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The clinical impact of tumour-infiltrating lymphocytes in colorectal cancer differs by anatomical subsite: A cohort study

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Accumulating evidence demonstrates an association between dense infiltration of lymphocytes and prognosis in colorectal cancer (CRC), but whether this prognostic impact differs by tumour location remains unknown. This study investigated the prognostic impact of cytotoxic and regulatory T cells in CRC, with particular reference to the anatomical subsite of the primary tumour. The density of $CD3^+$, $CD8^+$ and $FoxP3^+$ tumour-infiltrating T cells was calculated in tissue microarrays with tumours from 557 incident CRC cases from a prospective population-based cohort. Kaplan–Meier and Cox regression analyses were applied to determine the impact of high and low lymphocyte density on 5-year overall survival, in subgroup analysis of right colon, left colon and rectum. High $CD8^+$ cell density was a favourable prognostic factor for patients with right-sided colon tumours (hazard ratio [HR]=0.53, 95% confidence interval [CI] 0.29–0.95), independent of age, sex, TNM stage, differentiation grade and vascular invasion, with a significant prognostic interaction between $CD8^+$ cells and right-sidedness ($p = 0.031$). High $FoxP3^+$ cell density was an independent favourable prognostic factor only in patients with rectal tumours (HR = 0.54, 95% CI 0.30-0.99), and $CD3^+$ cell density was an independent favourable prognostic factor for tumours in the right colon and rectum, but there was no significant prognostic interaction between $CD3^+$ or $FoxP3^+$ cells and sidedness. These results demonstrate that the prognostic impact of tumour-infiltrating lymphocytes in CRC differs by primary tumour site, further indicating that tumour location may be an important factor to take into consideration in therapeutic decisions, including eligibility for immunotherapy.

Key words: T cells, colorectal cancer, tumour location, sidedness, prognosis

Abbreviations: CRC: colorectal cancer; CRT: classification and regression tree; HR: hazard ratio; IHC: immunohistochemical; MDCS: Malmö Diet and Cancer Study; OS: overall survival; TMA: tissue microarray; Treg: regulatory T cells

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With approximately 1.4 million new cases every year, colorectal cancer (CRC) is the third most common cancer globally.[1] Despite advances in treatment, CRC is still the third leading cause of cancer-related death and therefore, there is an urgent need to identify novel prognostic and predictive biomarkers.

Immuno-oncology is a rapidly emerging field that has elicited promise in cancer therapy. High levels of tumour-infiltrating ($CD3^+$) T cells and cytotoxic ($CD8^+$) T cells have been associated with auspicious clinical outcome in CRC,[2–9] occasionally outsmarting traditional tumour characteristics in predicating prognosis.[7,10] Paradoxically, high infiltration of $FoxP3^+$ regulatory T cells (Tregs), suppressing effector function of cytotoxic T cells, correlates with a dismal prognosis in numerous other cancers,[11] but is reported as a favourable prognostic factor in CRC.[12–15]

Increasing evidence suggests that CRC should be considered as a heterogeneous disease, with proximal and distal CRCs showing multiple clinicopathological and molecular distinctions, including the density of some immune cells.[16] However, to the best of our knowledge, no studies have hitherto investigated whether the prognostic significance of immune cell infiltration differs by primary tumour location. Therefore, the aim of this study was to examine the clinicopathological and molecular correlates and prognostic significance of the density of $CD3^+$, $CD8^+$ and $FoxP3^+$ T cells in CRC, with particular reference to

What's new?

In colorectal cancer, elevated levels of tumor-infiltrating lymphocytes in the tumor and its microenvironment are associated with improved survival. Whether this prognostic benefit differs according to tumor location, however, is unknown. Here, the prognostic impacts of $CD3^+$, $CD8^+$ and $FoxP3^+$ tumor-infiltrating T cells were examined with respect to tumor location. The data link high $CD8^+$ density with favorable prognosis for right-sided tumors, high $FoxP3^+$ density to improved prognosis for rectal tumors and $CD3^+$ density with improved prognosis for right colon and rectal tumors. Knowledge of variable immune system responses by tumor location could help inform the development of immune-modulating therapies.

the anatomical subsite of the primary tumour. Reanalysis of the prognostic value of previously investigated $CD20^+$ B cells and $CD138^+$ and $IGKC^+$ plasma cells[17] according to primary tumour subsite was also performed.

Methods

Patients

The study cohort consists of all incident cases of CRC in the Malmö Diet and Cancer Study (MDCS) from 1991 up until December 31, 2008 ($n = 626$). The MDCS is a prospective population-based cohort with the primary aim to investigate associations between various dietary factors and cancer incidence.[18] The project, including nonparticipants in the European Prospective Investigation into Cancer (EPIC) cohort, enrolled 18,326 women (60.2%) and 12,120 (39.8%) men, with a total of 30,446 participants (from a background population of 74,138).

Information on CRC incidence was obtained through the Swedish Cancer Registry up until December 31, 2007, and from The Southern Swedish Regional Tumour Registry for the period of January 1, 2008–December 31, 2008. Clinical and treatment data were obtained from medical charts. Histopathological data were obtained from pathology records. TNM staging was performed according to the American Joint Committee on Cancer. Right colon was defined as appendix, caecum, ascending and 2/3 of transverse colon, whereas left colon was defined as the left colic flexure, descending and sigmoid colon, corresponding to the midgut fetal origin versus the hindgut as well as different innervation and blood supply.

Median age at diagnosis was 71 (range 50–86) years. Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry up until December 31, 2013. Follow-up began at CRC diagnosis and ended at death, emigration or December 31, 2013, whichever came first. Median follow-up time was 5.97 (range 0–21.69) years for the full cohort ($n = 626$) and 10.05 (range 5.03–21.69) years for patients alive ($n = 274$). Microsatellite instability (MSI) screening status was assessed by immunohistochemistry as previously described,[19] and KRAS and BRAF mutation status was determined by pyrosequencing as previously described.[20]

Ethics approval and consent to participate

All EU and national regulations and requirements for handling human samples have been fully complied with during the conduct of this project; that is, decision no. 1110/94/EC of the European Parliament and of the Council (OJL126 18,5,94), the Helsinki Declaration on ethical principles for medical research involving human subjects and the EU Council Convention on human rights and Biomedicine. Ethical permission for the MDCS (LU 90–51) and the present study (LU 530–2008) was obtained from the Ethics Committee at Lund University. Written informed consent has been obtained from each subject at study entry.

Tissue microarray construction

All tumours with available slides or paraffin blocks were histopathologically re-evaluated on haematoxylin and eosin stained slides by a senior pathologist (KJ). Cases with an insufficient amount of tumour material were excluded, whereby a total number of 557 (89.0%) cases were available for tissue microarray (TMA) construction. Representative and non-necrotic areas were marked, and TMAs were constructed as previously described.[21] In brief, duplicate tissue cores (1 mm) were taken from each primary tumour and mounted in a recipient block, using a semi-automated arraying device (TMArrayer, Pathology Devices, Westminister, MD). Four μm sections from this block were subsequently cut using a microtome and mounted on glass slides.

Immunohistochemistry

For immunohistochemical (IHC) analysis of CD8 and FoxP3, 4 μm TMA-sections were pretreated using the PT Link system, and subsequently stained with the anti-CD8 antibody (clone C8/144B, mouse; dilution, 1:50; product M7103; Dako) and the anti-FoxP3 antibody (clone236A/E7, mouse, dilution 1:200, Abcam, Cambridge, UK) using the Autostainer Plus (Dako; Glostrup, Denmark).

For IHC analysis of CD3, 4 μm TMA-sections were pretreated using ULTRA Cell Conditioning Solution 1, pH 8.5 (Ventana Medical Systems Inc., Tucson, AZ) for heat induced epitope retrieval, and stained in a Ventana BenchMark stainer (Ventana Medical Systems) with the anti-CD3 antibody (clone 2GV6, prediluted, Ventana Medical Systems).

Evaluation of tumour-infiltrating lymphocytes

The total number of $CD3^+$ and $CD8^+$ lymphocytes in each core was calculated by automated analysis using the colocalization algorithm within the Halo image analysis software (Indica Labs, Corrales, NM). Automated analysis of FoxP3

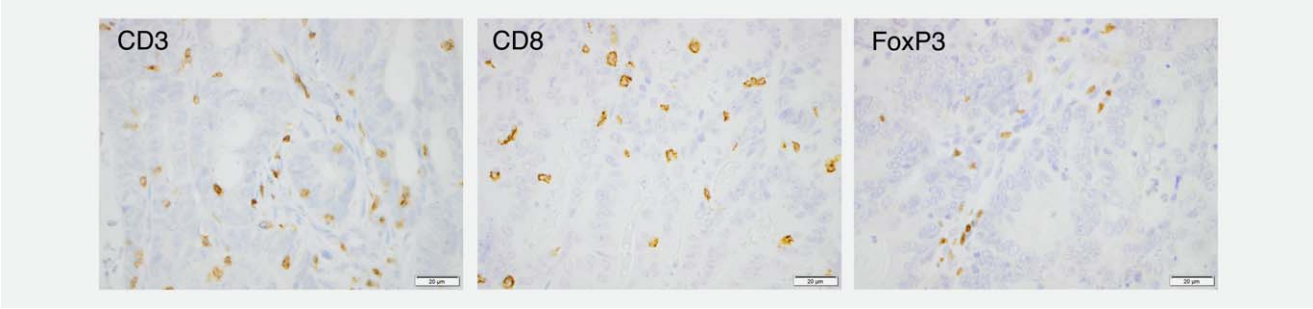

Figure 1. Immunohistochemical images of CD3, CD8 and FoxP3 staining in colorectal cancer. Sample images (10x magnification) representing immunohistochemical expression of CD3, CD8 and FoxP3 in colorectal cancer.

was not possible to undertake due to concomitant staining of the stroma, thus, the number of FoxP3 stained lymphocytes was manually counted. A mean value of the two cores was calculated and used in the analyses.

Furthermore, to validate the prognostic impact of the total number of $CD3^+$ and $CD8^+$ lymphocytes, an additional scoring system according to Dahlin *et al.*[22] and Ogino *et al.*[23] was performed. $CD3^+$ and $CD8^+$ lymphocyte infiltration was assessed as no/sporadic (Score 1), moderate (Score 2), abundant (Score 3) and highly abundant infiltration (Score 4) in three locations: (*i*) intratumoural (within the tumour nest), (*ii*) tumour-adjacent (defined as within one tumour cell diameter of the tumour) and (*iii*) within the distant stroma (defined as more than one tumour cell diameter away from the tumour). A total score for both CD3 and CD8 was calculated as the sum of the scores intratumourally, tumour-adjacent and within the distant stroma, generating a total score (lymphocytic reaction score) ranging from 3 to 12. A mean value of the total score from each core was calculated and used in the analysis. As FoxP3 was only sporadically expressed intratumourally and within the adjacent stroma, analysis according to lymphocytic reaction score was not meaningful.

Statistical analysis

Unadjusted nonparametric test was used to evaluate associations between infiltration of $CD3^+$, $CD8^+$ and $FoxP3^+$ cells and established clinicopathological characteristics and other investigative biomarkers. Spearmans Rho test was used to analyse the interrelationship between $CD3^+$, $CD8^+$ and $FoxP3^+$ cells. Classification and regression tree (CRT) analysis was used to determine the optimal prognostic cut-off for dichotomisation into high and low infiltration of $CD3^+$, $CD8^+$ and $FoxP3^+$ cells. Kaplan–Meier analysis and log rank test were applied to illustrate differences in five-year overall survival (OS) with respect to immune cell density. Cox regression proportional hazard models were used to estimate hazard ratios (HRs) for death from CRC in both univariable and multivariable analysis, adjusted for age, sex, T-stage, N-stage, M-stage, differentiation grade and vascular invasion.

The proportional hazard assumption was tested using Cox regression with a time-dependent covariate analysis, whereby the proportional hazard assumption was considered to be satisfied when the factor $\times$ time interaction was non-significant.

To estimate the interaction effect between tumour location and lymphocyte density, an interaction variable was constructed with tumour location (right/other, left/other or rectal/other, respectively) $\times$ lymphocyte density (low/high).

All calculations were performed using SPSS version 24.0 (SPSS, Chicago, IL). All statistical tests were two-sided and p-values < 0.05 were considered statistically significant.

Results

Associations of $CD3^+$, $CD8^+$ and $FoxP3^+$ lymphocyte count with clinicopathological factors according to primary tumour site

Information on tumour location was available for 555 (99.6%) cases in the TMA, with 201 (36.1%) right-sided colon tumours, 145 (26.0%) left-sided colon tumours and 209 (37.5%) rectal tumours.

Immune cell-specific $CD3^+$ lymphocyte count could be determined in 530 (95.2%) cases, and $CD8^+$ lymphocyte count in 539 (96.8%) cases. $FoxP3^+$ lymphocyte count was assessable in 547 (98.2%) cases. Sample IHC images are shown in Figure 1.

Associations between $CD3^+$, $CD8^+$ and $FoxP3^+$ lymphocyte count and clinicopathological factors in the entire cohort and by tumour subsite are shown in Tables (1–3), respectively. In general, high density of all investigated T lymphocyte subsets was associated with more favourable tumour characteristics. The majority of significant associations between high lymphocyte density and favourable clinicopathological factors were seen in the right colon for all types of investigated immune cells. High $CD3^+$, $CD8^+$ and $FoxP3^+$ cell infiltration were all significantly associated with MSI tumours, and only in the right colon. A significantly higher number of $CD8^+$ cells were observed in BRAF mutated tumours in the entire cohort, but not according to tumour subsite. No significant associations were observed between immune cell density and KRAS mutation status.

The clinicopathological correlates of $CD20^+$ B cells, and $CD138^+$ and $IGKC^+$ plasma cells in the full cohort have been described previously[17], and was, similar to the T lymphocytes, found to be associated with more favourable

Table 1. Associations between $CD3^+$ cell infiltration and clinicopathological and investigative factors stratified by primary tumour location

	Entire cohort			Right colon			Left colon			Rectum		
Factor	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*
Age												
< 75	134.00 (0.00–1,758.50)		369	276.00 (2.50–1,587.00)		110	237.75 (0.00–1,758.50)		104	213.50 (139.00–543.00)		158
=> 75	1,275.00 (4.50–2,280.00	0.090	165	278.00 (6.00–2,280.50)	0.239	87	246.50 (4.50–974.00)	0.634	36	265.75 (0.00–1,147.50)	0.057	42
Sex												
Female	288.50 (0.00–1,758.50)		283	332.50 (9.50–1,675.00)		110	281.00 (10.00–1,758.50)		75	362.25 (13.00–1,158.00)		97
Male	252.00 (0.00–2,280.00)	0.153	256	253.00 (2.50–2,280.0)	0.164	87	230.50 (0.00–886.50)	0.372	64	275.00 (0.00–1,077.50)	0.963	103
T-stage												
1	419.25 (57.00–1,675.00)		48	691.00 (154.50–1,675.00		9	369.75 (110.00–974.00)		22	382.00 (57.00–1,158.00)		17
2	380.00 (90.00–2,047.50)		63	388.25 (92.50–2,074.50)		18	307.50 (129.50–849.50)		9	405.13 (90.00–950.00)		36
3	0.00 (0.00–2,280.00)		325	299.00 (2.50–2,280.00)		121	230.25 (0.00–1,758.50)		88	219.50 (0.00–1,147.50)		115
4	176.00 (0.00–1,283.50)	**<0.001****	80	192.50 (6.00–1,283.50)	**<0.001****	46	184.00 (0.00–736.00)	**0.010**	19	83.00 (7.00–566.00)	**<0.001****	15
N-stage												
0	287.00 (0.00–2,280.00)		287	326.50 (38.50–2,280.00)		101	253.50 (0.00–910.50)		81	295.75 (0.00–1,136.00)		104
1	214.50 (4.50–1,675.00)		123	263.00 (9.50–1,675.00)		47	207.25 (4.50 (711.50)		34	207.00 (6.50–1,147.50)		42
2	237.75 (0.00–1,757.50)	**0.019***	82	247.00 (2.50–1,081.00)	0.072	41	230.50 (0.00–1,758.50)	0.800	13	257.50 (7.00–910.00)	0.064	28
M-stage												
0	290.50 (0.00–2,280.00)		441	356.50 (9.50–2,280.00)		157	273.50 (0.00–1,758.50)		113	276.00 (0.00–1,158.00)		170
1	158.00 (0.00–940.50)	**<0.001****	91	130.00 (2.50–940.50)	**<0.001****	38	167.25 (0.00–687.50)	**0.009***	26	163.00 (7.00–909.00)	0.190	27
Differentiation grade												
Low	273.25 (0.00–2,074.50)		410	126.00 (2.50–2,074.50)		126	259.00 (0.00–1,758.00)		115	272.00 (0.00–1,158.00)		168
High	234.75 (6.00–2,280.00)	0.521	118	339.00 (6.00–2,280.00)	0.689	68	176.50 (18.00–543.00)	**0.047***	23	234.00 (7.50–909.00)	0.478	27
Mucinous												
No	284.00 (0.00–2,280.00)		426	339.00 (2.50–2,280.00)		138	247.50 (0.00–1,758.50)		117	289.00 (0.00–1,158.00)		171
Yes	203.75 (0.00–1,130.00)	**0.004**	106	233.00 (9.50–1,130.00)	**0.010***	58	223.50 (0.00–886.50)	0.338	23	162.50 (7.50–910.00)	0.066	24
MSI status												
Stable	273.00 (0.00–1,758.00)		425	252.50 (6.00–1,587.00)		117	259.00 (0.00–1,758.50)		127	290.00 (6.50–1,158.00)		179
Unstable	431.33 (33.00–2,280.00)	**<0.001**	74	490.75 (33.00–2,280.00)	**<0.001****	68	145.75 (45.50–291.00)	**0.010**	4	351.75 (124.00–579.50)	0.983	2
KRAS												
Wild-type	279.50 (0.00–2,074.50)		322	323.00 (9.50–2,074.50)		114	234.50 (0.00–1,758.50)		78	290.00 (6.50–1,158.00)		127
Mutated	229.00 (0.00–1,587.00)	0.111	186	241.25 (2.50–1,587.00)	0.095	68	244.75 (0.00–886.50)	0.946	56	209.75 (0.00–950.00)	0.510	62

Table 1. Associations between $CD3^+$ cell infiltration and clinicopathological and investigative factors stratified by primary tumour location (Continued)

	Entire cohort			Right colon			Left colon			Rectum		
Factor	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*
BRAF												
Wild-type	269.00 (0.00–1,758.00)		431	269.50 (2.50–1,587.00)		116	242.00 (0.00–1,758.50)		129	276.26 (0.00–1,158.00)		186
Mutated	283.00 (9.50–2,074.50)	0.369	76	299.00 (9.50–2,074.50)	0.699	67	213.50 (139.00–543.00)	0.986	5	124.00 (14.00–234.00)	0.236	2
Tumour location												
Right	278.00 (2.50–2,280.00)		197									
Left	243.75 (0.00–1,758.50)		140									
Rectum	275.00 (0.00–1,158.00)	0.342	200									

*Significance at the 5% level.
**Significance at the 1% level. The analysis of $CD3^+$ cell infiltration was based on total lymphocyte count.
Abbreviations: MSI: microsatellite instability.

tumour characteristics. Corresponding analyses according to tumour subsite for these lymphocytes are shown in Supporting Information Tables 1–3. In general, fewer significant associations with clinicopathological factors were observed for B cells and plasma cells than for T cells, and only the density of $IGKC^+$ plasma cells was significantly higher in MSI right-sided tumours.

There were moderate to very strong intercorrelations between all investigated T- and B-lymphocytes, and plasma cells (Supporting Information Table 4).

Prognostic significance of $CD3^+$, $CD8^+$ and $FoxP3^+$ cell infiltration

For the entire cohort, CRT analysis established an optimal cut-off point for $CD3^+$ lymphocyte count at ≤ 292.75, which was used to stratify cases into groups of low ($\leq$292.75, $n = 338$) and high (>292.75, $n = 203$) count. Similarly, $CD8^+$ lymphocyte count was dichotomised into groups of low ($\leq$33.5, $n = 77$) and high (>33.50, $n = 453$) lymphocyte count. For $FoxP3^+$ lymphocyte count, cases were divided into groups of low ($\leq$9.25, $n = 300$) and high (>9.25, $n = 247$) count. Finally, according to the total CD3 and CD8 score, respectively, patients were divided into three groups of equal size with low (3–4), intermediate (5–6), or high (7–12) total score.

In the entire cohort, Kaplan–Meier analysis revealed significant correlations between all investigated T cell subsets and a prolonged 5-year OS ($p = 0.001$ for $CD3^+$ and $CD8^+$, and $p = 0.006$ for $FoxP3^+$, Supporting Information Fig. 1). Kaplan–Meier estimates of 5-year OS according to tumour subsite are shown in Figure 2. The prognostic impact of the pan T cell marker CD3 was stepwise decreased from the right colon ($p = 0.001$, Fig. 2*a*), left colon ($p = 0.036$, Fig. 2*b*), to the rectum ($p = 0.051$, Fig. 2*c*). High density of $CD8^+$ cells was significantly associated with a prolonged 5-year OS in right-sided tumours ($p < 0.001$, Fig. 2*d*), but was not prognostic in the left colon or in the rectum (Figs. 2*e* and 2*f*). $FoxP3^+$ cells were not prognostic in either subsite (Figs. 2*g*–2*i*).

Cox proportional hazards analyses of 5-year OS according to lymphocyte density and tumour subsite are shown in Table 4. The time-dependent covariate was non-significant for all investigated T cell subsets, and therefore, the factor × time interaction term was dropped from the model. The proportional hazard assumption was also considered to be satisfied with graphical evaluation using log-minus-log plots (data not shown).

The significant associations between $CD3^+$, $CD8^+$ and $FoxP3^+$ lymphocytes and an improved 5-year OS in the entire cohort were confirmed in univariable Cox regression analysis (HR = 0.51; 95% CI 0.37–0.70, HR = 0.56; 95% CI 0.40–0.79 and HR = 0.68; 95% CI 0.51–0.91, respectively), and all remained significant in multivariable analysis, after adjustment for age, sex, TNM stage, differentiation grade and vascular invasion (HR = 0.47; 95% CI 0.33–0.69, HR = 0.60; 95% CI 0.41–0.87 and HR = 0.67; 95% CI 0.49–0.94,

Table 2. Associations between $CD8^+$ cell infiltration and clinicopathological and investigative factors stratified by primary tumour location

	Entire cohort			Right colon			Left colon			Rectum		
Factor	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*
Age												
75	266.75 (0.00–1,758.50)		369	176.00 (1.50–1,130.50)		109	129.50 (6.50–970.00)		103	125.00 (0.00–992.00)		155
=> 75	131.00 (1.00–2,125.50)	0.455	161	153.50 (2.00–2,125.50)	0.239	83	102.50 (2.00–685.50)	0.138	38	142.00 (1.00–1,093.00)	0.402	40
Sex												
Female	136.00 (0.00–1,683.50)		282	173.75 (1.50–1,683.50)		110	128.00 (2.00–970.00)		77	120.75 (0.00–1,093.00)		94
Male	132.50 (0.00–2,125.50)	0.365	248	158.25 (2.00–2,125.50)	0.590	82	104.25 (3.00–834.50)	0.093	64	134.00 (0.00–875.00)	0.489	101
T-stage												
1	231.00 (26.00 −1,416.00)		47	412.75 (165.00–1,416.00)		8	199.50 (49.50–685.50)		22	172.00 (26.00–929.50)		17
2	155.25 (10.00–2,125.50)		64	246.00 (12.50–2,125.50)		18	62.00 (10.00–210.00)		9	162.00 (20.00–626.50)		37
3	129.75 (0.00–1,683.50)		320	187.50 (5.50–1,683.50)		119	121.50 (3.00–970.00)		89	116.00 (0.00–992.00)		111
4	91.00 (1.00–1,016.50)	**<0.001****	79	116.00 (1.50–1,016.50)	**0.002***	45	125.00 (2.00–480.50)	**0.035**	19	42.50 (1.00–350.00)	**<0.001****	15
N-stage												
0	138.00 (2.00–2,125.50)		283	163.00 (10.00–2,125.50)		99	113.50 (2.00–834.50)		82	138.00 (2.00–992.00)		101
1	105.20 (0.00–1,416.00)		122	176.00 (4.00–1,416.00)		45	123.25 (3.00–399.00)		34	76.50 (0.00–498.00)		43
2	128.00 (0.00 −970.00)	**0.026***	79	177.00 (1.50–940.00)	0.285	39	79.50 (32.00–970.00)	0.823	13	128.00 (0.00–824.00)	**0.012**	**27**
M-stage												
0	139.00 (0.00–2,125.50)		440	187.50 (6.00–2,125.50)		155	128.00 (2.00–970.00)		115	125.00 (0.00–992.00)		169
1	101.00 (1.00–1,093.00)	**0.004***	83	101.50 (1.50–757.50)	**0.001****	35	71.00 (24.50–793.50)	0.073	25	151.00 (1.00–1,093.00)	0.994	23
Differentiation grade												
Low	134.25 (0.00 −2,125.50)		404	163.00 (8.00–2,125.50)		126	125.00 (2.00–970.00)		117	130.00 (0.00–1,093.00)		165
High	125.75 (1.50–1,646.50)	0.835	116	213.50 (1.50–1,646.50)	0.687	68	106.50 (32.50–390.50)	0.929	22	90.75 (4.00–647.00)	0.099	26
Mucinous												
No	134.25 (0.00–2,125.50)		418	193.75 (1.50–2,125.50)		134	126.00 (2.00–970.00)		118	125.25 (0.00–1,093.00)		166
Yes	120.75 (4.00–932.00)	0.618	104	117.00 (8.00–932.00)	0.052	57	109.50 (6.50–483.00)	0.836	23	151.00 (4.00–824.00)	0.861	23
MSI status												
Stable	129.00 (0.00–1,130.50)		418	126.25 (1.50–1,130.50)		114	128.75 (2.00–970.00)		128	129.00 (0.00–1,093.00)		174
Unstable	305.00 (13.00–2,125.50)	**<0.001****	75	312.75 (13.00–2,125.50)	**<0.001****	68	95.25 (52.00–191.00)	0.515	4	571.00 (106–984.50)	0.084	3
KRAS												
Wild-type	140.00 (0.00–2,125.50)		317	171.50 (1.50–2,125.50)		113	114.00 (10.00–970.00)		79	125.00 (0.00–984.50)		123
Mutated	127.25 (1.00–2,230.50)	0.481	182	128.50 (2.00–2,230.50)	0.198	65	125.25 (2.00–834.50)	0.933	56	128.00 (1.00–1,093.00)	0.702	61

Table 2. Associations between $CD8^+$ cell infiltration and clinicopathological and investigative factors stratified by primary tumour location (Continued)

	Entire cohort			Right colon			Left colon			Rectum		
Factor	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*
BRAF												
Wild-type	128.00 (0.00–1,683.50)		423	147.00 (2.00–1,683.50)		111	124.50 (2.00–970.00)		130	126.75 (0.00–1,093.00)		182
Mutated	171.50 (1.50–2,125.50)	**0.049***	75	176.00 (1.50–2,125.50)	0.530	67	109.50 (79.50–390.50)	0.930	5	16.00 (16.00–16.00)	0.109	1
Tumour location												
Right	168.25 (1.50–2,125.50)		192									
Left	243.75 (0.00–1,758.50)		141									
Rectum	275.00 (0.00–1,158.000)	**0.004***	195									

*Significance at the 5% level.
**Significance at the 1% level.
The analysis of $CD8^+$ cell infiltration was based on total lymphocyte count.
Abbreviations: MSI: microsatellite instability.

respectively). In the right colon, the prognostic significance of $CD3^+$ and $CD8^+$ density was confirmed in univariable analysis (HR = 0.43; 95% CI 0.27–0.71, and HR = 0.35; 95% CI 0.21–0.60, respectively), and remained significant in multivariable analysis (HR = 0.53; 95% CI 0.29–0.95 and HR = 0.35; 95% CI 0.19–0.65, respectively). In the rectum, $CD3^+$ density was not prognostic in univariable analysis, but multivariable Cox regression analysis revealed a significant association with a prolonged 5-year OS (HR = 0.45; 95% CI 0.22–0.94). $FoxP3^+$ density was not prognostic in either tumour subsite in univariable analysis; however, dense $FoxP3^+$ cell infiltration was significantly associated with a prolonged 5-year OS in the rectum (HR= 0.54; 95% CI 0.30–0.99).

When MSI status was included in the adjusted model, $CD8^+$ cells remained an independent favourable prognostic factor in right-sided tumours (HR = 0.42; 95% CI 0.21–0.82), however, when BRAF mutation status also was included, the association did not remain significant (HR = 0.49; 95% CI 0.24–1.02).

There was a significant interaction between tumour location in the right colon and high density of $CD8^+$ lymphocytes (p for interaction = 0.031). No significant interactions were observed between $CD3^+$ or $FoxP3^+$ lymphocytes and any tumour location.

Using the lymphocytic reaction score,[22,23] Kaplan–Meier analysis revealed significantly prolonged survival for patients with right-sided tumours displaying high CD8 lymphocytic score ($p = 0.002$), and for patients with left-sided tumours displaying high CD3 ($p = 0.008$) and intermediate CD8 ($p = 0.012$) lymphocytic reaction score (Supporting Information Fig. 2). In Cox regression analysis, high and intermediate lymphocytic reaction score was included in one variable, whereby the prognostic significance of high CD8 lymphocytic score in right-sided tumours was confirmed in univariable (HR = 0.50; 95% CI 0.31–0.79) and multivariable (HR = 0.44; 95% CI 0.25–0.78) Cox regression analysis (Supporting Information Table 6). In left-sided tumours, the prognostic impact of high CD3 lymphocytic reaction score was confirmed in univariable (HR = 0.44, 95% CI 0.25–0.78) and multivariable (HR = 0.44, 95% CI 0.23–0.86) Cox regression analysis, whereas high CD8 lymphocytic reaction score was significant in univariable (HR = 0.46, 95% CI 0.25–0.86) but not in multivariable Cox regression analysis. There were no significant associations between neither CD3 nor CD8 lymphocytic reaction score and survival in rectal tumours.

Dense infiltration of $CD20^+$ B cells has in the herein investigated cohort been found to be an independent favourable prognostic factor.[17] Cox proportional hazards analyses of 5-year OS according to B cell and plasma cell density and tumour subsite are shown in Supporting Information Table 5. Only high $CD20^+$ density was found to be associated with an improved prognosis in right-sided tumours, in both univariable (HR= 0.51; 95% CI 0.27–0.97) and multivariable (HR = 0.38; 95% CI 0.18–0.83) analysis. In left-sided tumours, high $CD20^+$ was significantly associated with a

Table 3. Associations between FoxP3$^+$ cell infiltration and clinicopathological and investigative factors stratified by primary tumour location

	Entire cohort			Right colon			Left colon			Rectum		
Factor	**Median (range)**	***p*-value**	***n***	**Median (range)**	***p*-value**	***n***	**Median (range)**	***p*-value**	***n***	**Median (range)**	***p*-value**	***n***
Age												
< 75	6.00 (0.00–128.00)		375	6.50 (0.00–100.00)		108	5.50 (0.00–101.00)		105	6.00 (0.00–128.00)		160
=> 75	11.50 (0.00–140.00)	**0.002***	172	13.50 (0.00–116.00)	**0.022***	87	8.50 (0.00–140.00)	0.499	39	10.25 (0.00–73.50)	0.108	46
Sex												
Female	8.00 (0.00–140.00)		286	10.00 (0.00–116.00)		109	7.50 (0.00–140.00)		77	7.00 (0.00–128.00)		99
Male	7.00 (0.00–109.00)	0.425	261	8.75 (0.00–98.00)	0.445	86	5.00 (0.00–69.00)	0.995	67	7.00 (0.00–109.00)	0.490	107
T-stage												
1	13.50 (0.00–140.00)		48	10.00 (0.00–116.00)		9	9.00 (0.00–140.00)		22	28.00 (0.00–86.00)		17
2	19.50 (0.00–92.00)		64	29.00 (0.00–80.00)		18	30.00 (2.00–92.00)		9	11.00 (0.00–89.00)		37
3	8.00 (0.00–109.00)		330	12.67 (0.00–100.00)		119	5.50 (0.00–94.00)		91	5.00 (0.00–109.00)		119
4	2.00 (0.00–108.00)	**<0.001****	81	2.50 (0.00–108.00)	**0.016***	46	0.75 (0.00–23.00)	**0.003***	20	0.00 (0.00–41.50)	**0.002***	15
N-stage												
0	9 (0.00–140.00)		290	13.25 (0.00–102.00)		98	5.75 (0.00–140.00)		84	6.50 (0.00–128.00)		107
1	8.00 (0.00–116.00)		124	12.00 (0.00–116.00)		46	6.50 (0.00–94.00)		34	6.50 (0.00–109.00)		44
2	3.00 (0.00–57.00)	**0.027***	84	4.75 (0.00–41.50)	0.086	42	3.25 (0.00–39.50)	0.623	14	1.50 (0.00–57.00)	0.147	28
M-stage												
0	9.00 (0.00–140.00)		446	13.00 (0.00–116.00)		154	8.00 (0.00–140.00)		116	8.00 (0.00–128.00)		175
1	2.00 (0.00–108.00)	**<0.001****	94	2.00 (0.00–108.00)	**0.001****	39	2.50 (0.00–64.00)	0.055	27	1.50 (0.00–95.00)	0.142	28
Differentiation grade												
Low	8.50 (0.00–140.00)		415	12.00 (0.00–102.00)		123	8.00 (0.00–140.00)		115	8.00 (0.00–128.00)		172
High	5.00 (0.00–116.00)	0.172	121	6.50 (0.00–116.00)	0.388	69	2.50 (0.00–94.00)	0.192	23	4.00 (0.00–107.00)	0.246	29
Mucinous												
No	9.00 (0.00–140.00		433	13.50 (0.00–116.00)		137	8.00 (0.00–140.00)		121	7.00 (0.00–128.00)		175
Yes	4.75 (0.00–82.00)	**0.012***	106	5.00 (0.00–82.00)	**0.004***	57	1.50 (0.00–64.00)	**0.030***	23	9.00 (0.00–57.00)	0.951	25
MSI status												
Stable	8.00 (0.00–140.00)		432	8.00 (0.00–108.00)		116	8.00 (0.00–140.00)		131	8.00 (0.00–128.00)		183
Unstable	14.75 (0.00–116.00)	**0.010***	74	15.50 (0.00–116.00)	**0.012***	67	19.00 (0.00–30.00)	0.619	4	0.00 (0.00–0.50)	0.056	3
KRAS												
Wild-type	8.00 (0.00–140.00)		328	8.50 (0.00–116.00)		114	5.50 (0.00–140.00)		80	8.50 (0.00–107.00)		132
Mutated	7.00 (0.00–109.00)	0.336	187	10.75 (0.00–100.00)	0.410	66	7.50 (0.00–72.00)	0.946	58	6.00 (0.00–109.00)	0.475	63

Table 3. Associations between FoxP3$^+$ cell infiltration and clinicopathological and investigative factors stratified by primary tumour location (Continued)

	Entire cohort			Right colon			Left colon			Rectum		
Factor	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*	Median (range)	*p*-value	*n*
BRAF												
Wild-type	7.50 (0.00–140.00)		437	10.75 (0.00–108.00)		112	5.50 (0.00–140.00)		133	8.00 (0.00–109.00)		192
Mutated	8.50 (0.00–116.00)	0.145	77	8.25 (0.00–116.00)	0.680	68	24.00 (0.00–94.00)	0.167	5	4.25 (4.00–4.50)	0.745	2
Tumour location												
Right	9.00 (0.00–116.00)		195									
Left	5.75 (0.00–140.00)		144									
Rectum	7.00 (0.00–128.00)	0.185	206									

*Significance at the 5% level.
**Significance at the 1% level.
The analysis of FoxP3$^+$ cell infiltration was based on total lymphocyte count.
Abbreviations: MSI: microsatellite instability.

prolonged 5-year OS in univariable (HR= 0.37; 95% CI 0.16–0.87) but not in multivariable analysis, whereas high CD138$^+$ cell infiltration was significantly associated with a prolonged 5-year OS in multivariable (HR = 0.48, 95% CI 0.48–0.96) but not in univariable analysis. No significant associations were observed between B cells or plasma cells and prognosis in rectal cancer. There were no significant associations between density of IGKC$^+$ cells and prognosis in either tumour location.

Survival analysis in strata according to adjuvant chemotherapy in curatively treated Stage III patients revealed that the prognostic significance of CD3$^+$ cells was only evident in untreated patients, in the entire cohort as well as in the right colon, but there was no significant treatment interaction (data not shown). The prognostic impact of the other lymphocyte subsets did not differ significantly in strata according to adjuvant chemotherapy (data not shown).

Discussion

Numerous studies have thus far examined tumour infiltration of T cells in CRC and its relation to prognosis. However, this study is, to the best of our knowledge, the first to investigate the prognostic impact of immune cell infiltrates in colorectal cancer with specific emphasis on the anatomical localisation of the primary tumour.

In the entire cohort, dense infiltration of CD3$^+$ and CD8$^+$ lymphocytes was independently associated with an improved prognosis, which is in concordance with previous research.[24] Furthermore, high numbers of FoxP3$^+$ Tregs was found to be an independent auspicious prognostic factor. This is in line with previous studies in CRC; however, in the majority of human carcinomas, FoxP3 has been demonstrated to be mainly associated with a dismal prognosis, as reviewed in Ref. 9. The contrasting findings in CRC have been attributed to the microbiota in the colon, that triggers a carcinogenic cascade which FoxP3$^+$ cells inhibits.[9] Furthermore, as Tregs suppress other T cells,[25] they may also impair the function of pro-tumourigenic inflammatory Th17 cells,[26] thus inhibiting tumour progression. Albeit Tregs have been considered a potential target for immunotherapy,[27,28] the results from our study further indicate that Treg-depleting treatment might be detrimental in CRC.

When taking primary tumour subsite into consideration, only CD3$^+$ and CD8$^+$ T cells were independent prognostic factors in right-sided tumours, whereas FoxP3$^+$ T cells were independently associated with an improved prognosis in rectal tumours, but not in left- or right-sided tumours. A significant interaction with tumour subsite was only observed for CD8$^+$ lymphocyte density and the righ-sidedness. Furthermore, CD20$^+$ B cells were independently associated with an improved prognosis for patients with right-sided tumours, but not for those with left-sided or rectal tumours, whereas dense CD138$^+$ immune cell infiltration was an independent predictor of improved prognosis in left-sided tumours, but not in right-sided or rectal

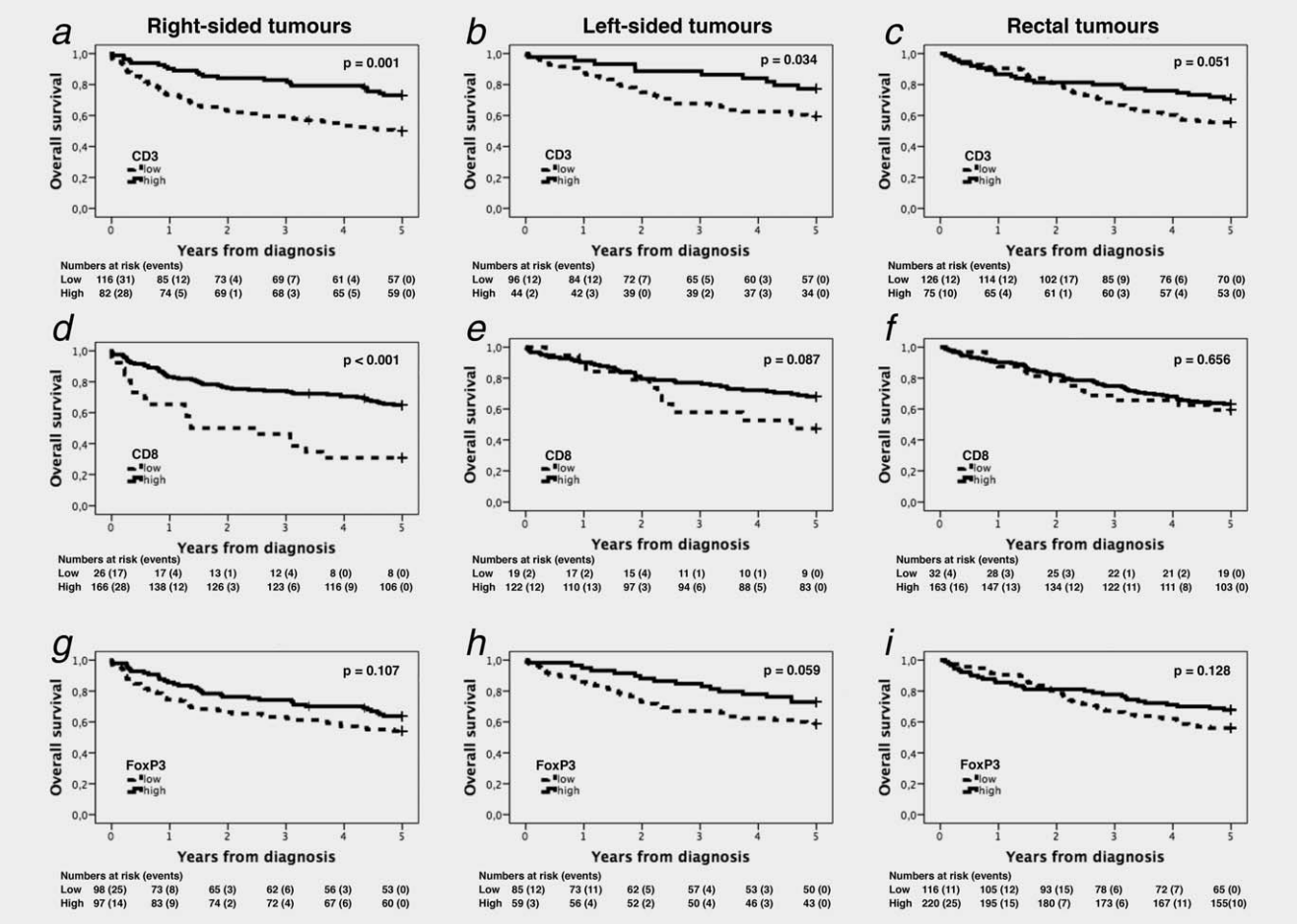

Figure 2. Kaplan–Meier estimates of overall survival according to CD3$^+$, CD8$^+$ and FoxP3$^+$ cell infiltration and primary tumour location. Kaplan–Meier analysis of 5-year overall survival in strata of low and high CD3$^+$ (*a, b, c*), CD8$^+$ (*d, e, f*) and FoxP3$^+$ (*g, h, i*) lymphocyte infiltration in right-sided (first row), left-sided (second row) and rectal (third row) tumours.

tumours. Of note, the segments in the colon have different fetal origin. The proximal part, from the appendix to the first two thirds of the transverse colon, originates from the midgut, whereas the distal part, from the left colic flexure to the rectum, originates from the hindgut. Increasing evidence suggests several differences between right-sided and left-sided CRC, including epidemiology, tumour characteristics and prognosis.[29] Furthermore, right-sided tumours demonstrate diverse genetic and molecular characteristics compared to left-sided tumours,[30] and these differences in biological behaviour have been suggested to induce different response to chemotherapy.[31] The findings from the present study provide further evidence that proximal and distal CRC may represent distinct disease entities, wherein the impact of the inflammatory tumour microenvironment on tumour progression, prognosis and prediction differs.

Interestingly, only CD8$^+$ T cell infiltration differed significantly according to anatomical subsite, with denser infiltration in rectal and left-sided tumours than in right-sided tumours. This is in contrast with a previous study, demonstrating no differences in CD8$^+$ cell infiltration according to tumour subsite, but a denser infiltration of FoxP3$^+$ cells in rectal than in right-sided or left-sided tumours.[16] As right-sided colon cancer generally carries a poorer prognosis than left-sided colon cancer,[29,32] our findings further emphasise the positive prognostic impact of CD8$^+$ lymphocyte infiltration.

An increased infiltration of lymphocytes has previously been found in MSI-high tumours,[33] mostly being proximally located. This was confirmed in the present study, with the vast majority of MSI tumours displaying a high density of CD3$^+$, CD8$^+$ and FoxP3$^+$ cells, in the full cohort as well as in right-sided tumours, despite the total number of lymphocytes not being higher in the latter. However, the favourable impact of CD8$^+$ T cells was independent of MSI status in the entire cohort as well as in right-sided tumours, further supporting that combined assessment of MSI status and tumour-infiltrating lymphocytes will provide a more accurate prognostication, in particular in patients with right-sided tumours. Similar findings have been observed using the immunoscore.[34]

Additionally, high density of CD3$^+$, CD8$^+$ as well as FoxP3$^+$ T cells was found to correlate significantly with

Table 4. Cox proportional hazards models for 5-year overall survival in relation to $CD3^+$, $CD8^+$ and $FoxP3^+$ cell infiltration

	Entire cohort			Right colon			Left colon			Rectum		
	HR (95% CI)	*p-value*	*n (deaths)*	HR (95% CI)	*p-value*	*n* (deaths)	HR (95% CI)	*p-value*	*n* (deaths)	HR (95% CI)	*p-value*	*n* (deaths)
Univariable												
CD3												
Low	1.00		335 (152)	1.00		116 (58)	1.00		96 (39)	1.00		126 (56)
High	0.51 (0.37–0.70)	**<0.001****	203 (54)	0.43 (0.27–0.71)	**0.001***	82 (22)	0.48 (0.24–0.96)	**0.038***	44 (10)	0.62 (0.38–1.01)	0.053	75 (22)
CD8												
Low	1.00		77 (41)	1.00		26 (18)	1.00		19 (10)	1.00		32 (13)
High	0.56 (0.40–0.79)	**0.001***	452 (157)	0.35 (0.21–0.60)	**<0.001****	166 (58)	0.55 (0.27–1.10)	0.092	122 (39)	0.83 (0.48–1.59)	0.656	163 (60)
FoxP3												
Low	1.00		299 (131)	1.00		98 (45)	1.00		85 (35)	1.00		116 (51)
High	0.68 (0.51–0.90)	**0.006***	257 (80)	0.70 (0.49–1.08)	0.109	97 (35)	0.57 (0.32–1.03)	0.063	59 (16)	0.70 (0.45–1.11)	0.130	90 (29)
Multivariable												
CD3												
Low	1.00		306 (132)	1.00		106 (49)	1.00		91 (37)	1.00		109 (46)
High	0.47 (0.33–0.69)	**<0.001****	176 (40)	0.53 (0.29–0.95)	**0.033***	78 (20)	0.50 (0.21–1.19)	0.117	36 (7)	0.45 (0.22–0.94)	**0.033***	61 (13)
CD8												
Low	1.00		73 (38)	1.00		26 (18)	1.00		18 (9)	1.00		29 (11)
High	0.60 (0.41–0.87)	**<0.001****	402 (129)	0.35 (0.19–0.65)	**0.001****	153 (48)	0.61 (0.25–1.47)	0.272	110 (35)	0.98 (0.49–1.96)	0.948	138 (46)
FoxP3												
Low	1.00		270 (115)	1.00		88 (38)	1.00		77 (32)	1.00		105 (45)
High	0.67 (0.49–0.94)	**0.018***	218 (61)	0.95 (0.56–1.59)	0.835	93 (31)	0.54 (0.27–1.07)	0.075	54 (14)	0.54 (0.30–0.99)	**0.048***	70 (16)
Multivariable including MSI status												
CD3												
Low	1.00		275 (121)	1.00		97 (43)	1.00		84 (33)	1.00		94 (45)
High	0.49 (0.33–0.73)	**<0.001****	173 (39)	0.58 (0.31–1.08)	0.085	78 (20)	0.43 (0.18–1.04)	0.060	35 (7)	0.52 (0.25–1.09)	0.083	59 (12)
CD8												
Low	1.00		62 (32)	1.00		23 (15)	1.00		15 (7)	1.00		24 (10)
High	0.69 (0.46–1.03)	0.072	380 (124)	0.42 (0.21–0.82)	**0.012***	148 (46)	0.69 (0.26–1.82)	0.448	105 (33)	1.13 (0.54–2.34)	0.753	126 (45)
FoxP3												
Low	1.00		242 (104)	1.00		81 (33)	1.00		69 (28)	1.00		92 (43)
High	0.74 (0.53–1.05)	0.088	211 (59)	1.19 (0.67–2.11)	0.554	91 (30)	0.55 (0.26–1.14)	0.107	54 (12)	0.81 (0.42–1.57)	0.533	65 (15)

*Significance at the 5% level.
**Significance at the 1% level. *p* values from multivariable analysis adjusted for age, sex, T-stage (I, II, III, IV), N-stage (0,1,2), M-stage (0, 1), differentiation grade (high-intermediate versus low) and vascular invasion (+/−/unknown), without and with inclusion of microsatellite instability (MSI) status. Information on age and sex was available for all cases. Cases with unknown information on TNM stage, differentiation grade and MSI status were not included in the multivariable model. The analysis of $CD3^+$, $CD8^+$ and $FoxP3^+$ cell infiltration was based on total lymphocyte count.

lower T- and M-stage both in the entire cohort and after stratifying for tumour location. However, the associations between cytotoxic as well as regulatory T cell infiltration and lower M-stage only remained significant in right-sided tumours, further suggesting that dense infiltration of these lymphocytes carries a greater clinical impact in right-sided tumours than in left-sided or rectal tumours.

A rather large proportion of the studies investigating the differences between right- and left-sided CRC have used alternative definitions, for example, defining the right colon from the appendix to the hepatic flexure and the left colon from the splenic flexure to the rectum, thus excluding the transverse colon altogether. We defined right colon as appendix, caecum, ascending and two thirds of the transverse colon, and left colon as left colic flexure, descending and sigmoid colon, corresponding to the midgut versus the hindgut fetal origin. However, research demonstrates a gradual transition through the multiple anatomic subsites, rather than abrupt changes as in the two-colon model.[35] Nonetheless, as a clinically practicable tool, the two-colon model might still be preferable.

The prognostic relevance of tumour subsite has hitherto mainly been acknowledged in patients with metastatic CRC and in relation to chemotherapy response.[32] In the present cohort, with incident cases spanning over several decades and a comparatively large proportion of curatively treated Stage II patients who did not receive adjuvant chemotherapy, no evident predictive value of any of the investigated T cells, B cells or plasma cells could be observed, neither in the entire cohort nor according to tumour subsite. However, the lack of a prognostic impact of $CD3^+$ lymphocytes in adjuvant Stage III treated patients who received chemotherapy in contrast to those who did not, supports that assessment of tumour-infiltrating lymphocytes may provide additional prognostic information, and, hence, be of value regarding choice of treatment in some situations.

As the study was performed retrospectively, there is an inherent risk of selection bias. Nevertheless, the herein investigated tumours are derived from a prospective, population-based cohort with clinically and histopathologically well-characterised incident CRC cases. Another potential weakness of the study is the use of the TMA technique. Although two 1 mm cores can be considered an adequate sampling and a plethora of validatory studies have previously concluded that findings based on large sections were fully reproducible in TMA-based studies,[36] the tumour stroma has not been specifically sampled in the herein used TMA. Future studies on the prognostic value of the inflammatory tumour microenvironment should ideally be performed on TMAs also including tissue samples from the stromal compartment. However, it should also be pointed out that the prognostic value of B-cells and plasma cells has been shown to be concordant between studies using whole tissue sections and studies using the TMA technique, including in the herein investigated cohort.[17]

Conclusion

This study provides a first demonstration of the prognostic impact of cytotoxic and regulatory T cells in colorectal cancer according to primary tumour location. Whereas a high density of cytotoxic T cells was an independent prognostic factor in right-sided tumours, but not in left-sided or rectal tumours, regulatory T cells predicted longer survival only in patients with rectal tumours. These findings further underline that tumour location may be an important factor to take into consideration when assessing immune cell density for the purpose of prognostication and possibly also for prediction of response to immunotherapy in patients with colorectal cancer.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

JB performed the immunohistochemical and statistical analyses and drafted the manuscript. MCS helped draft the manuscript. KL contributed with reagents and helped draft the manuscript. BN constructed the TMAs and performed immunohistochemical stainings. PM contributed with analysis tools and assisted with the automated analysis. AHL and JE collected clinical data. KJ conceived of the study, assisted with the statistical analyses and helped draft the manuscript. All authors read and approved the final manuscript.

References

1. Jemal A, Bray F, Center MM, *et al.* Global cancer statistics. *CA Cancer J Clin* 2011;61:69–90.
2. Deschoolmeester V, Baay M, Van Marck E, *et al.* Tumor infiltrating lymphocytes: an intriguing player in the survival of colorectal cancer patients. *BMC Immunol* 2010;11:19.
3. Galon J, Costes A, Sanchez-Cabo F, *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313:1960–4.
4. Naito Y, Saito K, Shiiba K, *et al.* CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998;58:3491–4.
5. Chiba T, Ohtani H, Mizoi T, *et al.* Intraepithelial CD8+ T-cell-count becomes a prognostic factor after a longer follow-up period in human colorectal carcinoma: possible association with suppression of micrometastasis. *Br J Cancer* 2004;91:1711–7.
6. Laghi L, Bianchi P, Miranda E, *et al.* CD3+ cells at the invasive margin of deeply invading (pT3-T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. *Lancet Oncol* 2009;10:877–84.
7. Sinicrope FA, Rego RL, Ansell SM, *et al.* Intraepithelial effector (CD3+)/regulatory (FoxP3+) T-cell ratio predicts a clinical outcome of human colon carcinoma. *Gastroenterology* 2009;137:1270–9.
8. Mei Z, Liu Y, Liu C, *et al.* Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis. *Br J Cancer* 2014;110:1595–605.
9. Ladoire S, Martin F, Ghiringhelli F. Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer. *Cancer Immunol Immunother* 2011;60:909–18.
10. Baxevanis CN, Papamichail M, Perez SA. Immune classification of colorectal cancer patients: impressive but how complete? *Expert Opin Biol Ther* 2013;13:517–26.
11. Curiel TJ, Coukos G, Zou L, *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942–9.
12. Salama P, Phillips M, Grieu F, *et al.* Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009;27:186–92.

13. Hanke T, Melling N, Simon R, *et al.* High intratumoral FOXP3(+) T regulatory cell (Tregs) density is an independent good prognosticator in nodal negative colorectal cancer. *Int J Clin Exp Pathol* 2015;8:8227–35.
14. Frey DM, Droeser RA, Viehl CT, *et al.* High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. *Int J Cancer* 2010;126:2635–43.
15. Nosho K, Baba Y, Tanaka N, *et al.* Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 2010;222:350–66.
16. Minoo P, Zlobec I, Peterson M, *et al.* Characterization of rectal, proximal and distal colon cancers based on clinicopathological, molecular and protein profiles. *Int J Oncol* 2010;37:707–18.
17. Berntsson J, Nodin B, Eberhard J, *et al.* Prognostic impact of tumour-infiltrating B cells and plasma cells in colorectal cancer. *Int J Cancer* 2016;139:1129–39.
18. Berglund G, Elmstahl S, Janzon L, *et al.* The Malmo Diet and Cancer Study. Design and feasibility. *J Intern Med* 1993;233:45–51.
19. Wangefjord S, Brandstedt J, Lindquist KE, *et al.* Associations of beta-catenin alterations and MSI screening status with expression of key cell cycle regulating proteins and survival from colorectal cancer. *Diagn Pathol* 2013;8:10.
20. Wangefjord S, Sundstrom M, Zendehrokh N, *et al.* Sex differences in the prognostic significance of KRAS codons 12 and 13, and BRAF mutations in colorectal cancer: a cohort study. *Biol Sex differ* 2013;4:17.
21. Kononen J, Bubendorf L, Kallioniemi A, *et al.* Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998;4:844–7.
22. Dahlin AM, Henriksson ML, Van Guelpen B, *et al.* Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. *Mod Pathol* 2011;24:671–82.
23. Ogino S, Nosho K, Irahara N, *et al.* Lymphocytic reaction to colorectal cancer is associated with longer survival, independent of lymph node count, microsatellite instability, and CpG island methylator phenotype. *Clin Cancer Res* 2009;15:6412–20.
24. Pernot S, Terme M, Voron T, *et al.* Colorectal cancer and immunity: what we know and perspectives. *World J Gastroenterol* 2014;20:3738–50.
25. Fridman WH, Pages F, Sautes-Fridman C, *et al.* The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012;12:298–306.
26. Stewart CA, Metheny H, Iida N, *et al.* Interferon-dependent IL-10 production by Tregs limits tumor Th17 inflammation. *J Clin Invest* 2013;123:4859–74.
27. Rech AJ, Vonderheide RH. Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells. *Ann N Y Acad Sci* 2009;1174:99–106.
28. Kurose K, Ohue Y, Wada H, *et al.* Phase Ia study of FoxP3+ CD4 Treg depletion by infusion of a humanized anti-CCR4 antibody, KW-0761, in cancer patients. *Clin Cancer Res* 2015;21:4327–36.
29. Benedix F, Kube R, Meyer F, *et al.* Comparison of 17,641 patients with right- and left-sided colon cancer: differences in epidemiology, perioperative course, histology, and survival. *Dis Colon Rectum* 2010;53:57–64.
30. Missiaglia E, Jacobs B, D'ario G, *et al.* Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features. *Ann Oncol* 2014;25:1995–2001.
31. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008;135:1079–99.
32. Holch JW, Ricard I, Stintzing S, *et al.* The relevance of primary tumour location in patients with metastatic colorectal cancer: a meta-analysis of first-line clinical trials. *Eur J Cancer* 2017;70:87–98.
33. Dolcetti R, Viel A, Doglioni C, *et al.* High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 1999;154:1805–13.
34. Mlecnik B, Bindea G, Angell HK, *et al.* Integrative analyses of colorectal cancer show immunoscore is a stronger predictor of patient survival than microsatellite instability. *Immunity* 2016;44:698–711.
35. Yamauchi M, Morikawa T, Kuchiba A, *et al.* Assessment of colorectal cancer molecular features along bowel subsites challenges the conception of distinct dichotomy of proximal versus distal colorectum. *Gut* 2012;61:847–54.
36. Schraml P, Kononen J, Bubendorf L, *et al.* Tissue microarrays for gene amplification surveys in many different tumor types. *Clin Cancer Res* 1999;5:1966–75.