



Retrospective Cohort Study

Distribution and prognostic value of macrophages in colorectal cancer and adjacent mucosa in patient stages I-III vs IV

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Abstract

BACKGROUND

Synchronous and metachronous liver metastases (LM) of colorectal cancer (CRC) drastically worsen the patient's survival. The biological and immunological mechanisms underlying these distinct metastatic trajectories remain incompletely understood. Prognostic impact of macrophages in primary CRC (pCRC) is uncertain, with discrepant findings reported for different macrophage subsets and different stage of the disease. Most of prior studies of tumor-infiltrating macrophages in CRC have focused primarily on the tumor core or invasive margin,

whereas less attention has been given to the adjacent nontumor mucosa (NM), which may harbor early immunological alterations that precede or accompany metastatic spread.

AIM

To evaluate distribution and prognostic value of macrophages in pCRC and NM in patients at stage I-III *vs* IV.

METHODS

Paired specimens of pCRC and NM were collected retrospectively from: (1) Stage IV ($n = 55$) patients with synchronous LM; and (2) Stage I-III ($n = 44$) patients who developed metachronous LM thereafter. After immunohistochemical staining CD68+ (M0), CD80+ (M1), CD206+ and CD163+ (M2) macrophages were quantified in NM and tumor center (TC) of pCRC. Cell densities in NM and TC and TC/NM ratios were tested as prognostic variables for overall survival since liver surgery. Cox-regression and Kaplan-Meier analyses were applied using the R environment.

RESULTS

Densities of macrophages followed the declining pattern from CD163+ through CD206+ and CD68+ to CD80+ in both NM and TC, with significantly smaller densities of all cell types in tumors. Greater densities of CD80+ cells were observed in NM in stage I-III over stage IV patients: 309 (24-1143) cells/mm² *vs* 208 (3-1084) cells/mm² [median (minimum-maximum), $P = 0.04$]. High CD163+ cell density in NM in stage IV [hazard ratio = 0.45 (95%CI: 0.22-0.95), $P = 0.04$] and CD80+ cell density in NM in stage I-III [hazard ratio = 0.24 (95%CI: 0.10-0.57), $P = 0.001$] were associated with longer overall survival.

CONCLUSION

Contrary to TC of pCRC, we found favorable prognostic implications of macrophages in NM, driven by distinct subsets of macrophages in stage IV (CD163+ M2) and stage I-III CRC (CD80+ M1).

Key Words: Macrophages; Primary colorectal cancer; Adjacent nontumor mucosa; Liver metastases; Survival

Core Tip: Limited evidence exists regarding the distribution and prognostic associations of different subsets of macrophages in colorectal cancer (CRC) and adjacent nontumor mucosa (NM) in patients stage IV with synchronous liver metastases or stage I-III who developed metachronous liver metastases thereafter. This retrospective cohort study included 55 stage IV and 44 stage I-III patients. M2-macrophages outnumbered M1 and M0 both in primary CRC and NM with significantly reduced cell densities in tumor tissue. Greater density of M1 macrophages is a hallmark of NM in stage I-III CRC. Higher densities of macrophages in NM demonstrate favorable associations with survival.

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INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality worldwide, with liver representing the most common and clinically significant site of distant metastases[1-4]. Approximately 20%-25% of patients present with synchronous liver metastases (LM) at the time of CRC diagnosis, while another 20%-30% will develop metachronous metastases during follow-up[5-7]. The biological and immunological mechanisms underlying these distinct metastatic trajectories remain incompletely understood[6-9], and there is a growing interest in the role of the tumor immune microenvironment (TIME) in shaping disease progression and patient outcomes[10].

In the TIME of CRC and other cancers macrophages are often recognized as the most populous group of tumor-infiltrating immune cells[11,12]. Macrophages demonstrate remarkable phenotypic plasticity in response to local signals, and based on their activation status, colonic macrophages are broadly classified into M0 (non-polarized), M1, classically activated by interferon-gamma, and M2, alternatively activated by interleukin-4[11,13,14]. M1 macrophages are highly phagocytic, cytotoxic for tumor cells, pro-inflammatory and therefore anti-tumorigenic, whereas M2 macrophages are immunosuppressive and promote tumor growth, angiogenesis, stromal activation and metastasis[11,14-16]. The balance between M1 and M2 macrophages in the TIME plays a pivotal role in the progression and treatment of cancer, as reviewed in[15-18].

Immunohistochemical markers commonly used to distinguish these subsets include CD68 (lysosomal/endosomal-associated membrane glycoprotein) for M0 macrophages, CD80 (a co-stimulatory molecule) for M1 macrophages, CD206 (a mannose receptor) and CD163 (a scavenger receptor) for M2 macrophages[13,14,17-19].

Macrophages have been generally associated with poor prognosis in most solid tumors[10,11]; however, in CRC, the prognostic impact appears more complex, with discrepant findings reported for different macrophage subsets[13,14,16-18].

Importantly, most prior studies of tumor-infiltrating macrophages in CRC have focused primarily on the tumor core or invasive margin[11,13,20,21], whereas less attention has been given to the adjacent nontumor mucosa (NM), which may harbor early immunological alterations that precede or accompany metastatic spread[22,23]. The immune landscape of tumor-adjacent NM may serve as a critical, yet underexplored, niche that reflects systemic immune surveillance and may influence tumor behavior and dissemination. Of note, macrophages constitute 10%-20% of mononuclear cells in the intestinal mucosa[11,14,19,24,25], where they play a significant role in clearing pathogens, regulating inflammatory responses and maintaining homeostasis. However, only a few studies have explored the distribution of macrophages in NM in CRC[13,22,23].

Given these gaps in knowledge, we hypothesized that the quantification and comparative analysis of CD68+, CD80+, CD206+, and CD163+ macrophages in both the tumor center (TC) of primary CRC (pCRC) and NM may provide prognostic insights in stage IV CRC patients with synchronous LM and stage I-III patients who developed metachronous LM later.

MATERIALS AND METHODS

Patients

All patients who underwent a curative-intent resection of both pCRC and LM in Pilsen University Hospital between 1999 and 2021 were identified from pathology archives and involved in the current retrospective cohort study. Paired formalin-fixed paraffin-embedded (FFPE) specimens of pCRC and NM were collected retrospectively from stage IV patients ($n = 80$), who presented with synchronous LM at the time of primary tumor surgery and from stage I-III patients ($n = 100$) who developed metachronous LM within 1-59 months after resection of pCRC (median, 17 months).

The following patient inclusion criteria were applied in the current study: (1) Only liver-first metastases; (2) Available complete clinical and survival data; and (3) Available FFPE tissue of both NM and pCRC of sufficient quality. We excluded from the study patients with multiple primary neoplasms, with presence of preoperative extrahepatic metastases, history of prior liver resections, those who received neoadjuvant chemoradiotherapy before pCRC surgery and those who underwent emergency surgery. Ninety-nine patients fulfilled all criteria (44 stage I-III and 55 stage IV).

Selected information on demographic, pathological and clinical data was extracted from the medical files of the patients. Location of the primary tumor, its size, TNM classification, stage, histological type and grade, *KRAS*, *BRAF*, microsatellite instability (MSI) status and carcinoembryonic antigen blood level were recorded (Table 1). Tumors were classified as the right-sided or left-sided with respect to the splenic flexure of the colon and they were staged according to the American Joint Committee on Cancer (8th edition) criteria. The two groups differed only in terms of size of LM, which was greater in the metachronous one, and in the frequency of patients who received FOLFOX chemotherapy, which was greater in stage IV (Table 1). This retrospective study was conducted in accordance with the ethical principles of Declaration of Helsinki (2013 version) and it was approved by the Ethics Committee of the Faculty of Medicine and University Hospital in Pilsen.

Pathology and immunohistology

FFPE tissues of pCRC and tumor-adjacent NM were retrieved for each patient and sectioned at 4- μ m thickness. NM samples were obtained from the oral or aboral resection margin closest to the tumor, with a median distance of 34 mm (range: 4-200 mm) from the tumor edge and were confirmed pathologically to be free of tumor infiltration.

One or two tissue sections were mounted onto BOND Plus Microscope Slides (Cat 00270, Leica Biosystems Newcastle Ltd., Newcastle, United Kingdom). Then, the immunohistochemical staining was performed using fully automated BOND RXm, Leica Biosystems. Ready-to-use monoclonal primary antibodies for CD68 (clone 514H12, Bond) and CD163 (clone 10D6, Bond) from Leica Biosystems (Newcastle Ltd., United Kingdom), monoclonal antibodies to CD80 (clone 37711, dilution 1:100) from R&D Systems (Minneapolis, MN, United States) and polyclonal antibodies to CD206 in dilution 1:1000 (Abcam Ltd., United Kingdom) were used. Binding of primary antibodies with their targets was visualized using horseradish peroxidase-linker antibody conjugate system (Leica Biosystems). Sections were counterstained with Mayer's hematoxylin and embedded into Micromount mounting medium (Leica Biosystems Newcastle Ltd., United Kingdom). Appropriate positive (tonsils) and negative tissue control samples were used throughout.

Image analysis

Whole-slide scans of NM and pCRC were obtained using Olympus VS200 scanner (Olympus, Shinjuku, Japan). Annotations of the regions of interest and estimation of immune cells densities were performed using an open-source software for image analysis (QuPath v.0.4.3). NM was annotated as a single region above muscularis mucosa, which encompasses surface epithelium, intestinal crypts and lamina propria. Lymphoid follicles if located above or transected by muscularis mucosa were included as well. Foci of dysplasia, lumina of crypts and artifacts were excluded. Tumor was annotated along the border separating the malignant cell nests and adjacent non-tumor tissue. After exclusion of a 500 μ m-width margin, the remaining tumor area represented TC. We limited the analysis of macrophages in pCRC to TC region for valid comparisons with NM because both regions are mainly composed of colonic epithelium; in addition, the desmoplastic reaction is less prominent in the TC. Luminal surface of the tumor, large vessels, normal mucosa, dysplastic epithelium, muscularis propria and supportive stroma > 2 mm in diameter, extracellular mucin, fat, necroses, abscesses,

Table 1 Clinical backgrounds of enrolled patients and histopathological features of primary colorectal cancer and liver metastases, *n* (%) / median (minimum-maximum)

Parameter		Stage I-III (<i>n</i> = 44)	Stage IV (<i>n</i> = 55)	<i>P</i> value
Age at the diagnosis (years)		64 (46-73)	62 (29-78)	0.31
Gender	Male	29 (65.9)	34 (61.8)	0.67
	Female	15 (34.1)	21 (38.2)	
Primary tumor				
Location	Right colon	8 (18.2)	12 (21.8)	0.65
	Left colon	36 (81.8)	43 (78.2)	
Size (cm)		3.5 (0.8-8.3)	4.3 (1.0-7.5)	
Pathologic T stage	T1	1 (2.3)	0 (0.0)	0.25
	T2	6 (13.6)	1 (1.8)	
	T3	35 (79.5)	48 (87.3)	
	T4	2 (4.5)	6 (10.9)	
Histological type	Not otherwise specified	39 (88.6)	51 (92.7)	
	Mucinous	4 (9.1)	2 (3.6)	
	Other	1 (2.3)	2 (3.6)	
Pathologic N stage	N0	13 (29.5)	13 (23.6)	0.51
	N1	16 (36.4)	22 (40.0)	
	N2	15 (34.1)	20 (36.4)	
American Joint Committee on Cancer 8 th staging	Stage I	1 (2.3)		
	Stage II	12 (27.3)		
	Stage III	31 (70.4)		
	Stage IV		55 (100)	
Grade	1	14 (31.8)	12 (21.8)	0.77
	2	26 (59.1)	37 (67.3)	
	3	4 (9.1)	6 (10.9)	
<i>KRAS</i> status	Mutated	11 (25.0)	20 (36.4)	0.26
	Wild-type	25 (56.8)	27 (49.1)	
	Not tested	8 (18.2)	8 (14.5)	
<i>BRAF</i> status	Mutated	1 (2.3)	0 (0.0)	0.27
	Wild-type	35 (79.5)	44 (80.0)	
	Not tested	8 (18.2)	11 (20.0)	
Microsatellite instability status	High	1 (2.3)	1 (1.8)	0.90
	Low	35 (79.5)	42 (76.4)	
	Not tested	8 (2.3)	12 (21.8)	
CEA ng/mL		8.9 (0.8-1649.0)	6.8 (1.0-945.0)	0.90
CEA	< 5 ng/mL	13 (46.4)	18 (46.2)	0.98
	> 5 ng/mL	15 (53.6)	21 (53.8)	
Number of examined lymph nodes		11 (0-43)	13 (1-34)	0.38
Lymph node ratio		0.13 (0-0.32)	0.22 (0-0.57)	0.14
Liver metastases				
Number		1 (1-7)	2 (1-24)	0.08

Size (cm)		2.7 (0.6-7.1)	2.0 (0.4-26.0)	0.02
Grade	1	18 (42.9)	21 (39.6)	0.75
	2	24 (57.1)	32 (60.4)	
Metastasis resection	R0	29 (69.0)	40 (76.9)	0.39
R status	R1	13 (31.0)	12 (23.1)	
Chemotherapy ± targeted therapy	CHT alone	30 (68.2)	28 (50.9)	0.07
	CHT + anti-vascular endothelial growth factor	4 (9.1)	11 (20.0)	
	CHT + anti-epidermal growth factor receptor	5 (11.4)	9 (16.4)	
	No treatment	2 (4.5)	4 (7.3)	
	Not known	3 (6.8)	3 (5.4)	
Chemotherapy regimens	FOLFOX	16 (36.4)	31 (56.4)	0.05
	Dle Gramont	9 (20.5)	7 (12.7)	
	FOLFIRI	2 (4.5)	4 (7.3)	
	Others	12 (27.3)	6 (10.9)	

CEA: Carcinoembryonic antigen; CHT: Chemotherapy.

hemorrhages and artefacts were excluded before analysis.

The density of immune cells in NM and TC was estimated as the number of nucleated immunopositive cell profiles divided by the total area of the region. To eliminate skewness in the distribution, for subsequent survival analysis, we converted the raw data into corresponding percentile values and categorized them into low (below the 25%) *vs* high (25%-100%). To get insight into how effectively tumor exclude, recruit, or reprogram immune cells we also calculated the ratio of TC/NM for each cell type and categorized them as high and low using the median as a cutoff.

Follow-up

Patients were followed until December 2023, with a median observation time 61 months after resection of metachronous LM and 84 months after resection of synchronous LM. Postoperative treatment was carried out in accordance with generally accepted recommendations.

Outcomes

The date of liver surgery was chosen as the reference point for survival analysis. The primary endpoint of the study was overall survival (OS) that was defined as the interval from the date of liver resection to the date of death from any cause. Secondary outcomes were time to recurrence (TTR) and disease-free survival (DFS). TTR was defined as the interval from the date of liver metastasectomy to the date of diagnosis of any site of recurrence. DFS was considered as the time from resection of LM to the date of diagnosis of recurrence or death from any cause. OS probabilities at 5 years after LM surgery were 34.8% in stage I-III group and 31.3% in stage IV group ([Supplementary Table 1](#)).

Statistical analysis

Continuous non-normally distributed data are expressed as median (minimum-maximum); their comparison was made by Mann-Whitney *U*-test to compare cell densities between stage IV *vs* I-III or by Friedman analysis of variance, followed by Dunn's test to compare densities of 4 cell types either in NM or in TC. Wilcoxon test was used for comparisons between NM and TC of pCRC. Proportions are expressed as raw data (%). The associations between pairs of ordinal or quantitative variables were assessed using Spearman correlation due to nonparametric distribution of most of the variables. For those analyses GraphPad Prism 9.0 (GraphPad Software LLC) was used. Survival analysis was performed in the R environment. Using the Finalfit package[26] we performed a univariable Cox regression analysis to determine the prognostic value of individual predictors for OS followed by backward stepwise multivariable analysis. Hazard ratios showing the relative risk compared with 1 for the "low group", were calculated. Taking into account number of patients in both groups and assumptions for correct multivariable analysis, we tested prognostic associations of CD80+ and CD163+ macrophages in 3 multivariable models adjusted for: (1) N stage and grade of pCRC; (2) Number, size and grade of LM; and (3) Chemotherapy regimen (other *vs* FOLFOX and use of targeted therapy). Because of very low frequency of *BRAF* mutations and "high" MSI instability status as well as missing data for a significant proportion of patients, MSI status and presence of *KRAS* and *BRAF* mutations were not included into the multivariable models. OS was estimated using the Kaplan-Meier method and compared between groups with the log-rank test. Kaplan-Meier analyses were conducted using the survival package, and survival curves were visualized with the survminer package[27,28]. A two-sided *P* value < 0.05 was considered statistically significant.

RESULTS

Distribution of macrophages in NM and pCRC: Morphology and localization of macrophages in NM and TC of pCRC

In NM all types of macrophages were located exclusively in lamina propria, with no cells observed within the crypts or surface epithelium. In a majority of cases, CD163+, CD68+ and CD206+ macrophages were found scattered throughout the mucosa thickness with an obvious predomination in the superficial layer, whereas the CD80+ cells were more frequently located in the middle third of the mucosa (Figure 1A-D). CD163+ and CD206+ macrophages were the largest in size with shapes varying from rounded/polygonal to spindle or stellate-shaped. CD68+ and CD80+ macrophages were more frequently rounded or polygonal, and the CD80+ ones were also somewhat smaller in size. CD163, CD206 and CD80 putative antigens had rather membranous than cytoplasmic expression, whereas CD68 was expressed in cytoplasmic granules.

Macrophages were also found in mucosa-associated lymphoid follicles where CD163+ cells outnumbered CD206+ cells in the mantle zone, whereas CD68+ cells were located in the germinative centres of secondary lymphoid follicles.

A vast majority of macrophages in the TC of pCRC were located in the stromal compartment (Figure 1E-H). Some cells were observed in the lumina of tumor glands or in pseudo-luminal spaces and few cells of each type were in direct contact with the tumor cells.

We did not observe any correlation between macrophage densities in NM and the distance from the tumor, except for a negative correlation with CD206+ cells (Supplementary Table 2).

Comparative distribution of macrophages

Densities of macrophages within the NM and TC of pCRC followed the order: CD163+ > CD206+ = CD68+ > CD80+ ($P < 0.05$ for the differences; Figure 2) in both groups. Similar trend was observed in lymphoid follicles. Besides that, the densities of all 4 cell types were significantly smaller in pCRC TC compared to NM ($P < 0.0001$).

The only difference between patients at stage IV *vs* I-III was a greater density of CD80+ cells observed in the NM in stage I-III CRC: 309 (24-1143) cells/mm² *vs* 208 (3-1084) cells/mm² [median (minimum-maximum)], $P = 0.04$.

Associations between subsets of macrophages within and between NM and pCRC

In NM, the densities of CD68+ cells positively correlated with those of CD163+ and CD80+ cells in both groups (Supplementary Table 3). In the TC, the densities of CD68+ cells correlated with those of CD163+ macrophages and additionally with CD206+ cells in stage I-III.

Prognostic associations of macrophages and clinical and pathological variables

In stage I-III CRC high densities of CD80+ macrophages in NM and higher ratio of CD68+ cells between TC and NM were associated with lower risk of death after resection of LM in univariable Cox analysis (Table 2), which was confirmed by longer OS in Kaplan-Meier analysis (Figure 3A and Supplementary Figure 1). Only CD80+ macrophages in NM remained predictive for OS in multivariable analysis after adjustment for CD68 TC/NM ratio: Hazard ratio = 0.31 (95%CI: 0.14-0.71), $P = 0.006$.

In stage IV patients, high densities of CD163+ macrophages in NM were associated with lower risk of death in univariable Cox analysis (Table 2) and longer OS in Kaplan-Meier analysis (Figure 3B). Concordant associations were observed for DFS and TTR (Supplementary Tables 4 and 5). Notably, the ratios between M1 and M2 putative markers (CD80+/CD206+, CD80+/CD163+) neither in NM nor in TC of pCRC were not associated with OS (data are not shown).

Stage I-III patients receiving FOLFOX regimen of adjuvant chemotherapy demonstrated longer OS after resection of LM, whereas other clinical or pathological variables were not associated with OS (Supplementary Table 6).

Both CD163+ cells in NM of stage IV patients and CD80+ cells in NM of stage I-III patients retained their significant associations with OS in multivariable models after adjustment for clinically meaningful confounding variables (Supplementary Table 7).

Associations of clinical and pathology variables with macrophage densities

The densities of CD80+ cells were smaller in NM of stage IV females and TC of larger tumors in stage I-III patients. Also, in patients at stage I-III, the densities of CD68+ cells in NM were greater in stage N2 *vs* N1 and were greater in TC of patients with multiple LM. The densities of CD163+ cells were greater in TC of right-sided tumors in stage IV (Supplementary Table 8 for all findings in this paragraph).

DISCUSSION

This study provides novel insights into the spatial distribution and prognostic significance of key macrophage phenotypes in the NM and TC of pCRC, with respect to metastatic or non-metastatic stage of CRC. The results demonstrate a differential spatial distribution and density of macrophage subtypes, supporting the hypothesis that the immune microenvironment, particularly macrophage polarization, may influence the metastatic pattern and survival outcomes in CRC.

Macrophage distribution patterns and phenotypes in NM and pCRC

According to results presented above, CD163+, CD68+, and CD206+ macrophages predominated in the superficial layers

Table 2 Hazard ratios for overall survival between high vs low cell density of macrophages in colorectal cancer patients' stage I-III vs IV, *n* (%) /hazard ratio (95%CI)

	Stages I-III		Stage IV	
CD163 NM	33 (75.0)	1.09 (0.43-2.76), <i>P</i> = 0.89	40 (74.1)	0.45 (0.22-0.95), <i>P</i> = 0.04
CD163 TC	31 (73.8)	0.94 (0.39-2.30), <i>P</i> = 0.90	40 (75.5)	0.96 (0.47-1.93), <i>P</i> = 0.90
CD163 TC/NM	21 (50.0)	0.82 (0.36-1.89), <i>P</i> = 0.65	26 (50.0)	1.26 (0.66-2.39), <i>P</i> = 0.48
CD206 NM	32 (74.4)	0.90 (0.37-2.20), <i>P</i> = 0.81	39 (75.0)	1.30 (0.62-2.72), <i>P</i> = 0.48
CD206 TC	32 (76.2)	1.49 (0.50-4.41), <i>P</i> = 0.47	40 (74.1)	0.73 (0.36-1.46), <i>P</i> = 0.37
CD206 TC/NM	21 (50.0)	1.41 (0.60-3.31), <i>P</i> = 0.43	25 (49.0)	0.54 (0.28-1.04), <i>P</i> = 0.06
CD68 NM	33 (75.0)	0.79 (0.34-1.87), <i>P</i> = 0.60	40 (74.1)	0.65 (0.33-1.30), <i>P</i> = 0.22
CD68 TC	31 (75.6)	0.62 (0.26-1.51), <i>P</i> = 0.30	40 (75.5)	0.78 (0.39-1.57), <i>P</i> = 0.48
CD68 TC/NM	20 (48.8)	0.31 (0.12-0.82), <i>P</i> = 0.02	26 (50.0)	0.84 (0.45-1.59), <i>P</i> = 0.59
CD80 NM	31 (73.8)	0.24 (0.10-0.57), <i>P</i> = 0.001	39 (75.0)	0.61 (0.29-1.30), <i>P</i> = 0.20
CD80 TC	30 (73.2)	1.00 (0.40-2.48), <i>P</i> = 0.10	39 (75.0)	0.84 (0.41-1.74), <i>P</i> = 0.64
CD80 TC/NM	20 (48.8)	1.04 (0.45-2.41), <i>P</i> = 0.93	24 (49.0)	1.04 (0.54-2.00), <i>P</i> = 0.92

Densities of macrophages (per mm²) were converted into percentiles and then categorized into low (0%-24%) and high (25%-100%). Ratio of tumor center (TC)/adjacent nontumor mucosa (NM) was calculated by dividing the densities of macrophages in TC by their densities in NM. Median of TC/NM ratios were used as cutoffs to define high and low ratio groups. Hazard ratios represent the relative risk compared with low density/ratio group (hazard ratio = 1.00). The *n* (%) refers to patients classified as the high-density/ratio group for that specific marker in each individual region. NM: Adjacent nontumor mucosa; TC: Tumor center.

of the mucosa with densities declining from CD163+ to CD80+, suggesting a M2-like polarization profile[11,14,19,24]. The prevalence of M2-macrophages and M0-macrophages in healthy colon mucosa especially below the apical epithelium, was described earlier and allows the macrophages to serve as the first line of defense against commensal microorganisms and pathogens[14,19,20,24,25]. The low prevalence of CD80+ M1 macrophages in NM is in line with the previously reported downregulation of costimulatory molecule CD80 and other potentially proinflammatory molecules in intestinal macrophages, which prevents them from exhibiting the potentially life-threatening, proinflammatory transcriptional response[24].

The macrophage densities quantified in our study were consistent with those reported by Hernández *et al*[29] in normal mucosa adjacent to colorectal tumors, namely 480/mm² for CD68+ cells and 228/mm² for CD206+ macrophages. In addition, we examined the relationship between macrophage densities in NM and the distance from the tumor and found no association, except for a negative correlation with CD206+ cells. Taken together, these findings suggest that the macrophage densities observed in NM in our cohort likely represent a "normal" baseline state; however, further studies are required to confirm this.

Similarly to NM, the TC of pCRC showed macrophage densities following a declining trend from CD163+ to CD80+ cells. This aligns with prior immunohistochemical analyses reporting that M2 macrophages outnumber the M1 ones in CRC and other gastrointestinal malignancies[11,13,20,21,24]. Contrarily to NM, the densities of M0 macrophages were associated only with M2, but not with M1 macrophages in the TC. A majority of patients in our cohort were in advanced stages (IV and III) of CRC, when the tumor cells induce polarization of M0-macrophages and the conversion of proinflammatory M1 macrophages into anti-inflammatory and cancer-promoting M2 phenotype[11].

Reduced macrophage densities in TC of pCRC

Strikingly, in both stage IV and I-III, the densities of all macrophage subtypes were significantly lower in the TC compared to tumor-adjacent mucosa. This may reflect an immune exclusion phenomenon within the tumor core, potentially driven by tumor-derived factors that can suppress macrophage recruitment and polarize them toward the M2 phenotype [30]. Stroma surrounding the tumor may impede traffic of monocytes, which can explain the relative paucity of macrophages in the tumor core[31]. Our findings are supported by data from other groups who showed lower expression of M2 macrophages in pCRC *vs* adjacent NM using immunohistochemistry[22,23], flow cytometry[32] and gene expression analysis[23,33].

Differential prognostic implications in stage IV vs stage I-III patients

In stage IV patients, a high density of CD163+ macrophages in the adjacent NM was significantly associated with improved OS after LM resection. It is, therefore, possible to hypothesize that a subset of CD163+ macrophages in the NM retains phagocytic and antigen-presenting functions, which, together with their anti-inflammatory and reparative capacity can restrain tumor dissemination[34]. Moreover, interleukin-10-secreting M2-like macrophages contribute to intestinal tissue repair and are essential for maintaining epithelial integrity[35]. The anti-tumor effect of M2 macrophages

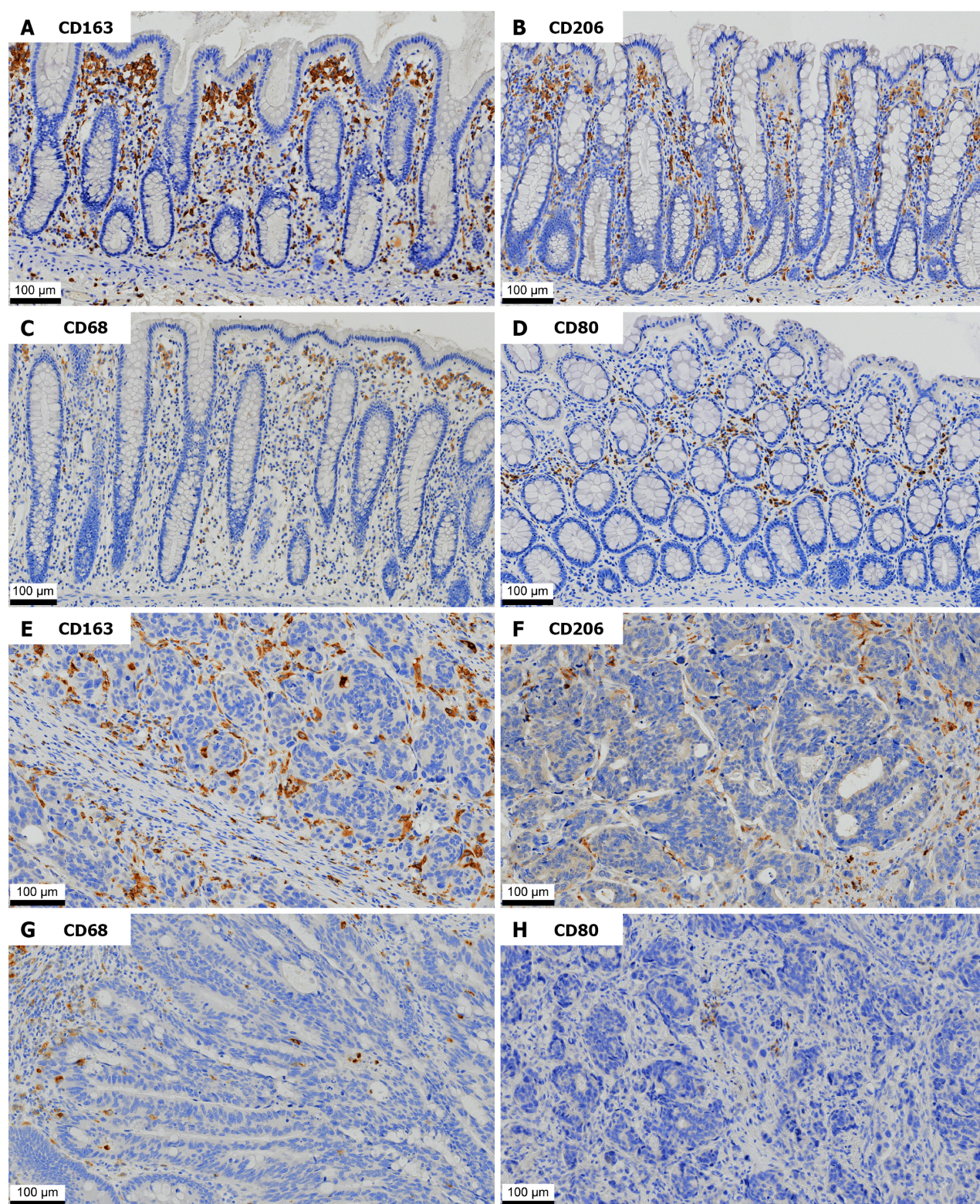


Figure 1 Immunoperoxidase staining for macrophages in adjacent nontumor mucosa and tumor center of primary colorectal cancer. A-D: Adjacent nontumor mucosa; E-H: Tumor center of primary colorectal cancer.

might also be attributed to their role in vascular maturation, *i.e.* full coverage of the vasculature by pericytes, which can prevent intravasation of tumor cells and limit metastatic spread in CRC patients[36,37]. In addition, these M2 macrophages may be re-polarized by therapy or systemic signals into phenotypes that further promote anti-tumour immunity, therefore improving post-operative control of eventual residual tumor cells[38]. Notably, some other authors have also associated higher CD163+ cell density within pCRC[39,40] and LM of CRC with better outcomes[41].

In stage I-III patients, a higher density of CD80+ cells in the adjacent NM was associated with longer OS, which aligns with the protective role of M1 macrophages in CRC[11,13,14,42] and cancer in general[15]. Specifically, Pinto *et al*[13] demonstrated the association between a higher expression of CD80+ macrophages in the tumor margin and improved OS in

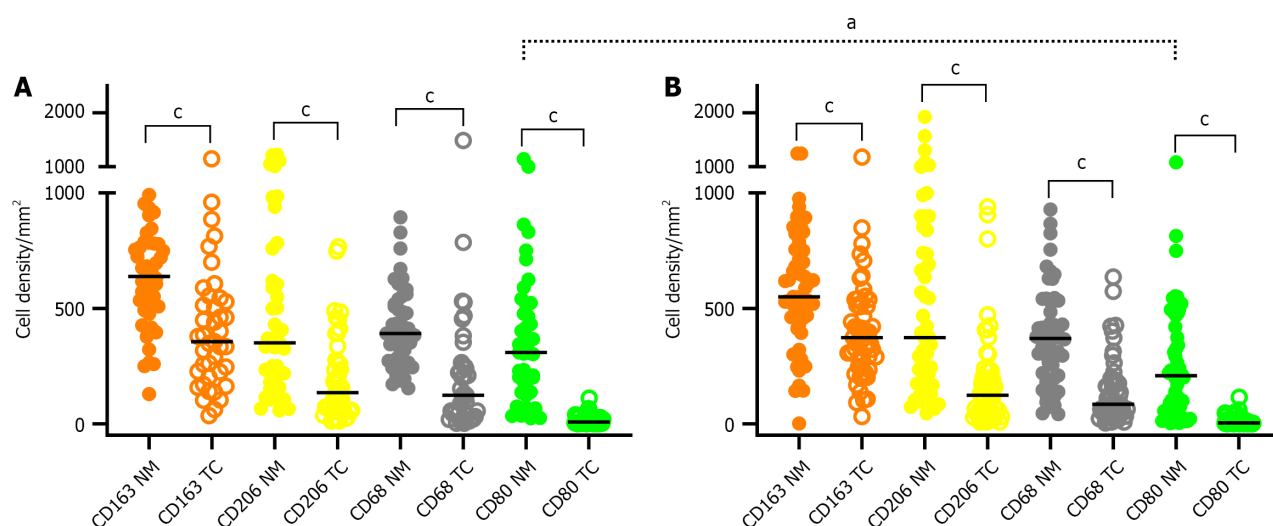


Figure 2 Statistics depicting the spatial distribution of macrophages in adjacent nontumor mucosa and tumor center. A: Stage I-III; B: Stage IV. Statistics depicting the spatial distribution of CD68+, CD163+, CD206+ and CD80+ tumor infiltrating macrophages per mm² of the section in the adjacent nontumor mucosa and tumor center of primary colorectal cancer in patients of stage I-III and IV. Black lines: Medians. ^a $P < 0.05$, ^c $P < 0.001$. NM: Adjacent nontumor mucosa; TC: Tumor center.

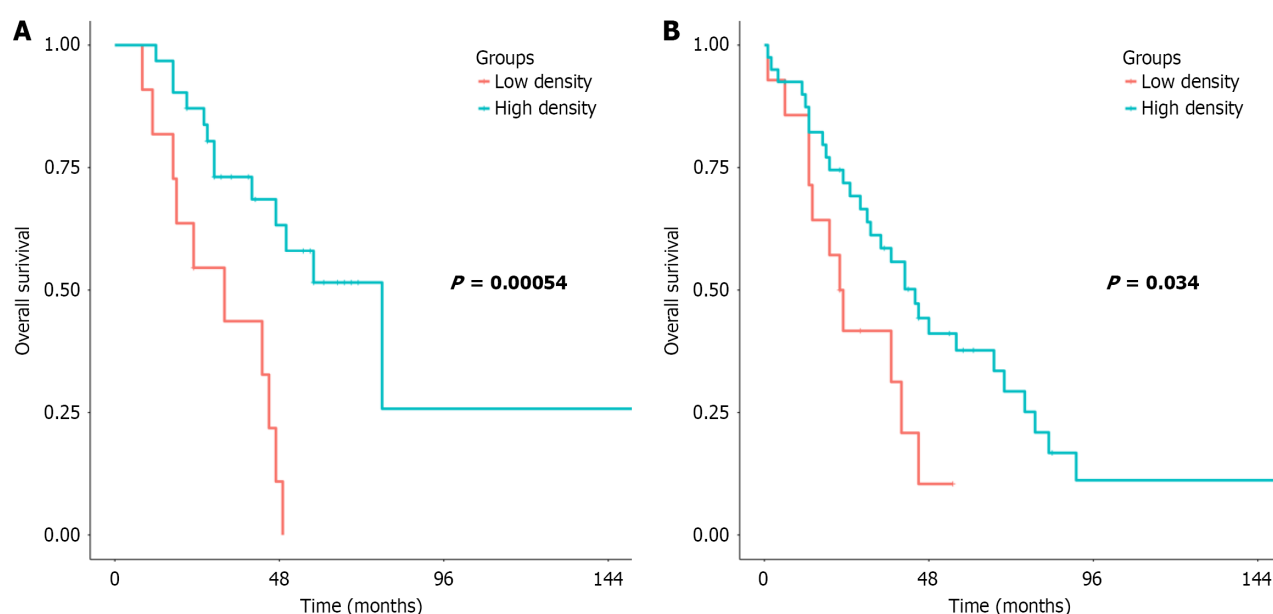


Figure 3 Kaplan-Meier analysis for overall survival according to high vs low densities of macrophages in adjacent nontumor mucosa. A: Stage I-III; B: Stage IV. Kaplan-Meier analysis for overall survival according to high vs low densities of CD80+ macrophages in adjacent nontumor mucosa in stage I-III patients and CD163+ macrophages in adjacent nontumor mucosa in stage IV patients.

stage III CRC. A plausible explanation for this effect can be M1-macrophages-induced recruitment of CD8+ T cells and natural killer cells into the tumor with a parallel enhancement of their ability to eliminate tumor cells[11,14,15,43]. Association of macrophages in NM with OS after resection of metachronous LM also assume their role in systemic inflammatory tone, baseline immune competence, and the patient's ability to tolerate or respond to subsequent treatments[44, 45]. Nonetheless, CD80+ macrophages in NM were not associated with TTR or DFS. We believe this is because recurrence after resection of CRC LM is primarily driven by biological features of the LM themselves and by established clinical risk factors, particularly in the case of metachronous LM[46].

Of notes, the FOLFOX regimen was also associated with longer OS after resection of metachronous LM. Its key distinguishing feature is the inclusion of oxaliplatin, which induces immunogenic cell death and skews macrophages toward a pro-inflammatory, antitumor M1-like phenotype[47-50]. Furthermore, M1 macrophages have been shown to synergize with oxaliplatin in cancer therapy by enhancing tumor-cell phagocytosis[51]. Our findings therefore provide indirect evidence for a potential synergism between M1-like macrophages in NM and the FOLFOX chemotherapy regimen, a relationship that merits further investigation in future studies.

Noteworthy, the greater density of CD80+ M1 macrophages in NM in stage I-III over stage IV further supports the hypothesis that immune surveillance may be more effective or preserved in patients with later-occurring metastases[12]. M1 macrophages, marked by CD80, secrete pro-inflammatory cytokines such as interleukin-12 and tumour necrosis factor alpha and enhance cytotoxic T cell activation, which may delay the onset of liver metastasis[52,53]. Greater prevalence of M1 macrophages in non-metastatic pCRC[33] and of M2 macrophages in stage IV pCRC[54] were reported earlier. Accordingly, decreased densities of CD80+ macrophages in the NM may delineate a subgroup of patients with an elevated risk of distant metastasis, who could benefit from intensified surveillance and macrophage-directed immunotherapeutic strategies[12,13,21,55].

Our multivariable analysis confirmed that immune-based indicators, specifically CD163+ and CD80+ macrophages in NM, outperformed clinical and pathological variables as prognostic factors for OS. These findings are consistent with results from a large international study in pCRC, where the T-cell-based immunoscore contributed more to the prediction of recurrence and mortality than any clinical parameter, including the TNM classification[56].

Differential prognostic implications in NM vs pCRC

In contrast to most of the previous studies, we highlighted the prognostic associations of macrophages in tumor adjacent NM, or between TC and NM, but not in the TC itself. The same CD molecule can mark distinct macrophage programs depending on the tissue context[57]. Therefore, it is possible that, in contrast to the lack of prognostic impact of CD163+ macrophages in pCRC in our study, or the pro-tumor associations reported by others[58-60], CD163+ macrophages in NM instead carry out homeostatic functions. As shown by Strasser *et al*[22] macrophages in the tumor compared to those in tumor-distant normal tissue appear to have an altered phenotype, presumably because of impaired maturation and polarization. M2 macrophages in NM may be more readily reprogrammed by peri-operative systemic cues (chemotherapy, cytokines) into anti-tumor phenotypes than tumor-embedded macrophages that are entrenched in an immunosuppressive niche. We may hypothesize that tumor-derived signals may "educate" the adjacent mucosa, for instance, through altering macrophage polarization, even before overt histological changes occur. Such signals can be proteins, growth factors, cytokines, lipids, miRNA, mRNA, and DNAs loaded into exosomes[61,62] as well as soluble chemokines and growth factors or hypoxia-driven metabolites[63-66]. Adjacent NM is an often-overlooked compartment in cancer research; however, our findings collectively underscore that the immune landscape of NM may serve as a surrogate for host-tumor immune interactions that are not always apparent in the tumor core. This supports the growing concept of "immunological field cancerization", wherein the immune cell alterations in histologically normal tissues adjacent to tumors reflect host-tumor interactions and may influence disease outcomes[22,67,68]. Understanding changes in tumor adjacent NM may help explain why some normal-looking tissue gives rise to metachronous lesions even after tumor resection.

Theoretical and clinical implications

The identification of an ideal pro-inflammatory and anti-inflammatory macrophage markers has been challenging[13]. Contrary to CD163, the other M2 marker CD206 did not show any prognostic associations in our study. This implies that CD163 and CD206 are expressed by distinct subsets of macrophages, what is supported by absence of correlation between those markers in our study, and is in line with findings from another study[32]. Leveraging macrophage markers such as CD80 and CD163 as tissue-based prognostic biomarkers may augment current prognostic models based on histopathology.

The differences in prognostic macrophage profiles between the metastatic and non-metastatic stages of CRC emphasize different immunological trajectories of metastatic disease and may inform the development of stratified surveillance or immunotherapeutic strategies. For instance, enhancing M1 polarization or reprogramming M2 macrophages could represent a viable approach to improve outcomes[11-13,21].

Along with our earlier findings on the same cohort as for antitumor effects of CD8+ T cells[53], we suggest that absolute and relative macrophage densities must be interpreted in conjunction with adaptive immune cells for accurate prognostic modelling. Altogether our findings have a promise to stratify cancer risk, tailor surveillance or design early preventive interventions.

Discordant findings in the literature

Some CRC immunoprofiling studies including several large-scale ones reported results discordant with ours, which nevertheless provide important context for our findings. Väyrynen *et al*[69] conducted a multiplex immunofluorescence study of TAMs in 931 CRC samples, of which 16% were stage IV, with survival assessed from CRC diagnosis. They associated higher stromal M2-like macrophages with worse cancer-specific survival[69]. Notably, their study relied on tissue microarray, which may not fully capture tumor heterogeneity, additionally CD206 and MAF were used as M2 markers. Pinto *et al*[13] reported greater expression of CD68 compared with CD80 and CD163 in NM, however, abundance of immune cell was quantified using immunoreactive area derived from selected microscopic fields of view rather than from cell counts in whole-slide analysis. Koelzer and co-authors observed predominance of M1 over M2 macrophages in pCRC, but the analysis was performed on tissue microarrays using different M1 marker (inducible nitric oxide synthase), which can explain differences[40]. Feng and coworkers found greater expression of CD163 in pCRC *vs* NM in a large cohort of 984 CRC patients, although their NM samples were taken 3-5 cm from the tumor margin and the assessment was performed semiquantitatively in five fields of view[54]. Using a CIBERSORT Zhong *et al* found higher expression of M2 macrophages in normal tissues, along with higher expression of M0 and M1 macrophages in tumors [33]; this analysis was based on bulk gene expression profiles from GEO and TCGA databases. Ma *et al*[23] did not observe any associations between CD163 gene expression in tumor-adjacent NM and survival, relying exclusively on

TCGA/GEO transcriptomic data. In addition, several studies have described protumor associations of CD163+ macrophages in CRC[58-60], although these investigations varied widely in the composition of cohorts, treatment background and methodology. Overall, the discrepancies between studies likely reflect differences in cohort composition, tumor stage, mutational status, and treatments regimens, as well as substantial methodological variation in sample selection, macrophage marker panels, and quantification approaches.

Study strengths and limitations

The strengths of this study include the comprehensive, side-by-side quantification of four key macrophage markers across the tumor and adjacent NM in CRC patient stage IV *vs* stage I-III. Macrophage densities were evaluated objectively and quantitatively using digitized high-resolution images in combination with specialized software, thereby minimizing observer bias.

This study is limited by its retrospective design, which introduces potential selection biases and restricts the ability to establish causal relationships. In addition, the single-institution data source and moderate sample size reduce statistical power and may limit the generalizability of the findings. We omitted from the analysis other regions of pCRC and LM because we believe TC is the most relevant region to be compared with NM. Moreover, abundance and prognostic associations of macrophages within different ROI of pCRC and LM in the same cohort is the scope of our ongoing research. Similarly, paper exploring associations between macrophages and different subsets of adaptive immune cells in NM, pCRC and LM is in preparation.

Although immunohistochemical markers are widely used, they do not fully capture the dynamic functional spectrum of macrophages, necessitating more advanced techniques (*e.g.*, multiplex imaging, spatial transcriptomics) in future work. Functional assays on isolated mucosal macrophages could help to validate our hypotheses as for their role in antigen presentation, T-cell activation and tissue-repair.

Furthermore, the heterogeneity of adjuvant anti-cancer therapies precluded a reliable evaluation of the relationships between treatment effects and macrophages. The limited availability of molecular and mutational data across a substantial proportion of patients constrained the robustness of our analyses regarding the associations between specific variables and survival outcomes. Nevertheless, cell density data will be integrated with the recently published whole exome sequencing results from the same cohort[70], providing a valuable framework for further mechanistic insights.

CONCLUSION

Our study demonstrates the prevalence of M2-macrophages over M1 and M0 phenotypes in both pCRC and NM with significantly smaller cell densities in the tumor, probably concordant with immune-exclusion. Contrary to TC of pCRC, we found prognostic implications of macrophages in NM, which were distinct between stage IV and stage I-III CRC. Survival benefit was associated with high densities of CD163+ M2 macrophages in NM in stage IV and CD80+ M1 macrophages in NM in stage I-III. Furthermore, high CD80+ cell density in NM of stage I-III patients may provide a mechanistic basis for the delay of metastatic spread. These findings underscore the importance of evaluating the tumor microenvironment beyond the tumor itself for prognostic biomarker discovery in CRC. Upon validation, these macrophage-based prognostic markers hold promise for integration into clinical decision-making for both stage IV and I-III CRC patients.

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