The intratumoural subsite and relation of CD8+ and FOXP3+ T lymphocytes in colorectal cancer provide important prognostic clues

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Background: To find improved tools for prognostic evaluation in patients with colorectal cancer (CRC), we have analysed how infiltration of cytotoxic T lymphocytes (CD8+) and regulatory T lymphocytes (FoxP3+) correlates to prognosis, not only according to quantity and relation, but also to subsite within tumours of different molecular characteristics (microsatellite instability and CpG island methylator phenotype status).

Methods: CD8 and FOXP3 expression was evaluated by immunohistochemistry in 426 archival tumour tissue samples from patients surgically resected for CRC. The average infiltration of CD8+ and FOXP3+ cells was assessed along the tumour invasive front, in the tumour centre and within the tumour epithelium (intraepithelial).

Results: We found that infiltration of CD8+ T lymphocytes within the tumour epithelium provided the strongest prognostic information (P<0.001). At the tumour invasive front and tumour centre, FOXP3 expression withheld the strongest association to prognosis (P<0.001), suggesting FOXP3+ T-lymphocyte infiltration to be a better prognostic tool than CD8+ T lymphocytes at these intratumoural subsites. We further analysed the possible prognostic impact of the relation between these T-cell subsets, finding that a high intraepithelial CD8 expression was associated with a better patient outcome, independent of FOXP3 infiltration. In groups of low intraepithelial CD8 expression, however, a high infiltration rate of FOXP3+ cells at the tumour invasive front, significantly improved prognosis.

Conclusions: Analyses of intraepithelial infiltration of CD8+ T lymphocytes, infiltration of FOXP3+ T lymphocytes at the tumour front or centre, and the relation between these subsets, may be a valuable tool for predicting prognosis in colon cancer.

Colorectal cancer (CRC) is one of the four most common types of cancer in the western world (Jemal *et al*, 2011). Overall 5-year survival rates are reported to be between 50 and 60%, corresponding closely to disease progression. The success scores for accurate prediction of patient prognosis remain discouraging (Mlecnik *et al*, 2011). One variable that has been shown critical to prognosis in CRC is the state of the tumour-associated immune response (Hanahan and Weinberg, 2011), where a high number of tumour-infiltrating lymphocytes (TILs) has been associated with

a better prognosis (Maccarty, 1922; Svennevig *et al*, 1984; Ropponen *et al*, 1997). The interactions between the local immune infiltration and tumour cells are complex and describe a balance between tumour-controlling and tumour-promoting effects (Deschoolmeester *et al*, 2011). Also, an increased number of TILs can be counteracted by immune deviation and/or lymphatic inactivation. The immune infiltrates are found to be heterogeneous between tumour types and patients (Fridman *et al*, 2012) and their effect on prognosis varies in different cancers.

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The prognostic importance regarding intratumoural subsite, T-cell subsets and infiltration rate has been investigated in earlier studies (Naito *et al*, 1998; Galon *et al*, 2006), but questions still remain as to which variables bear the strongest prognostic information (Ogino *et al*, 2011; Fridman *et al*, 2012). The introduction of immune scoring into clinical practice of CRC classification is under progress, work initiated by Galon *et al* (2012), striving to find a standardised methodology. In the light of this it is of utmost importance to find the most informative prognostic factor/factors regarding immune cell infiltration in CRC.

Cytotoxic T lymphocytes (CTLs; CD8^{+}) are established as important players in anti-tumour immunity in CRC (Naito *et al*, 1998; Deschoolmeester *et al*, 2011). Cytotoxic T lymphocytes have the ability to kill target cells upon being exposed to a tumour cell antigen/HLA1 complex for which their T-cell receptor is specific (Deschoolmeester *et al*, 2011). Regulatory T lymphocytes (Tregs; FOXP3^{+}) can suppress the activity of CTLs and have therefore been thought to down-modulate tumour-specific immunity. In some studies, Tregs have been shown to correlate to reduced activation of conventional T cells (Svensson *et al*, 2012), but the data are conflicting. In line with their suggested suppressive functions, Tregs have been associated with adverse outcomes in ovarian, breast and hepatocellular carcinomas but were not found to be significantly prognostic in others, like oesophageal carcinomas (Ladoire *et al*, 2011a; Deschoolmeester *et al*, 2011). A number of studies have even found FOXP3^{+} T lymphocytes to be associated with a favourable prognosis in some cancers, notably CRC (Ladoire *et al*, 2011b).

Host cells and cancer cells interact constantly with each other in the tumour microenvironment so to understand the immunologic milieu of the cancer, both host and tumour factors need to be considered (Ogino *et al*, 2011). Three major pathways of CRC tumorigenesis have been well described (Jass, 2007), the microsatellite stable (MSS), the microsatellite instability (MSI) (Boland *et al*, 1998; Pino and Chung, 2010) and, more recently, the CpG island methylator phenotype (CIMP) pathways. Immune infiltration has been shown to vary according to MSI status, with MSI tumours more often being highly infiltrated by immune cells, which might explain the better prognosis in these patients (Popat *et al*, 2005; Deschoolmeester *et al*, 2011). However, most studies on the prognostic impact of immune infiltrates have generally not accounted for the molecular status of the tumour.

We have previously studied the relation between lymphocytic infiltration (CD3^{+}) and molecular characteristics (i.e., MSI screening status and CIMP status) in a cohort of CRC patients (Dahlin *et al*, 2011). In the present study, tumour progression in this CRC cohort is related to, not only quantity and the relationship between subsets of T lymphocytes (CD8^{+} and FOXP3^{+}), but also to their subsite within the tumour, to find out where the most important prognostic information of these lymphocyte markers can be found and how they best could be implemented in a future clinical setting. In addition, we compare the prognostic importance of CD8^{+} and FOXP3^{+} T lymphocytes in CRC of different MSI screening and CIMP status.

MATERIALS AND METHODS

Study population. The tumour tissue used in this study comes from The Colorectal Cancer in Umeå study (CRUMS) (Dahlin *et al*, 2010). Clinical specimens from patients surgically resected for CRC were collected between 1995 and 2003 at the department of Surgery, Umeå University Hospital, Umeå, Sweden. An informed consent was retrieved from the patients and the handling of patient data as well as tissue samples was approved by the research ethical committee at Umeå University Hospital (Regional Ethical Review

Board in Umeå, Sweden). Formalin-fixed paraffin-embedded tissue was sampled from all patients and pathological variables were characterised by one pathologist by reviewing routinely stained sections. By reviewing the patient records and the Swedish population registry during autumn 2012, clinical data, including survival data, were obtained by a surgeon. The median follow-up time for patients still alive at the end of follow-up was 113 months. Exclusion criteria included unavailable or insufficient tumour sample, or lack of clinical information. A total of 426 patients (268 colon cancers, 155 rectal cancers, and 3 not specified site within the colorectum) were included in the study. Because of missing data of either CD8 or FOXP3 expression, 409 specimen for CD8 and 412 specimens for FOXP3 were available for analysis. Preoperative radiation therapy was administered to 90 (58%) rectal cancer patients of whom 70 received 5×5 Gy, and 20 received 25×2 Gy. For survival analyses, 31 patients were excluded due to incomplete follow-up data or due to death by perioperative complications (death within 30 days of and due to operation).

Immunohistochemistry. According to routine procedures, CRC specimens obtained after primary tumour resection were fixed in 4% formaldehyde and embedded in paraffin. From each patient, one 4-μm section was cut, dried, de-waxed and rehydrated. For immunohistochemical procedures, a staining machine (Ventana BenchMark Ultra, Ventana Medical Systems, Inc., Tucson, AZ, USA) was used with the CC1 standard pretreatment and the iVIEW DAB detection kit (Ventana Medical Systems, Inc.) for visualization. Anti-CD8 polyclonal antibody (Clone C8/144B: Dako, Stockholm, Sweden) was used at a dilution of $1:50$, and anti-FOXP3 monoclonal antibody (Clone 236A/E7: AbCam, Cambridge, UK) was used at a dilution of $1:100$. The slides were counterstained with haematoxylin.

Immunohistochemical staining was evaluated under light microscope as most representative area at different subsites: the invasive tumour front, tumour centre and within the tumour epithelium (intraepithelial expression), as illustrated in Figure 1. In more detail, tumour front was identified as the stromal area along the invasive margin defined by a depth of two high-power fields ($\times 40$ objective magnification) underneath the invasive margin. Tumour centre was identified as the stromal area within the
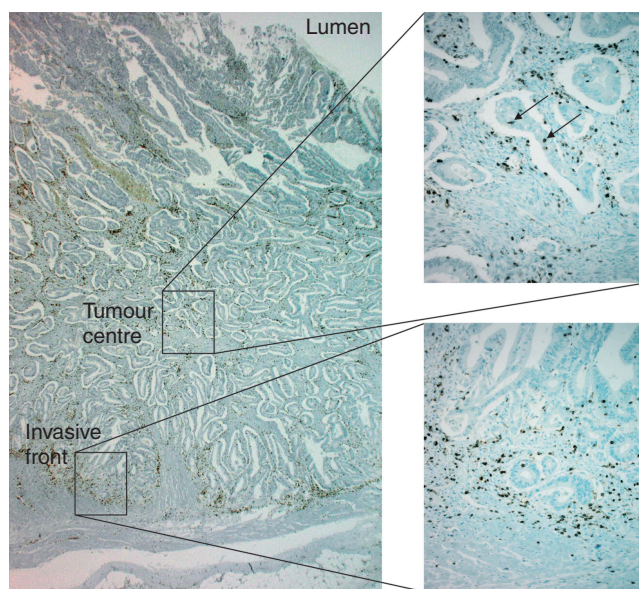

Figure 1. Evaluation of immune marker expression at intratumoural subsites of colon carcinoma. Expression of CD8^{+} cells determined by immunohistochemistry at the invasive front, tumour centre and within the tumour epithelium (intraepithelial expression, indicated by arrows).

tumour mass between, and clearly separated from, the luminal border and the invasive front. Intraepithelial lymphocytes were defined as lymphocytes located within tumour cell nests. Areas of necrosis were avoided. They were assessed as no/sporadic (score 1), moderate (score 2), abundant (score 3) and highly abundant infiltration (score 4) according to Dahlin *et al* (2011). The specimens were evaluated two times by the same observer (kappa-values ranging from 0.75–0.90) and discordant cases were reviewed a third time, followed by a conclusive judgment. A subset of 50 consecutively selected tumours was re-examined by a second observer to assess interobserver agreement (kappa-values 0.66–0.87). As FOXP3 was not or only sporadically expressed in the tumour epithelium, all tumours got score 1 for that marker, in the intraepithelial subsite. A total score was also obtained for each tumour, adding together the scores from each subsite. Total score was then divided into three groups (according to S Ogino and A Dahlin (Ogino *et al*, 2009; Dahlin *et al*, 2011)): 3–4 (low), 5–6 (moderate) and 7–12 (abundant) expression.

MSI screening status and CIMP status. MSI screening status of this cohort was determined by immunohistochemistry and has been previously published (Dahlin *et al*, 2010). A positive MSI screening status (MSI) was assigned to tissue samples with tumour cells lacking nuclear staining for one or more of the proteins MLH1, MSH2, MSH6 or PMS2, this in contrast to a negative screening status (MSS), where positive tumour nuclei were present expressing all four markers. CpG island methylator phenotype status has previously been assessed in this cohort and published (Dahlin *et al*, 2010). In brief, CIMP status was determined according to hypermethylation of an eight-gene panel (*CDKN2A, MLH1, CACNA1G, NEUROG1, RUNX3, SOCS1, IGF2* and *CRABP1*) (Toyota *et al*, 1999) by the MethyLight method (quantitative real-time PCR) with previously described primer and probe sequences (Weisenberger *et al*, 2006; Dahlin *et al*, 2010). The following number of hypermethylated genes defined CIMP-negative tumours, 0 genes; CIMP-low tumours, 1–5 genes; and CIMP high tumours, 6–8 genes.

Statistical analysis. Statistical analyses were performed using PASW Statistics 20 (SPSS Inc., Chicago, IL, USA). Cross-tabulations were analysed with χ^2 tests, or Fischer's exact test when expected frequencies were less than five for categorical variables and linear relationship with the exact linear-by-linear association test. Kaplan–Meier survival analysis was used to estimate cancer-specific survival, and comparisons between groups were performed with the log-rank test. The definition of cancer-specific survival was death with known disseminated or recurrent disease. Multivariate survival analyses were performed by using Cox proportional hazard models. $P < 0.05$ was considered statistically significant.

RESULTS

Infiltration of CD8$^+$ and FOXP3$^+$ cells at the invasive tumour front, tumour centre and within the tumour epithelium of CRC. The presence of CTL and Treg subsets in CRC was determined by immunohistochemical evaluation of CD8 and FOXP3 expression, respectively. CD8$^+$ and FOXP3$^+$ cells were semi-quantitatively evaluated in specimens from 426 CRC patients according to a previously documented four-graded scale (Dahlin *et al*, 2011). The T-lymphocyte markers were assessed at three different subsites: invasive tumour front, tumour centre and within the tumour epithelium (intraepithelial expression) (Figure 1). Approximately 85% of all tumours displayed a modest to massive infiltration of CD8$^+$ and FOXP3$^+$ cells (total score 5–12), whereas the remaining showed weak or no infiltration (total score 3–4). The majority of CD8$^+$ and FOXP3$^+$ cells were found at tumour

stromal sites (invasive tumour front and tumour centre), with the highest density along the invasive tumour front. CD8 expression was detected intraepithelially in 100% of the cases. FOXP3 expression was not, or just sporadically, present within the tumour epithelium.

The frequencies of infiltrating CD8$^+$ and FOXP3$^+$ cells using total score as a representative of all subsites, are presented in a cross-tabulation in Table 1A. Infiltrating cells expressing CD8 or FOXP3 were highly and positively correlated ($P < 0.001$). The amount of CD8$^+$ cells, however, was frequently higher than that of FOXP3$^+$ cells.

Associations between infiltrating CD8$^+$ and FOXP3$^+$ cells and clinicopathological parameters. The scores of infiltrating CD8$^+$ and FOXP3$^+$ cells in CRC specimens were correlated to clinicopathological variables (Table 1B). As no great difference could be seen when relating these parameters to lymphocyte infiltration at different sites compared with infiltration as a total, we chose to present the results for total score (clinicopathological characteristics in relation to CD8 intraepithelial expression and FOXP3 expression at the tumour front can be found in Supplementary Information Table S1). CD8 expression was significantly associated with age ($P = 0.001$), localisation ($P = 0.001$) and growth pattern ($P = 0.003$). CD8 expression further showed a strong association with preoperative radiation therapy ($P < 0.001$), where radiated tumours more often were less infiltrated. An inverse association with tumour stage was found for both CD8$^+$ ($P = 0.030$) and FOXP3$^+$ ($P < 0.001$) infiltration.

Prognostic importance of infiltrating CD8$^+$ and FOXP3$^+$ cells in colon cancer. We compared overall cancer-specific survival in patients with different scores of infiltrating CD8$^+$ or FOXP3$^+$ cells. As several of the patients in the rectal cancer group had received preoperative radiation therapy, which affects the infiltration rate significantly for CD8$^+$ cells (Table 1B), we separated this group from the colon cancer patients. Figure 2 shows Kaplan–Meier plots of cancer-specific survival in colon cancer patients with different levels of infiltrating CD8$^+$ and FOXP3$^+$ cells. An increased infiltration of CD8$^+$ cells (total score) was highly significantly associated with an improved prognosis (Log-rank $P = 0.008$) (Figure 2A). A similar, but even stronger association was seen for FOXP3$^+$ cells (Log-rank $P < 0.001$) (Figure 2A). When analysed as continuous variables in a multivariable model adjusting for stage, age, sex and localisation (proximal *vs* distal), the prognostic significance of CD8 total score was lost (HR 0.82; 95% CI 0.60–1.13; $P = 0.220$), whereas for FOXP3 total score remained significant (HR 0.68; 95% CI 0.51–0.90; $P = 0.008$).

When comparing infiltration rate and overall cancer-specific survival according to intratumoural subsite, the most prominent association for CD8$^+$ cells was seen for a high intraepithelial infiltration (Log-rank $P < 0.001$) (Figure 2B). CD8 infiltration at the invasive tumour front or tumour centre showed weaker associations to prognosis (Log-rank $P = 0.015$ and 0.021, respectively). When analysed as a continuous variable in the multivariable model, the risk estimate decreased significantly with

Table 1A. Cross-tabulation between total score for CD8 and FOXP3 expression in CRC

CD8	FOXP3			P-value
	3–4	5–6	7–12	
3–4	17 (34.7)	28 (57.1)	4 (8.2)	<0.001[a]
5–6	25 (20.7)	69 (57.0)	27 (22.3)	
7–12	27 (12.6)	98 (45.6)	90 (41.9)	

[a]Exact linear-by-linear association test.

Table 1B. Total score for CD8 and FOXP3 expression in relation to clinicopathologic characteristics in CRC

	CD8				FOXP3			
	3–4	5–6	7–12	P-value	3–4	5–6	7–12	P-value
Frequency (%)	55 (13.7)	128 (31.8)	219 (54.5)		79 (19.5)	201 (49.6)	125 (30.9)	
Gender, n (%)				0.781				0.178
Male	28 (12.6)	71 (32.0)	123 (55.4)		42 (18.7)	105 (46.7)	78 (34.7)	
Female	27 (15.0)	57 (31.7)	96 (53.3)		37 (20.6)	96 (53.3)	47 (26.1)	
Age, n (%)				0.001/0.086[a]				0.147/0.446[a]
⩽59	6 (8.3)	22 (30.6)	44 (61.1)		16 (22.2)	30 (41.7)	26 (36.1)	
60–69	23 (21.9)	38 (36.2)	44 (41.9)		26 (25.2)	45 (43.7)	32 (31.1)	
70–79	19 (14.0)	50 (36.8)	67 (49.3)		27 (19.1)	76 (53.9)	38 (27.0)	
⩾80	7 (7.9)	18 (20.2)	64 (71.9)		10 (11.2)	50 (56.2)	29 (32.6)	
Localisation, n (%)				0.001/0.003[a]				0.356/0.857[a]
Right colon	15 (11.6)	28 (29.5)	76 (58.9)		26 (20.0)	59 (45.4)	45 (34.6)	
Left colon	7 (5.7)	38 (31.1)	77 (63.1)		27 (21.6)	68 (54.4)	30 (24.0)	
Rectum	32 (21.6)	50 (33.8)	66 (44.6)		26 (17.7)	72 (49.0)	49 (33.3)	
Stage, n (%)				0.030/0.047[a]				<0.001/<0.001[a]
I	8 (12.7)	20 (31.7)	35 (55.6)		4 (6.3)	23 (36.5)	36 (57.1)	
II	16 (10.5)	42 (27.6)	94 (61.8)		25 (15.9)	78 (49.7)	54 (34.4)	
III	17 (20.0)	23 (27.1)	45 (52.9)		15 (18.1)	50 (60.2)	18 (21.7)	
IV	12 (12.8)	42 (44.7)	40 (42.6)		35 (37.2)	44 (46.8)	15 (16.0)	
Grade, n (%)				0.077				0.321/0.193[a]
Low	21 (10.3)	63 (31.0)	119 (58.6)		38 (18.7)	96 (47.3)	69 (34.0)	
High	33 (17.1)	65 (33.7)	95 (49.2)		41 (20.9)	102 (52.0)	53 (27.0)	
Growth pattern, n (%)				0.003				0.866
Pushing	16 (12.3)	28 (21.5)	86 (66.2)		26 (19.7)	63 (47.7)	43 (32.6)	
Infiltrating	37 (14.0)	98 (37.0)	130 (49.1)		52 (19.4)	135 (50.4)	81 (30.2)	
Histology type, n (%)				0.231				0.260
Mucinous	5 (9.3)	22 (40.7)	27 (50.0)		15 (27.3)	25 (45.5)	15 (27.3)	
Non-mucinous	50 (14.5)	103 (29.9)	191 (55.5)		62 (17.9)	174 (50.3)	110 (31.8)	
Preoperative radiation therapy[b], n (%)				<0.001				0.923
No	5 (7.7)	18 (27.7)	42 (64.6)		11 (17.5)	32 (50.8)	20 (31.7)	
Yes	27 (32.5)	32 (38.6)	24 (28.9)		15 (17.9)	40 (47.9)	29 (34.5)	

Unless otherwise indicated, χ^2 test was used for categorical variables.

[a] Exact linear-by-linear association test was used to test for linear relationship between variables.

[b] Preoperative radiation therapy in rectal cancers only.

increasing intraepithelial infiltration of CD8^{+} cells (HR 0.71; 95% CI 0.55–0.92; $P = 0.010$). Significance for a high CD8 infiltration at the tumour invasive front and centre was lost in multivariate models. This further supports the importance of intratumoural subsite for evaluation of the prognostic impact of CD8^{+} infiltration. Such discrepancy between sites could not be seen, when comparing infiltration of FOXP3^{+} cells in the front to that in the centre. An increased infiltration of FOXP3^{+} cells in both front and centre was highly and significantly associated with an improved prognosis (Log-rank $P < 0.001$) (Figure 2B). The prognostic importance of infiltrating FOXP3^{+} cells, according to intratumoural subsite retained significant in the multivariate analysis (FOXP3 at the tumour front; HR 0.73; 95% CI 0.55–0.96; $P = 0.022$; and tumour centre; HR 0.62; 95% CI 0.49–0.80; $P < 0.001$). This suggests that FOXP3 is a stronger indicator of prognosis than CD8 at these subsites.

Prognostic impact of CD8^{+} cells in relation to FOXP3^{+} cells in colon cancer. In order to find out whether the proportion of infiltrating CTLs in relation to that of the Tregs had any impact on prognosis in our colon cases, we compared overall cancer-specific survival in patients with different CD8 to FOXP3 density ratios.

We related immune cell density to prognosis both according to total score and intratumoural subsite. We restricted the analyses to comparison of CD8 intraepithelial expression (due to its most pronounced prognostic effect). FOXP3 infiltration showed a significant correlation to prognosis both in the centre and the front, but we chose to henceforth look at the front as regulatory T cells are generally distantly acting. When comparing total score, there was no association between the amounts of CD8^{+} and FOXP3^{+} cells and survival (Log-rank $P = 0.707$) (Figure 3A). Interestingly, when we instead compared intratumoural subsites, we saw that a higher intraepithelial CD8 to FOXP3 front ratio had the better prognostic association (Log rank $P = 0.039$) (Figure 3B), which further strengthens the importance of intratumoural subsite assessment. We also compared the prognostic value of different groups of CD8 or FOXP3 low (scores 1 and 2) or high (scores 3 and 4) expression according to subsites. The best prognosis was found in patients with a high density of intraepithelial CD8^{+} cells, irrespective of FOXP3 infiltration. FOXP3 density at the tumour invasive front was on the other hand of prognostic significance in cases with a low number of intraepithelial CD8^{+} T cells (Log-rank $P = 0.017$) (Figure 3C), suggesting that FOXP3 by itself holds important prognostic information. The poorest prognosis was
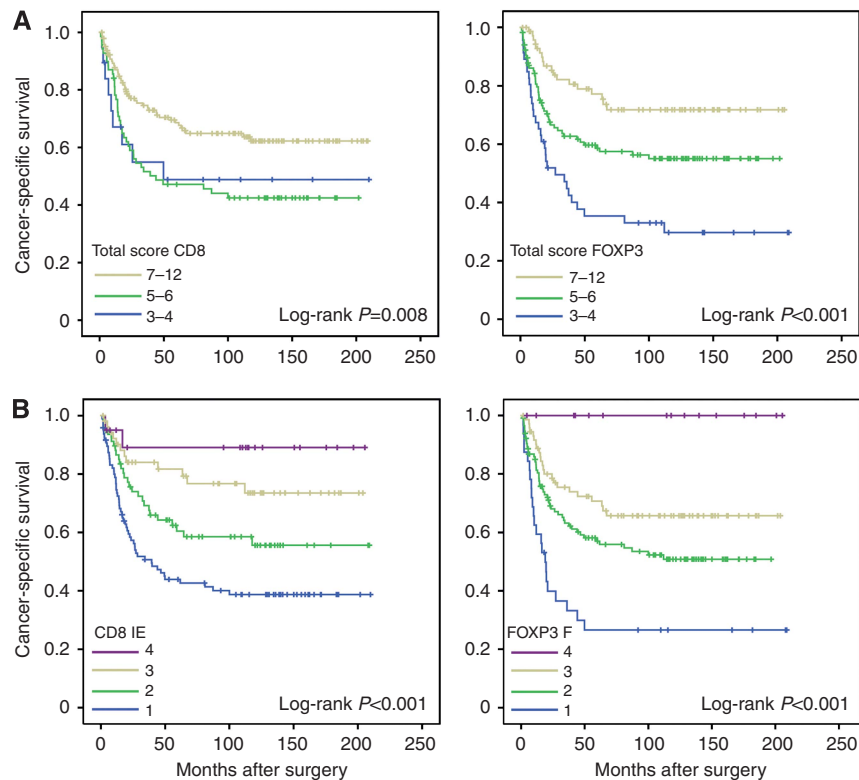
Figure 2. Cancer-specific survival in colon cancers. Cases scored (**A**) for expression of CD8 and FOXP3 total score: score 3–4 (low), 5–6 (moderate) or 7–12 (abundant) expression; or (**B**) at intratumoural subsites, CD8 IE (intraepithelial) and FOXP3 F (front), score 1–4: score 1 (no/sporadic), score 2 (moderate), score 3 (abundant) and score 4 (highly abundant). Shown are Kaplan–Meier plots. Log-rank tests were used to calculate P values.

found in cases with a low infiltration rate of both CD8$^+$ and FOXP3$^+$ cells.

Prognostic importance of infiltrating CD8$^+$ and FOXP3$^+$ cells in rectal cancer. No significant association was seen between infiltrating CD8$^+$ or FOXP3$^+$ cells (total score) and overall survival, in rectal cancer patients (Log-rank $P = 0.338$ and 0.106, respectively) (Supplementary Information Figure S1a). A significant correlation of CD8 and FOXP3 expression to prognosis was however seen when comparing sites. A high intraepithelial infiltration rate of CD8$^+$ cells was associated with an improved prognosis (Log-rank $P = 0.034$) as was a high infiltration rate of FOXP3$^+$ cells at the tumour front (Log-rank $P = 0.008$) (Supplementary Information Figure S1b).

Association between infiltrating CD8$^+$ and FOXP3$^+$ cells and molecular parameters. When relating infiltrating CD8$^+$ or FOXP3$^+$ cells to molecular parameters (Table 2), CD8 expression presented as total score was not found to be significantly associated with MSI screening status (Log-rank $P = 0.128$). However, significances were found at the different subsites, with the highest significance found for MSI tumours more often being highly infiltrated by intraepithelial CD8$^+$ T lymphocytes (Log-rank $P < 0.001$). FOXP3 expression (total score) was significantly associated with MSI screening status (Log-rank $P = 0.035$), whereas no association was found at the different subsites. Furthermore, no correlation of CD8$^+$ or FOXP3$^+$ infiltration was found to CIMP status.

Prognostic importance of infiltrating CD8$^+$ and FoxP3$^+$ cells according to MSI screening status and CIMP status. To further analyse the prognostic value of infiltrating T-lymphocyte subsets, we compared overall cancer-specific survival within different subgroups of colon cancer defined by MSI screening status and

CIMP status. We restricted the analyses to comparison of CD8 intraepithelial expression and FOXP3 expression at the tumour invasive front.

CD8$^+$ intraepithelial infiltration was not found to be a prognostic factor in MSI cases (Log-rank $P = 0.832$), however, it was so in MSS cases (Log-rank $P < 0.001$) (Figure 4A and B). FOXP3$^+$ infiltration at the tumour front was significant for prognosis in subgroups of both MSI (Log-rank $P < 0.001$) and MSS (Log-rank $P < 0.001$) (Figure 4A and B), where MSI or MSS cases with low infiltration of FOXP3$^+$ cells displayed the worst prognosis.

A high amount of CD8$^+$ and FOXP3$^+$ cells was found to be associated with a better prognosis in subgroups of colon cancer arranged by CIMP status, particularly in groups of CIMP-negative and CIMP-low cases (Supplementary Information Figure S2).

In a multivariate model adjusting for MSI and CIMP status, the prognostic importance of a high infiltration rate of intraepithelial CD8$^+$ cells and FOXP3$^+$ cells at the tumour invasive front respectively, remained significant and hazard ratio was mainly unaffected. This suggests that T-cell infiltration is independent of these molecular characteristics.

DISCUSSION

Tumours of CRC are generally immunogenic and often infiltrated by T lymphocytes (Sherwood et al, 2013). An increased amount of T lymphocytes infiltrating the tumour in CRC has repeatedly been proven to be associated with a better prognosis (Galon et al, 2006; Dahlin et al, 2011; Roxburgh and McMillan, 2012). Additional information regarding different intratumoural subsites, T-lymphocyte subsets and how infiltration correlates to prognosis in different
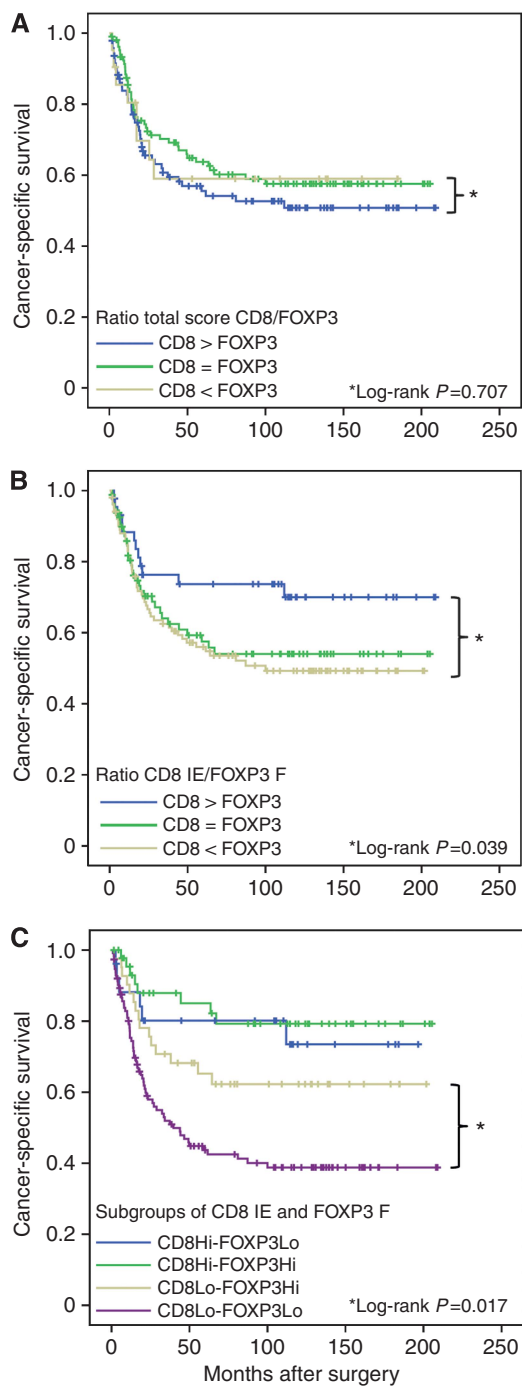
Figure 3. Cancer-specific survival of colon cancers. Cases scored for the quotient of (**A**) the total score: score 3–4 (low), 5–6 (moderate) or 7–12 (abundant) of CD8/FOXP3 expression; or (**B**) CD8 IE/FOXP3 F, score 1–4: score 1 (no/sporadic), score 2 (moderate), score 3 (abundant) and score 4 (highly abundant). (**C**) Cancer-specific survival of cases scored for subgroups of CD8Lo, score 1–2; CD8Hi, score 3–4; FOXP3Lo, score 1–2; and FOXP3Hi, score 3–4. Shown are Kaplan–Meier plots. Log-rank tests were used to calculate P values.

molecular subgroups of CRC, could further illuminate its importance both regarding prognosis and prediction of response to different treatment therapies (Ogino *et al*, 2011; Fridman *et al*, 2012). Studies on different subsets of T lymphocytes have shown that a high infiltration rate of one subset often associates strongly to a high infiltration of another T-cell subset, the correlation to prognosis equally convincing (Frey *et al*, 2010; deLeeuw *et al*, 2012).

In this study, we analyse the T-cell subsets likely to bear the most important effector functions in tumour control, that is the cytotoxic T cells and their counterpart, the regulatory T cells. We combine analyses of density, relation, different intratumoural subsites and tumour molecular characteristics to try to find out how these prognostic tools best could be implemented in the clinical setting.

By using an immunohistochemical approach we evaluated the degree of infiltrating CD8^{+} and FOXP3^{+} cell subsets in 426 archival tumour tissue samples from patients surgically resected for CRC. Colon carcinomas were separated from rectal cancers due to the reducing effect that radiation therapy given to many patients in the latter group, has on T-cell infiltration. In univariate analyses we observed significant associations of a high amount of infiltrating CD8^{+} cells with improved survival in patients with colon cancer. In multivariate analysis, adjusting for stage, age, sex and localisation (proximal *vs* distal), the prognostic effect of intraepithelial infiltration of CD8 cells remained significant whereas the significance for CD8 total score, tumour invasive front and centre was lost.

No prognostic discrepancy between intratumoural subsites could be seen when comparing infiltration of FOXP3^{+} cells in the tumour invasive front to that in the centre, a high infiltration rate in both subsites being significantly associated with a better prognosis. The prognostic effect of FOXP3^{+} cell infiltration for total score, at the tumour invasive front and in the centre remained significant in multivariate analysis, suggesting that at these intratumoural subsites, evaluation of FOXP3 is the better prognostic indicator. When comparing a possible effect of different relations between CD8^{+} and FOXP3^{+} cells on prognosis, we found that having a high infiltration rate of intraepithelial CD8^{+} cells was associated with a good overall survival, regardless of FOXP3^{+} density at the tumour front. However, in colon cancers with low infiltration of intraepithelial CD8^{+} cells, a better overall survival was found in patients with a high infiltration of FOXP3^{+} cells compared with those having a low infiltration rate. These results are in line with a study by Yoon *et al* (2012) who also observed FOXP3^{+} cells to be a prognostic indicator in patients with a low infiltration of CD8 positive lymphocytes, in CRC.

The biological explanation to why intraepithelial CTLs would have a greater impact on prognosis than the ones located in the front or centre is likely due to their direct anti-tumour effects. The CTLs infiltrating the tumour epithelium, being in the immediate proximity, are most likely specifically directed against the tumour, and could therefore be considered more effective. Earlier work by Pages *et al* (2005) has shown a correlation between high tumour immune cell infiltration and absence of perineural invasion and lymphatic emboli. The hypothesis that the sublocalisation of CTLs bears prognostic importance has gained momentum from studies by, for example, Naito *et al* (1998) and Galon *et al* (2006). An intra-epithelial location of CTLs could possibly mean a more effective protection in that aspect. Tregs have been known to counteract the anti-tumour immune response in some cancers (Deschoolmeester *et al*, 2011; Mathai *et al*, 2012), but its role cannot be assessed as unambiguous as we still cannot see the whole picture. In this study, a high infiltration of FOXP3^{+} cells seems to be a protective factor, associated with a very good patient outcome, whereas the reverse can be said for patients with a very low infiltration, results that are consistent with the works of Salama *et al* (2009), Ladoire *et al* (2011b) and Frey *et al* (2010). This raises the question of whether there is something that singles out the FOXP3^{+} cells in the colon from their non-colon counterparts. In a study by Martin *et al* (2010), it was shown that some tumour-infiltrating FOXP3^{+} cells are not Tregs but conventional T cells transiently expressing FOXP3 upon TCR activation. In another study, by Miyara *et al* (2009), they identified subgroups of FOXP3^{+} cells secreting pro-inflammatory cytokines IL-2 and IFN-γ.

Table 2. CD8 and FOXP3 total score and expression at intratumoural subsites in relation to molecular characteristics in colon cancers

	CD8					FOXP3				
Total score	**3–4**	**5–6**	**7–12**		*P*-value	**3–4**	**5–6**	**7–12**		*P*-value
MSI screening status[a], n (%)					0.128					0.035
MSI	3 (6.1)	10 (20.4)	36 (73.5)			14 (26.9)	18 (34.6)	20 (38.5)		
MSS	19 (9.7)	64 (32.7)	113 (57.7)			37 (18.8)	108 (54.8)	52 (26.4)		
CIMP status[b], n (%)					0.983					0.927
CIMP-negative	10 (9.7)	30 (29.1)	63 (61.2)			19 (18.4)	54 (52.4)	30 (29.1)		
CIMP-low	9 (8.7)	32 (30.8)	63 (60.6)			24 (22.9)	51 (48.6)	30 (28.6)		
CIMP-high	3 (6.8)	14 (31.8)	27 (61.4)			10 (21.3)	22 (46.8)	15 (31.9)		
Front	**1**	**2**	**3**	**4**		**1**	**2**	**3**	**4**	
MSI screening status[a], n (%)					0.004					0.201
MSI	1 (2.0)	5 (10.2)	21 (42.9)	22 (44.9)		9 (17.3)	21 (40.4)	16 (30.8)	6 (11.5)	
MSS	13 (6.6)	57 (29.1)	79 (40.3)	47 (24.0)		29 (14.7)	102 (51.8)	57 (28.9)	9 (4.6)	
CIMP status[b], n (%)					0.244					0.849
CIMP-negative	9 (8.7)	26 (25.2)	39 (37.9)	29 (28.2)		13 (12.6)	55 (53.4)	29 (28.2)	6 (5.8)	
CIMP-low	4 (3.8)	27 (26.0)	48 (46.2)	25 (24.0)		19 (18.1)	48 (45.7)	33 (31.4)	5 (4.8)	
CIMP-high	1 (2.3)	10 (22.7)	15 (34.1)	18 (40.9)		7 (14.9)	22 (46.8)	14 (29.8)	4 (8.5)	
Centre										
MSI screening status[a], n (%)					0.023					0.175
MSI	3 (6.0)	14 (28.0)	21 (42.0)	12 (24.0)		13 (24.5)	19 (35.8)	17 (32.1)	4 (7.5)	
MSS	16 (8.0)	82 (40.8)	85 (42.3)	18 (9.0)		27 (13.4)	98 (48.8)	64 (31.8)	12 (6.0)	
CIMP status[b], n (%)					0.336					0.422
CIMP-negative	6 (5.6)	42 (39.3)	50 (46.7)	9 (8.4)		13 (12.4)	53 (50.5)	29 (27.6)	10 (9.5)	
CIMP-low	10 (9.5)	38 (36.2)	44 (41.9)	13 (12.4)		20 (18.5)	45 (41.7)	38 (35.2)	5 (4.6)	
CIMP-high	3 (6.7)	19 (42.2)	14 (31.1)	9 (20.0)		9 (19.1)	20 (42.6)	16 (34.0)	2 (4.3)	
Intraepithelial										
MSI screening status[a], n (%)					<0.001					
MSI	14 (28.0)	11 (22.0)	14 (28.0)	11 (22.0)						
MSS	93 (46.3)	59 (29.4)	39 (19.4)	10 (5.0)						
CIMP status[b], n (%)					0.553					
CIMP-negative	44 (41.1)	32 (29.9)	24 (22.4)	7 (6.5)						
CIMP-low	48 (45.7)	29 (27.6)	20 (19.0)	8 (7.6)						
CIMP-high	17 (37.8)	10 (22.2)	11 (24.4)	7 (15.6)						

Abbreviations: CIMP = CpG island methylator phenotype; MSI = microsatellite unstable; MSS = microsatellite stable.
Unless otherwise indicated, χ^2 test was used for categorical variables.
[a]Cases lacking nuclear staining of tumour cells for at least one of MLH1, MSH2, MSH6 or PMS2 were considered to have a positive MSI screening status.
[b]Phenotype determined according to hypermethylation of an eight-gene panel with the following number of hypermethylated genes found for CIMP-negative, 0 genes; CIMP-low, 1–5 genes; and CIMP-high, 6–8 genes.

Other studies though have reached the conclusion that CRC-derived FOXP3^{+} cells, do mediate immune suppression (Kryczek *et al*, 2009; Sherwood *et al*, 2013). Possibly the answer partly lies in the organ specific differences, attributed to the intestinal environment. The fact that the colon is a barrier organ, constantly confronted with foreign antigens, could make it an exception to the rule, with regulatory T cells blocking tumour promoting inflammation (Ladoire *et al*, 2011b). In gastric cancer, a high amount of Tregs proved to be associated with a better prognosis, leading Haas *et al* (2009) to suggest this potentially protective role. Gastrointestinal bacteria can trigger cascades of pro-inflammatory cytokines, with tumour promoting effects (Whiteside, 2012). Having a high amount of regulatory T cells, repressing microbe induced inflammation, could mean a protection not only by hindering the development of cancer in the colorectal epithelium, but also by preventing tumour growth. Bacteria and bacterial products could also potentially aid to sustain the activity of the tumour immune response. As there seem to be a strong correlation between the different subsets, regarding infiltration rate, a high

amount of FOXP3^{+} cells can be an indication for an active and potent immune response, thereby explaining the association to better prognosis.

In this study, we can see that a high infiltration rate of FOXP3^{+} cells is associated with a high infiltration of CD8^{+} cells. Both subsets express ligands enabling T-cell extravasation and tumour infiltration (Ohmichi *et al*, 2011; West *et al*, 2013), possibly potentiating immune cell infiltration synergistically.

With some exceptions, many other studies on the prognostic impact on immune infiltrates have generally not accounted for the molecular status of the tumour. In this study, lymphocyte infiltration was evaluated in molecular subgroups of CRC defined by MSI screening and CIMP status. We found that even though MSI tumours are more highly infiltrated, the prognostic importance of lymphocyte infiltration is likely independent of these molecular characteristics.

Due to the convincing prognostic importance of immune cell infiltration, Galon *et al* (2012) have taken on the urgent task to implement an immunoscore as a component of cancer
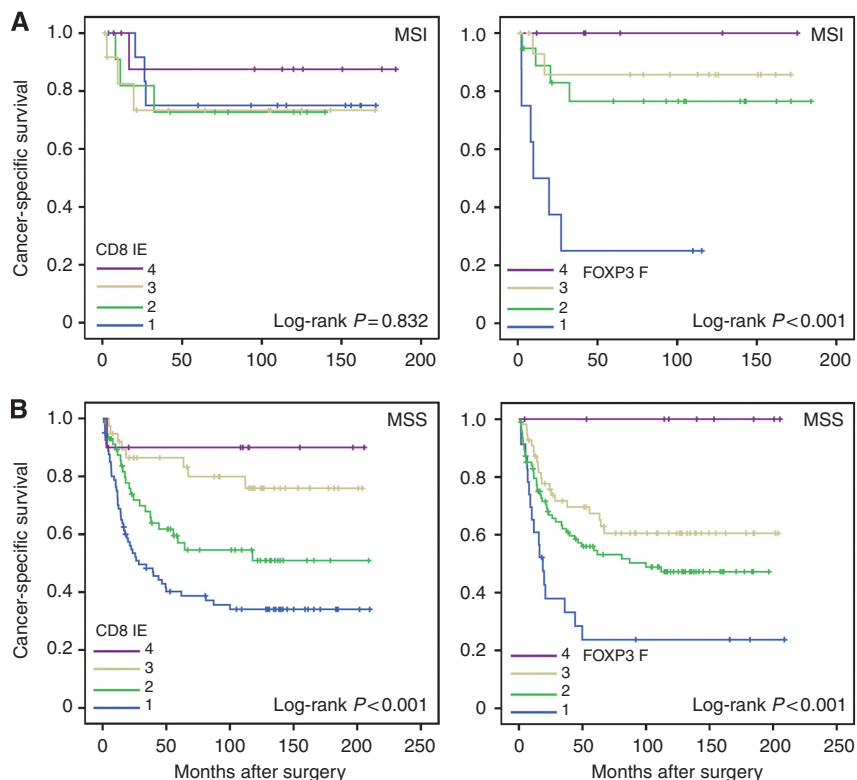
Figure 4. Cancer-specific survival in subgroups of colon cancer arranged according to MSI screening status. Cases scored for CD8 IE or FOXP3 F expression, score 1–4: score 1 (no/sporadic), score 2 (moderate), score 3 (abundant) and score 4 (highly abundant). Shown are Kaplan–Meier plots of specimens of (A) MSI cases and (B) MSS cases. Log-rank tests were used to calculate P values.

classification. The method advocated is a quantitative automated immunohistochemistry score that grades $CD3^+$ and $CD8^+$ infiltration at the invasive margin and tumour centre. By this Galon and colleagues wish to pursue assay uniformity and in the future a globally accepted rapid, inexpensive and powerful scoring method of immune cell infiltration. In this study, we have instead analysed the tumour sections semi-quantitatively, which has the disadvantage of observer variability. On the other hand, considering the heterogenic density of T cells within a single section, our method has the benefit of more accurately identifying tumour compartments and excluding necrotic areas (Richards *et al*, 2014). Furthermore, our method enables a more comprehensive view of the prognostic importance of immune cells at different intratumoural subsites, which might add important information as to how different markers are best implemented in a standardised immunoscore used in clinic.

In conclusion, analysis of the intratumoural subsite of infiltrating T cells is valuable for predicting prognosis in CRC. Here we found that evaluation of CD8 expression within the tumour epithelium of colon cancers bears the most important prognostic information. At other intratumoural subsites, such as tumour front and centre, evaluation of FOXP3 is potentially a stronger prognostic indicator. Furthermore, the relationship between intraepithelial CD8 expression and FOXP3 infiltration at the tumour front adds additional prognostic information. These results may be valuable when defining a prognostic immunoscore in the clinical setting.

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REFERENCES

Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S (1998) A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res* **58**(22): 5248–5257.

Dahlin AM, Henriksson ML, Van Guelpen B, Stenling R, Oberg A, Rutegard J, Palmqvist R (2011) Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. *Mod Pathol* **24**(5): 671–682.

Dahlin AM, Palmqvist R, Henriksson ML, Jacobsson M, Eklof V, Rutegard J, Oberg A, Van Guelpen BR (2010) The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. *Clin Cancer Res* **16**(6): 1845–1855.

deLeeuw RJ, Kost SE, Kakal JA, Nelson BH (2012) The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature. *Clin Cancer Res* **18**(11): 3022–3029.

Deschoolmeester V, Baay M, Lardon F, Pauwels P, Peeters M (2011) Immune cells in colorectal cancer: prognostic relevance and role of MSI. *Cancer Microenviron* **4**(3): 377–392.

Frey DM, Droeser RA, Viehl CT, Zlobec I, Lugli A, Zingg U, Oertli D, Kettelhack C, Terracciano L, Tornillo L (2010) High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. *Int J Cancer* **126**(11): 2635–2643.

Fridman WH, Pages F, Sautes-Fridman C, Galon J (2012) The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 12(4): 298–306.

Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313(5795): 1960–1964.

Galon J, Pages F, Marincola FM, Thurin M, Trinchieri G, Fox BA, Gajewski TF, Ascierto PA (2012) The immune score as a new possible approach for the classification of cancer. *J Transl Med* 10: 1.

Haas M, Dimmler A, Hohenberger W, Grabenbauer GG, Niedobitek G, Distel LV (2009) Stromal regulatory T-cells are associated with a favourable prognosis in gastric cancer of the cardia. *BMC gastroenterol* 9: 65.

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5): 646–674.

Jass JR (2007) Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 50(1): 113–130.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. *CA Cancer J Clin* 61(2): 69–90.

Kryczek I, Liu R, Wang G, Wu K, Shu X, Szeliga W, Vatan L, Finlayson E, Huang E, Simeone D, Redman B, Welling TH, Chang A, Zou W (2009) FOXP3 defines regulatory T cells in human tumor and autoimmune disease. *Cancer Res* 69(9): 3995–4000.

Ladoire S, Arnould L, Mignot G, Coudert B, Rebe C, Chalmin F, Vincent J, Bruchard M, Chauffert B, Martin F, Fumoleau P, Ghiringhelli F (2011a) Presence of Foxp3 expression in tumor cells predicts better survival in HER2-overexpressing breast cancer patients treated with neoadjuvant chemotherapy. *Breast Cancer Res Treat* 125(1): 65–72.

Ladoire S, Martin F, Ghiringhelli F (2011b) Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer. *Cancer Immunol Immunother* 60(7): 909–918.

Maccarty WC (1922) Longevity in cancer: a study of 293 cases. *Ann Surg* 76(1): 9–12.

Martin F, Ladoire S, Mignot G, Apetoh L, Ghiringhelli F (2010) Human FOXP3 and cancer. *Oncogene* 29(29): 4121–4129.

Mathai AM, Kapadia MJ, Alexander J, Kernochan LE, Swanson PE, Yeh MM (2012) Role of Foxp3-positive tumor-infiltrating lymphocytes in the histologic features and clinical outcomes of hepatocellular carcinoma. *Am J Surg Pathol* 36(7): 980–986.

Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochov G, Sakaguchi S (2009) Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30(6): 899–911.

Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, Bruneval P, Trajanoski Z, Fridman WH, Pages F, Galon J (2011) Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol* 29(6): 610–618.

Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H (1998) CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 58(16): 3491–3494.

Ogino S, Galon J, Fuchs CS, Dranoff G (2011) Cancer immunology–analysis of host and tumor factors for personalized medicine. *Nat Rev Clin Oncol* 8(12): 711–719.

Ogino S, Nosho K, Irahara N, Meyerhardt JA, Baba Y, Shima K, Glickman JN, Ferrone CR, Mino-Kenudson M, Tanaka N, Dranoff G, Giovannucci EL, Fuchs CS (2009) 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* 15(20): 6412–6420.

Ohmichi Y, Hirakawa J, Imai Y, Fukuda M, Kawashima H (2011) Essential role of peripheral node addressin in lymphocyte homing to nasal-associated lymphoid tissues and allergic immune responses. *J Exp Med* 208(5): 1015–1025.

Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Galon J (2005) Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 353(25): 2654–2666.

Pino MS, Chung DC (2010) The chromosomal instability pathway in colon cancer. *Gastroenterology* 138(6): 2059–2072.

Popat S, Hubner R, Houlston RS (2005) Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 23(3): 609–618.

Richards CH, Roxburgh CS, Powell AG, Foulis AK, Horgan PG, McMillan DC (2014) The clinical utility of the local inflammatory response in colorectal cancer. *Eur J Cancer* 50(2): 309–319.

Ropponen KM, Eskelinen MJ, Lipponen PK, Alhava E, Kosma VM (1997) Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. *J Pathol* 182(3): 318–324.

Roxburgh CS, McMillan DC (2012) The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer. *Cancer Treat Rev* 38(5): 451–466.

Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B (2009) Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 27(2): 186–192.

Sherwood AM, Emerson RO, Scherer D, Habermann N, Buck K, Staffa J, Desmarais C, Halama N, Jaeger D, Schirmacher P, Herpel E, Kloor M, Ulrich A, Schneider M, Ulrich CM, Robins H (2013) Tumor-infiltrating lymphocytes in colorectal tumors display a diversity of T cell receptor sequences that differ from the T cells in adjacent mucosal tissue. *Cancer Immunol Immunother* 62(9): 1453–1461.

Svennevig JL, Lunde OC, Holter J, Bjorgsvik D (1984) Lymphoid infiltration and prognosis in colorectal carcinoma. *Br J Cancer* 49(3): 375–377.

Svensson H, Olofsson V, Lundin S, Yakkala C, Bjorck S, Borjesson L, Gustavsson B, Quiding-Jarbrink M (2012) Accumulation of CCR4(+)CTLA-4 FOXP3(+)CD25(hi) regulatory T cells in colon adenocarcinomas correlate to reduced activation of conventional T cells. *PLoS One* 7(2): e30695.

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP (1999) CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 96(15): 8681–8686.

Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 38(7): 787–793.

West NR, Kost SE, Martin SD, Milne K, Deleeuw RJ, Nelson BH, Watson PH (2013) Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. *Br J Cancer* 108(1): 155–162.

Whiteside TL (2012) What are regulatory T cells (Treg) regulating in cancer and why? *Semin Cancer Biol* 22(4): 327–334.

Yoon HH, Orrock JM, Foster NR, Sargent DJ, Smyrk TC, Sinicrope FA (2012) Prognostic impact of FoxP3+ regulatory T cells in relation to CD8+ T lymphocyte density in human colon carcinomas. *PLoS One* 7(8): e42274.

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