determined by median of percent immunoreactivity values.

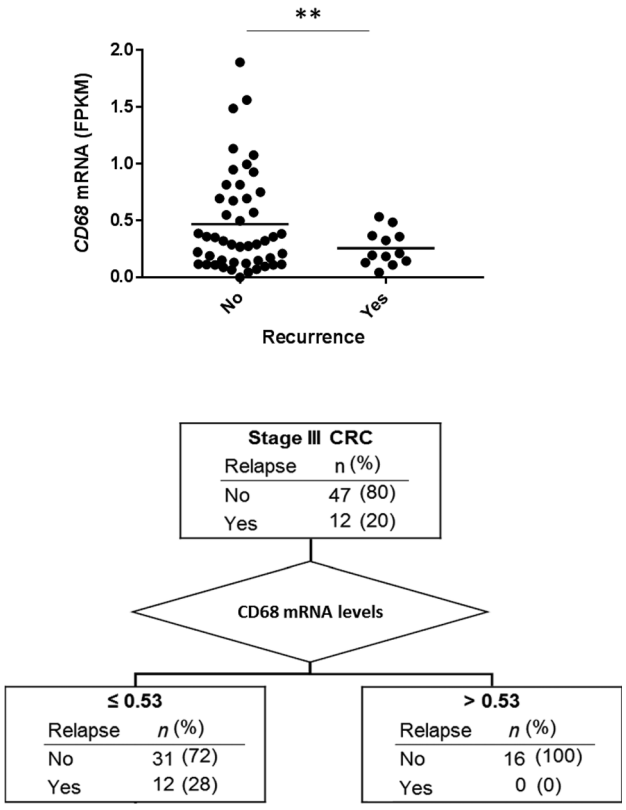

Figure 2. Tumor CD68 mRNA levels and postoperative recurrence in an *in silico* cohort of patients resected for stage III CRC and treated with FOLFOX. Upper panel: mRNA levels were significantly higher in patients with no recurrence (Fisher's exact, $p = 0.02$). Line bar: mean of CD68 mRNA. CD68 mRNA expression (high versus low expression) was reported as FPKM. Lower panel: at CART analysis, higher mRNA levels were associated with no recurrence. **p value < 0.01.

resected for stage III CRC as a suitable population for validating the clinical utility of TAM measurement. The results propose TAM densities as candidate biomarkers of CRC outcome after adjuvant therapy.

First, the predictive role of macrophage densities at the tumor front, originally detected in a historic cohort of 5FU-treated patients [12], is now confirmed in a new longitudinal series of patients resected for stage III CRC and treated with current standard of care, i.e. FOLFOX combination therapy. Second, but most importantly, the results are now supported by the association of macrophage-related mRNA levels with the outcome of an *in silico* cohort of stage III CRC patients receiving adjuvant FOLFOX. Finally, the study clearly indicates that the favorable impact of TAMs on prognosis of stage III CRC is essentially due to the interaction with adjuvant chemotherapy, with negligible or marginal effect among patients receiving no post-surgical treatment.

It is worthy of note that the association between lower TAM densities and poorer DFS (up to a 40% recurrence rate in patients with first quartile TAM density) was observed in a series whose overall DFS was 75%, which is in full agreement with most recent literature [14]. Due to the scalar effect of TAM densities on the recurrence rate, the optimal cut-off to maximize the predictive role of TAMs remains to be established in larger series.

Noticeably, the interaction between TAMs and adjuvant therapy was observed not only using a pan-macrophage marker such as CD68 but also targeting the CD163 epitope, typical of M2-like pro-tumor polarization. These findings are consistent with the hypothesis that TAMs, due to their plasticity, can

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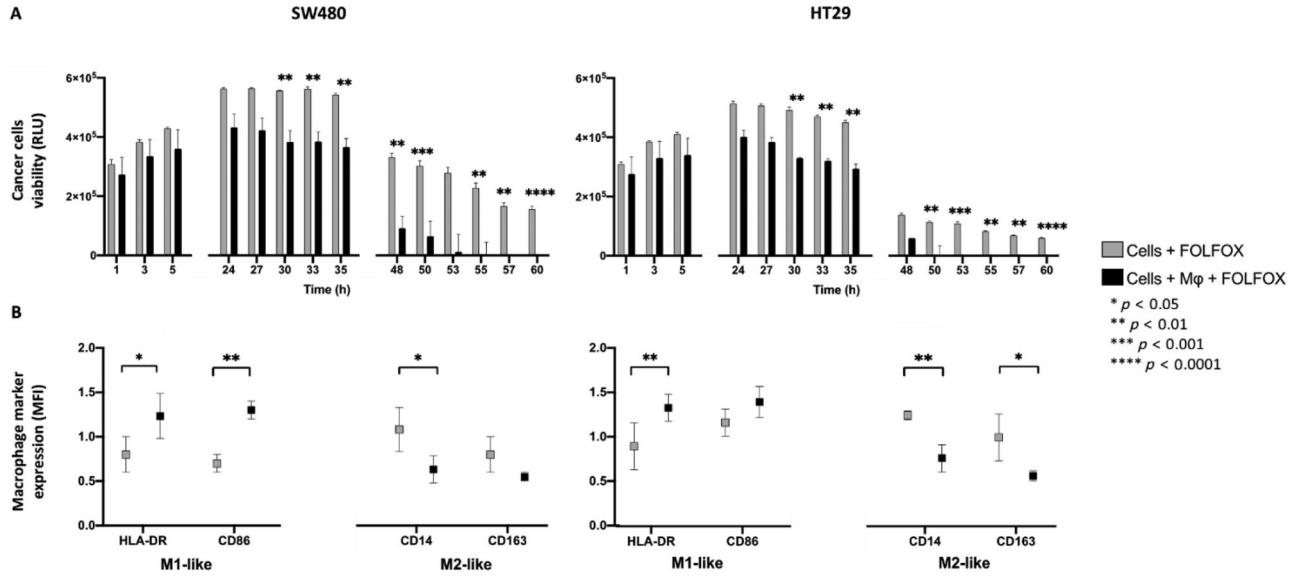

Figure 3. Effects of FOLFOX exposure in co-cultures of CRC cells and macrophages. (A) Viability of colon cancer cells after exposure to FOLFOX agents. Loss of SW480 and HT29 cells viability, as measured in relative luminescence units (RLUs), was significantly enhanced by macrophage co-culture. (B) When co-cultured with cancer cells, macrophages exposed to FOLFOX had higher expression of surface markers typical of the M1-like phenotype and lower expression of M2-like markers. The median of fluorescence intensity (MFI) for each marker was measured by fluorescence-activated cell sorting (FACS) in triplicate-run experiments.

repolarize toward the M1-like pro-inflammatory phenotype when engaging with CRC circulating cells or micro-metastasis exposed to chemotherapeutic agents. Fully supporting this concept, we found that the macrophage-enhanced cytotoxic effect of FOLFOX on colon cancer cells in co-culture models was paralleled by reshaping of macrophages from the M2- to the M1-phenotype [8,15,16]. From this perspective, the efficacy of adjuvant regimens might benefit from this association by using drugs capable of re-educating macrophages to a tumoricidal activity [9].

As to the mechanism modeling the predictive impact of TAMs, both clinical and experimental data support a true biological interaction between macrophages and chemotherapeutic drugs. In contrast, the negligible prognostic role for high TAMs in untreated patients argues against the hypothesis of an autonomous histolytic effect of macrophages on tumor cells.

This study has obvious limitations including the small number of investigated cohorts and the lack of a recognized molecular mechanism underlying the interaction between TAMs and FOLFOX. Nevertheless, the data strongly recommend the assessment of TAMs in cancers from patients entering large controlled studies aimed at validating the efficacy of adjuvant therapy in stage III CRC.

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Author contributions statement

TC made substantial contributions to conceptualization, project administration, visualization, writing the original draft (equal), and data curation, formal analysis, investigation and methodology (lead). LG was involved in methodology, formal analysis, writing the original draft and editing (lead). FR was responsible for methodology, writing, review and editing (supporting). TH contributed to supervision, writing and review (supporting). MQ was responsible for

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investigation and methodology (supporting). FG made a substantial contribution to methodology (supporting). ES was involved in formal analysis (supporting). VC was responsible for data curation (supporting). PB made substantial contribution to investigation (supporting). SV was responsible for methodology (supporting). LR contributed to writing, review and editing (supporting). VT was involved in writing review and editing (supporting). RB was responsible for formal analysis (supporting). AMan contributed to writing, review and editing (supporting). SO was involved in supervision and writing the original draft (supporting). AMal was responsible for writing, review and editing (lead). LL made substantial contributions to conceptualization, data curation, formal analysis, funding acquisition, project administratotion, visualization, and writing the original draft (lead).

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Examples of different TAM densities stained by immunohistochemistry and then measured using a RGB histogram-segmentation algorithm

Figure S2. Disease-free survival by adjuvant FOLFOX and TAM densities (high versus low)

Figure S3. Viability of colon cancer cells and macrophages, alone and in co-culture, exposed or not to FOLFOX

Figure S4. Examples of histologic correlates for the TCGA cohort

Table S1. $CD68^+$ and $CD163^+$ TAM densities in stage III CRC, by demographic, pathological, and molecular features

Table S2. CRC postoperative recurrences by TAM and TIL densities, clinico-pathological features, and FOLFOX adjuvant therapy

Table S3. Risk of recurrence in FOLFOX-treated and -untreated stage III CRC patients

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