FULL PAPER

BJC

British Journal of Cancer (2015) 113, 1677–1686 | doi: 10.1038/bjc.2015.427

Keywords: rectal cancer; regulatory T cells; chemoradiotherapy; treatment response

Low stromal Foxp3$^+$ regulatory T-cell density is associated with complete response to neoadjuvant chemoradiotherapy in rectal cancer

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Background: Foxp3$^+$ regulatory T cells (Tregs) play a vital role in preventing autoimmunity, but also suppress antitumour immune responses. Tumour infiltration by Tregs has strong prognostic significance in colorectal cancer, and accumulating evidence suggests that chemotherapy and radiotherapy efficacy has an immune-mediated component. Whether Tregs play an inhibitory role in chemoradiotherapy (CRT) response in rectal cancer remains unknown.

Methods: Foxp3$^+$, CD3$^+$, CD4$^+$, CD8$^+$ and IL-17$^+$ cell density in post-CRT surgical samples from 128 patients with rectal cancer was assessed by immunohistochemistry. The relationship between T-cell subset densities and clinical outcome (tumour regression and survival) was evaluated.

Results: Stromal Foxp3$^+$ cell density was strongly associated with tumour regression grade ($P=0.0006$). A low stromal Foxp3$^+$ cell density was observed in 84% of patients who had a pathologic complete response (pCR) compared with 41% of patients who did not (OR: 7.56, $P=0.0005$; OR: 5.27, $P=0.006$ after adjustment for presurgery clinical factors). Low stromal Foxp3$^+$ cell density was also associated with improved recurrence-free survival (HR: 0.46, $P=0.03$), although not independent of tumour regression grade.

Conclusions: Regulatory T cells in the tumour microenvironment may inhibit response to neoadjuvant CRT and may represent a therapeutic target in rectal cancer.

Neoadjuvant (preoperative) chemoradiotherapy (CRT) is used in locally advanced rectal cancer to reduce tumour volume, making it more amenable to resection, and to decrease the risk of local recurrence (Bosset *et al*, 2006; Gerard *et al*, 2006; Sauer *et al*, 2012). Although neoadjuvant CRT followed by surgery is now standard treatment in many centres around the world, response to CRT varies considerably between individuals. Pathological complete response (pCR), defined as no remaining viable tumour cells in the surgical specimen on histopathologic assessment, is obtained in ~20% of patients, and is associated with a reduced risk of distant recurrence and improved long-term survival (de Campos-Lobato *et al*, 2011; Martin *et al*, 2012; Zorcolo *et al*, 2012). However, ~40% of patients experience only mild to moderate tumour regression, or even no regression following

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Revised 5 November 2015; accepted 12 November 2015; published online 8 December 2015

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neoadjuvant CRT (Huebner *et al*, 2012; Agarwal *et al*, 2013; Fokas *et al*, 2014), and factors associated with CRT response remain poorly understood.

The presence of tumour-infiltrating lymphocytes (TILs) is associated with improved clinical outcome in many cancers (Cho *et al*, 2003; Zhang *et al*, 2003; Hiraoka *et al*, 2006; Loi *et al*, 2013). In colorectal cancer, the density of tumour-infiltrating T cells is a strong prognostic indicator, even after adjustment for clinical and molecular risk factors (Pages *et al*, 2005; Galon *et al*, 2006; Nosho *et al*, 2010). The recently developed Immunoscore, which involves assessment of T-cell density in the central tumour and invasive margin based on expression of pairs of T-cell markers (CD3 and CD8, CD3 and CD45RO or CD8 and CD45RO) (Pages *et al*, 2009), may be more prognostic than histopathologic TNM stage for patients with stage I–III disease (Mlecnik *et al*, 2011), and is currently undergoing international validation with a view to its incorporation into routine histopathologic practice (Galon *et al*, 2012). In rectal cancer, the CD3/CD8 Immunoscore is independently prognostic in patients treated with surgery alone (Anitei *et al*, 2014).

The intratumoural or peritumoural density of total T cells and CD8 T cells has been almost universally associated with good prognosis, whereas tumour infiltration by Foxp3$^+$ regulatory T cells (Tregs) is associated with poorer outcome in most solid cancers (reviewed in Fridman *et al*, 2012). This is consistent with their well-established role as suppressors of the antitumour immune response (reviewed in Zou 2006). However, in colorectal cancer, the presence of Foxp3$^+$ cells in the local tumour microenvironment has been associated with good prognosis in several independent studies (Salama *et al*, 2009; Correale *et al*, 2010; Frey *et al*, 2010). One explanation for apparent paradox is that expansion of Tregs within the tumour occurs in response to the activation and expansion of tumour-specific CD8$^+$ T cells, thereby representing an indirect measure of an antitumour immune response. The strong positive correlation between CD8$^+$ and Foxp3$^+$ TILs in colorectal cancer supports this theory (Michel *et al*, 2008; Frey *et al*, 2010; Yoon *et al*, 2012), as does the association between Foxp3$^+$ TILs and good prognosis in oestrogen receptor-negative breast cancer, seen only in patients who also had a high density of CD8$^+$ TILs (West *et al*, 2013). Alternatively, Tregs may limit the tumourigenic effects of Th17 cell-mediated inflammation (Liu *et al*, 2011; Tosolini *et al*, 2011).

There is now abundant evidence that both chemotherapy and radiotherapy can enhance antitumour immunity through a variety of mechanisms (Nowak *et al*, 2003; Ghiringhelli *et al*, 2007; Zhang *et al*, 2007; Ramakrishnan *et al*, 2010; Burnette *et al*, 2011; Gupta *et al*, 2012; Kim *et al*, 2014). Notably, 5-fluorouracil (5-FU), the radiosensitising agent currently used in neoadjuvant CRT for locally advanced rectal cancer, has been shown to induce immunogenic tumour cell death (Frey *et al*, 2012), and selective Treg depletion has been reported in patients with metastatic colorectal cancer following 5-FU-based chemotherapy (Maeda *et al*, 2011). Modulation of T-cell subsets may therefore play a significant role in response to neoadjuvant CRT. Indeed, high CD3 and CD8 expression in pretreatment rectal cancer biopsy samples is associated with tumour regression following CRT (Yasuda *et al*, 2011; Anitei *et al*, 2014), and radioresponsiveness has an immune-mediated component in animal models of colon, breast and other solid cancers (Apetoh *et al*, 2007; Liang *et al*, 2013; Gerber *et al*, 2014). In breast cancer, the disappearance of Tregs following neoadjuvant CRT is associated with complete pathological response (Ladoire *et al*, 2008). However, the impact of Tregs in the local tumour microenvironment during standard long-course CRT for locally advanced rectal cancer remains unknown.

In the current study we evaluated the density of Foxp3$^+$, CD3$^+$ CD8$^+$, CD4$^+$ and IL-17$^+$ cells in surgical resection samples from 128 patients with locally advanced rectal cancer treated with long-course 5-FU-based CRT. Our *a priori* hypotheses were that response to CRT has an immune-mediated component and that Tregs play an inhibitory role.

MATERIALS AND METHODS

Patients, treatment and follow-up. Patients who underwent neoadjuvant radiotherapy and surgery for rectal adenocarcinoma at our institution between August 2006 and December 2010, and who gave written informed consent for their biospecimens and health information to be used for research purposes, were identified from our prospectively maintained database. The consent rate for research participation at our institution is 98%. Clinical tumour and nodal stage (cT and cN), and the presence or absence of distant metastases (cM) was determined by computed tomography (CT) and magnetic resonance imaging (MRI) before commencement of CRT.

Neoadjuvant radiotherapy consisted of 50.4 Gy administered in 28 fractions over 5 weeks. Fluoropyrimidine-based chemotherapy was given concurrently via intravenous infusion 5-FU or oral capecitabine (Xeloda, Roche Products Pty Ltd, Dee Why, NSW, Australia). Surgical resection of the primary tumour was performed a minimum of 4 weeks after completing neoadjuvant treatment (6–8 weeks post CRT in most cases). Fluoropyrimidine-based adjuvant chemotherapy was offered to all patients (including those who attained a pCR), as per current Australian guidelines (ACN (Australian Cancer Network) Colorectal Cancer Guidelines Revision Committee, 2005), and was administered for up to 6 months postoperatively. Patients with distant metastases were progressively managed with neoadjuvant CRT, rectal surgery, potentially curative resection of metastatic lesions where possible and adjuvant chemotherapy.

Follow-up included 6-monthly visits, carcinoembryonic antigen (CEA) blood test and digital rectal examination. At 12-monthly intervals, patients had a repeat CT scan. A colonoscopy was performed at 12 months and then 4 years. Survival information was obtained from the Western Australian Cancer Registry every 6 months. Surviving patients were censored at the date of last survival update (1 May 2015). Cancer-specific survival (CSS) was defined as the time between the date of surgery and date of death from colorectal cancer. Recurrence-free survival (RFS) was defined as the time between the date of surgery and the date of first recurrence (local recurrence, development of first distant metastases or development of recurrent distant metastases after potentially curative resection) or date of death from colorectal cancer without prior documented recurrence (patients with unresected metastatic disease).

The study was approved by the St John of God Healthcare Human Research Ethics Committee and access to state cancer registry data was approved by the Department of Health Western Australia. The study was conducted in accordance with the Declaration of Helsinki.

Histopathologic assessment. Tumour regression grading was performed as part of routine pathological review using the Dworak system (Dworak *et al*, 1997), whereby grade 4 corresponds to pCR and grade 0 to no regression. Other variables obtained from the pathology report were pathologic T stage (ypT), pathologic N stage (ypN), resection margin status (R status), lymphovascular invasion (LVI), extramural venous invasion (EMVI) and perineural invasion (PNI).

Construction of tissue microarrays (TMAs). Formalin-fixed, paraffin-embedded (FFPE) tumour samples and corresponding H&E slides were retrieved from the institutional pathology archive. A specialist gastrointestinal histopathologist (CH) reviewed the slides, verifying Dworak grade and selecting target areas of tumour and stroma. Tumour areas (Dworak 0–3 patients only) targeted central tumour, or residual malignant glands/tumour nests,

depending on the degree of response. For the stromal cores, an area of nonepithelial tissue adjacent to the residual tumour was selected. In cases of pCR, areas within the fibrotic irradiated tumour bed, judged to most accurately represent the tumour stroma, were selected. Cylindrical tissue cores 1 mm in diameter were punched from the target area and inserted into a recipient paraffin block using an MTA-1 manual tissue arrayer (Beecher Instruments, Estigen, Tartu, Estonia). For each case, two tumour cores (where available), two stromal cores and one core of normal colonic epithelium (taken from a separate block of normal tissue) were included. Sections 4 μm in thickness were cut from the TMAs and mounted on positively charged slides for H&E staining and immunohistochemistry (IHC).

Immunohistochemistry. Staining for Foxp3, CD8 and IL-17 was performed manually. Manual staining protocols were carefully optimised with regard to antigen-retrieval, primary antibody concentration/incubation time and optimal detection system before staining the TMAs. Sections were dewaxed and rehydrated through graded xylene and alcohol washes. Antigen retrieval was performed in a DakoCytomation pressure cooker (Dako, Copenhagen, Denmark) at 121 °C for 6 min in Tris-EDTA buffer (pH 9.0). Peroxidase blocking solution (CINtec Histology Kit; Roche Australia, Castle Hill, NSW, Australia) was applied according to the manufacturer's instructions. For Foxp3 and CD8 staining, sections were incubated with serum-free protein block (Dako) for 10 min, followed by the primary antibody (Foxp3 clone 236A/E7; Abcam, Melbourne, VIC, Australia, 1 : 100 dilution for 60 min or CD8 clone C8/144B; Dako, 1 : 100 dilution for 30 min). After washing in TBS-Tween, sections were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 30 min (CINtec Histology Kit), washed again in TBS-Tween and incubated with DAB solution (CINtec Histology Kit). The IL-17 staining was performed using a goat polyclonal anti-human IL-17 antibody (R&D Systems, Burlingame, MN, USA, 1 μg ml^{-1} diluted in blocking serum for 60 min) and an avidin/biotin system (Avidin/Biotin Blocking Kit, Vectastain Elite ABC kit and ImmPACT DAB; all from Vector Labs, Burlingame, CA, USA), according to the manufacturer's instructions.

CD3 and CD4 staining was performed on an Autostainer Link 48 (Dako) using the EnVision FLEX+ high pH visualisation system (Dako). Rehydration and antigen-retrieval steps were performed in a PT Link pretreatment module (Dako). Primary antibodies used were a rabbit polyclonal anti-human CD3 and a mouse monoclonal anti-human CD4 clone 4B12 (both from Dako FLEX, ready-to-use).

Each TMA was stained on two separate occasions for each marker of interest. One staining run was selected as the primary run for analysis, based on overall staining quality. For cases where staining was judged to be inadequate (primarily because of detachment or folding of cores), the core from the alternative run was analysed where available.

For triple staining, an alkaline phosphatase (AP) and HRP multiplex detection system was used according to the manufacturer's instructions (Biocare Medical, Concord, CA, USA). Following our standard antigen-retrieval process described above, sections were incubated for 15 min with a universal blocking reagent (Background Sniper), then for 30 min with a CD4/CD8 ready-to-use multiplex cocktail (Biocare Medical). Endogenous peroxidase was then blocked using Peroxidazed 1 and antibody detection was performed using the MACH 2 Doublestain 2 system with Betazoid DAB and Warp Red (Biocare Medical). Sections were then washed and incubated for 60 min with the primary Foxp3 antibody (clone 236A/E7; Abcam). Detection was performed using MACH 2 mouse HRP polymer and Vina Green. All triple stain reagents other than the Foxp3 antibody were purchased from Biocare Medical.

All sections were lightly counterstained in Mayers haematoxylin (Hurst Scientific, Perth, WA, Australia) before dehydration and mounting. Sections of human tonsil were included as positive and negative controls in every staining run. An isotype control was used in place of the primary antibody on negative control sections.

Assessment of T-cell density. Slides were scanned using a high-resolution digital scanner (Aperio Scanscope XT; Leica Biosystems, North Ryde, NSW, Australia) at $\times 40$ magnification. Evaluation of T-cell density (cells per mm^2 tissue) was performed using image analysis software (Aperio Imagescope version 11; Leica Biosystems) after careful manual annotation of the cores to ensure that the area of analysis matched the histology type to which the core was assigned, and to exclude areas of damaged or missing tissue. Data presented represent the average of two cores where possible.

Statistical analysis. Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism version 6.0 (GraphPad software Inc., San Diego, CA, USA). Differences in T-cell density between response groups were assessed using general linear models. Associations between clinical/immunological variables and pCR were assessed using logistic regression. Time-to-event outcomes (CSS and RFS) were estimated using the Kaplan–Meier method with curves compared using the log-rank test. Hazard ratios (HRs) were determined using Cox proportional hazards regression (Cox, 1972). Multivariate analyses were performed using backwards stepwise selection of variables $P<0.2$ in univariate analysis. Differences and associations were considered statistically significant where $P<0.05$.

RESULTS

Patient characteristics and treatment response. A total of 135 eligible patients were identified. Samples from 128 of these patients were included in the TMAs. Reasons for exclusion included concurrent malignancy (one patient), prior polypectomy of the primary tumour (one patient), minimal neoadjuvant treatment received (one patient) and insufficient or unavailable material (four patients). Characteristics of the 128 patients are shown in Table 1. Mean age at the time of surgery was 63 years and 72% were male. The majority of patients (81%) were clinical T stage 3 and 74% were assessed as having nodal involvement on pretreatment CT scan and MRI. Eleven patients (9%) had metastatic disease at diagnosis. This included lesions in the liver (eight patients), lung (one patient), liver and lung (one patient) and peritoneal disease (one patient). The median interval between the end of CRT and surgery was 7 weeks. One elderly patient (93 years of age) elected to have neoadjuvant long-course radiotherapy (RT) without concurrent chemotherapy. The most commonly performed surgical procedures performed were ultra-low anterior resection (67%) and abdominoperineal resection (27%). Other procedures included low anterior resection (three patients), Hartmann's procedure (two patients) and total colectomy (two patients). Of the 11 patients with distant metastases, 5 underwent resection of liver/lung lesions and 1 patient with liver metastases received selective internal radiation therapy (SIRT).

In all, 26 patients (20%) achieved a pCR (Dworak grade 4) following neoadjuvant CRT (Table 1). In addition, 46 patients (36%) were assigned Dworak grade 3, 47 patients (37%) Dworak grade 2 and 9 patients (7%) Dworak grade 1. No patient was classed as Dworak grade 0.

T-cell subsets in the tumour microenvironment after CRT. Foxp3$^+$, CD3$^+$, CD8$^+$, CD4$^+$ and IL-17$^+$ cells were identified in the tumour, the stroma and the normal epithelium cores following CRT (Figure 1). The density of Foxp3$^+$ cells was highest in the tumour cores, followed by the normal cores and then

Table 1. Patient characteristics

	n = 128
Age, mean (s.d.)	63 (12.3)
Sex, *n* (%)	
Male	92 (71.9)
Female	36 (28.1)
Distance from anal verge (cm), mean (s.d.)	7 (3.7)
Weeks between end CRT and surgery, median (IQR)	7 (6, 8)
Pretreatment clinical T stage, *n* (%)	
cT2	8 (6.3)
cT3	104 (81.3)
cT4	14 (10.9)
NR	2 (1.6)
Pretreatment clinical N stage, *n* (%)	
cN0	31 (24.2)
cN1–2	95 (73.6)
NR	2 (1.6)
Pretreatment clinical M stage, *n* (%)	
cM0	115 (89.8)
cM1-2	11 (8.6)
NR	2 (1.6)
Dworak grade, *n* (%)	
4 (pCR)	26 (20.3)
3	46 (35.9)
2	47 (36.7)
1	9 (7.0)
Pathologic T stage, *n* (%)	
ypT0	27[a] (21.1)
ypTis/T1	9 (7.0)
ypT2	31 (24.2)
ypT3	56 (43.8)
ypT4	4 (3.1)
ypTx[b]	1 (0.8)
Pathologic N stage, *n* (%)	
ypN0	90 (70.3)
ypN1–2	38 (29.7)
Resection margin status, *n* (%)	
R0	123 (96.1)
R1–2	5 (3.9)
Lymphovascular invasion, *n* (%)	
Yes	11 (8.6)
No	117 (91.4)
Perineural invasion, *n* (%)	
Yes	21 (16.4)
No	107 (83.6)
Extramural venous invasion, *n* (%)	
Yes	9 (7.0)
No	119 (93.0)

Abbreviations: CRT = chemoradiotherapy; IQR = interquartile range; NR = not recorded; pCR = pathologic complete response; Tis=carcinoma *in situ*.
[a]One Dworak grade 3 patient had no residual tumour in bowel, but 5/15 nodes positive.
[b]Small clusters of malignant cells present in resection doughnut.

the stroma (median 125 *vs* 88 *vs* 28 cells per mm^2 respectively; Figure 1B). We observed a similar distribution of CD8$^+$ and CD4$^+$ T cells, with IL-17$^+$ cells most abundant in or around the normal epithelium (median 12 *vs* 11 *vs* 29 in tumour *vs* stroma *vs* normal cores; Figure 1B). To further characterise Foxp3$^+$ cells, we performed concurrent staining for CD4, CD8 and Foxp3 using a multiplex IHC system. Manual scoring of a subset of 25 stromal cores, selected to include a range of Foxp3$^+$ cell densities, demonstrated, as expected, that 99% of Foxp3$^+$ cells with visible surface staining were CD4$^+$ (Figure 1A, and data not shown).

Low stromal Foxp3$^+$ T-cell density is associated with response to CRT. Analysis of T-cell subset density by Dworak grade revealed a strong inverse correlation between Foxp3$^+$ cell density in the stroma and tumour regression ($P = 0.0006$; Figure 2A). Of patients who had a pCR, 84% had a stromal Foxp3$^+$ cell density lower than the median value as compared with 41% of noncomplete responders (OR: 7.56, $P = 0.0005$; Figure 2B and Table 2). Stromal Foxp3$^+$ cell density was strongly correlated with stromal CD3$^+$ and CD4$^+$ T-cell density, consistent with the co-expression of these molecules on Tregs (Supplementary Figure 1). A significant positive correlation was also observed between Foxp3$^+$ and CD8$^+$ cells in the stroma, the tumour and the normal cores. Foxp3$^+$ and IL-17$^+$ cell density positively correlated in the normal mucosa, but not in the tumour or stroma, where no significant correlation was seen (Supplementary Figure 1, and data not shown).

Stromal Foxp3$^+$ cell density was the only variable independently associated with pCR in multivariate analysis (OR: 5.27, $P = 0.0058$; Table 2). Although low total T-cell and CD4$^+$ T-cell densities were also associated with pCR in univariate analysis, these associations were no longer significant after adjustment for Foxp3$^+$ T-cell density and presurgery clinical and demographic factors (Table 2). The ratio between Foxp3$^+$ Tregs and other T-cell subsets was also investigated, but no correlation with CRT response was stronger than for stromal Foxp3$^+$ cell density alone.

No association between tumour regression grade and the density of Foxp3$^+$ Tregs, or other T-cell subsets, in the tumour (available for Dworak 1–3 patients only) or normal cores was found (Supplementary Figure 2A and B, and data not shown). However, there was no significant correlation between the density of Foxp3$^+$ Tregs in the stroma and that in the tumour or normal cores (Supplementary Figure 2C and D).

Foxp3$^+$ T-cell density as a prognostic marker in neoadjuvantly treated rectal cancer. A total of 104 patients (81%) were alive on the censor date and median follow-up was 76 months from the date of surgery. There were 4 deaths from causes other than colorectal cancer during the follow-up period (1 in each of Dworak 1, 2, 3 and 4 groups). Of these patients, 92 (72%) remained alive without disease recurrence. Only 3 patients (2.3%) had a local recurrence (2 in the Dworak grade 2 and 1 in the Dworak grade 3 groups), and in 1 case this was preceded by development of liver metastases. Median time to disease recurrence was 15.2 months (range 1.9–53 months). Distant recurrence events included development of new metastases (26 patients) and recurrence of previously resected metastatic disease (2 patients).

There were no deaths from rectal cancer in the Dworak 4 group (pCR). Dworak grade was significantly associated with both CSS and RFS (Figure 3A and B and Table 3). Stromal Foxp3$^+$ cell density was also significantly associated with survival; the probability of 5-year CSS and RFS being 92% *vs* 81% and 80% *vs* 63% in the Foxp3 low *vs* high groups respectively (Figure 3C and D and Table 3). In multivariate analysis, adjusting for clinical and pathologic variables, cM status, Dworak grade and the presence or absence of PNI were significantly associated with RFS (Table 3). When the analysis was limited to patients without distant metastases at time of primary surgery, Dworak grade and resection margin status were the only independent prognostic factors (data not shown). No statistically significant interactions between variables included in the multivariate analyses were observed.

DISCUSSION

This study investigated the relationship between Foxp3$^+$ Treg density in the local tumour microenvironment following

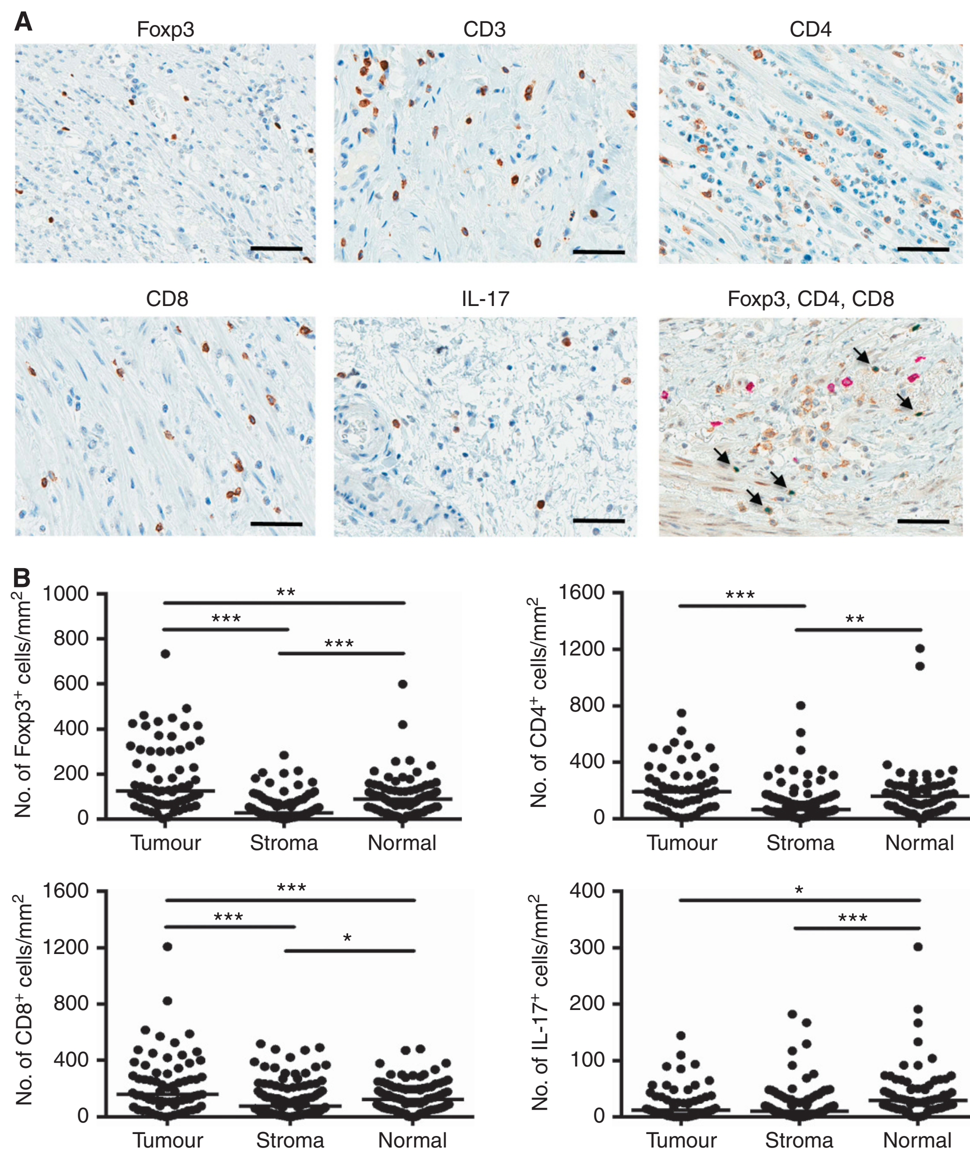

Figure 1. Identification of T-cell subsets. (A) Representative immunohistochemical staining of stromal cores for Foxp3 (top left), CD3 (top centre), CD4 (top right), CD8 (bottom left), IL-17 (bottom centre) and multiplex detection of Foxp3 (green), CD4 (brown) and CD8 (pink) (bottom right). Arrows indicate $CD4^+Foxp3^+$ cells. Scale bar, 50 μm. (B) Frequencies of $Foxp3^+$, $CD4^+$, $CD8^+$ and $IL\text{-}17^+$ cells in tumour (Dworak 1–3 patients only), stroma and normal cores. Line at median. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

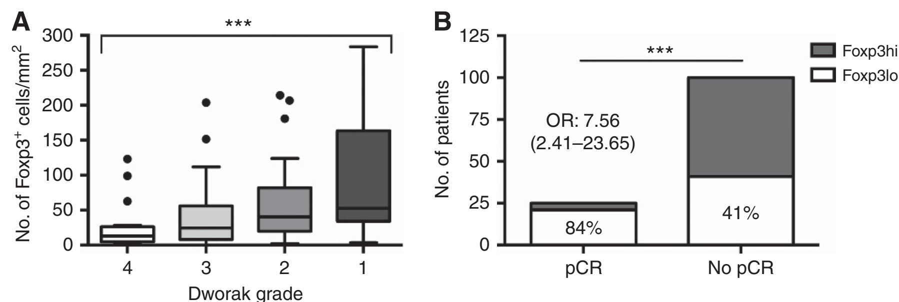

Figure 2. Low stromal $Foxp3^+$ cell density is associated with response to CRT. (A) Stromal $Foxp3^+$ cell density by Dworak grade ($P=0.0006$; general linear model). Circles represent outliers using Tukey's method (> the 75th percentile plus 1.5 times the interquartile range). (B) Low *vs* high stromal $Foxp3^+$ cell density (split at the median value) by pCR ($P=0.0005$; logistic regression). ***$P<0.001$.

neoadjuvant CRT and treatment response in locally advanced rectal cancer. Given the increasing evidence for an immune-mediated component to chemotherapy and radiotherapy efficacy (Apetoh *et al*, 2007; Ladoire *et al*, 2008; Yasuda *et al*, 2011; Liang *et al*, 2013; Anitei *et al*, 2014; Gerber *et al*, 2014), and the well-established role of Tregs as suppressors of antitumour immunity, we hypothesised that Tregs inhibit the response of rectal carcinomas to neoadjuvant CRT.

Table 2. Relationship between stromal T-cell densities and pCR adjusting for presurgery clinical and demographic factors

	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*	OR	95% CI	*P*
Foxp3						
High	1.00			1.00		
Low	7.56	2.41–23.65	0.0005	5.27	1.62–17.16	0.0058
CD3						
High	1.00					
Low	2.97	1.13–7.79	0.0270			
CD4						
High	1.00					
Low	3.11	1.11–8.73	0.0312			
CD8						
High	1.00					
Low	1.88	0.78–4.54	0.1615			
IL-17						
High	1.00					
Low	2.31	0.91–5.84	0.0780			
Age						
<65 Years	1.00					
⩾65 Years	1.04	0.44–2.46	0.9289			
Sex						
Male	1.00					
Female	1.47	0.59–3.69	0.4111			
Distance from anal verge						
<7 cm	1.00					
⩾7 cm	0.82	0.35–1.95	0.6547			
Pretreatment clinical T stage						
cT4	1.00		0.0839			
cT3	3.29	0.41–26.58				
cT2	13.00	1.11–152.4				
Pretreatment clinical N stage						
cN1–2	1.00					
cN0	0.68	0.23–1.98	0.4774			
Pretreatment clinical M stage						
cM1–2	1.00					
cM0	1.19	0.24–5.86	0.8335			
Interval between CRT and surgery						
⩽7 Weeks	1.00					
>7 Weeks	1.25	0.52–3.01	0.6223			

Abbreviations: CI = confidence interval; CRT = chemoradiotherapy; Foxp3 = forkhead box P3; IL-17 = interleukin 17; OR = odds ratio; pCR = pathologic complete response. Variables included in multivariate analysis: stromal Foxp3, CD3, CD4 and cT stage.

We report a strong inverse correlation between stromal Foxp3$^+$ cell density and tumour regression following CRT. Patients with a low stromal Foxp3$^+$ cell density were over 5 times more likely to have a pCR, after adjustment for pretreatment clinical and demographic factors. Stromal Foxp3$^+$ density was also significantly associated with improved RFS in univariate analysis. Although stromal Foxp3$^+$ cell density was not independently prognostic in multivariate analysis, Dworak grade remained significantly associated with RFS, after adjustment for clinical and pathological variables, highlighting the clinical significance of response to CRT, even in the context of potentially curative resection.

In a recent study, Shinto *et al* (2014) found post-CRT stromal CD8$^+$ T-cell density to be associated with improved RFS and CSS in a cohort of 93 rectal cancer patients who received short-course CRT (20 Gy given over 5 days with concurrent uracil over 7 days) and surgery 30 days later. They also assessed Foxp3$^+$ cell density in resection samples, and found no significant association with survival. However, CD8/Foxp3 ratio in pre-CRT biopsies was predictive of improved tumour regression. An important difference between this and the current study is treatment schedule. Complete response is less common after short-course CRT (just 3% of the cohort studied by Shinto *et al* (2014) were classed as Dworak grade 4), and tumour regression can continue for up to 12 weeks post CRT (Glimelius, 2014). As such, although highlighting the potential importance of local antitumour immunity in rectal cancer response to CRT, these results may not be relevant to long-course CRT.

The negative association between the presence of Foxp3$^+$ Tregs and clinical outcome observed in this study appears contrary to several studies in colorectal cancer, including our own previous work (Salama *et al*, 2009; Correale *et al*, 2010; Frey *et al*, 2010) that have demonstrated a positive association between Foxp3$^+$ TILS and prognosis. However, the majority of samples assessed in these previous studies were from chemo/radiotherapy-naive colon cancer patients. Multiple preclinical studies have now demonstrated that radiotherapy efficacy requires an intact immune system and that tumour radioresponsiveness is associated with

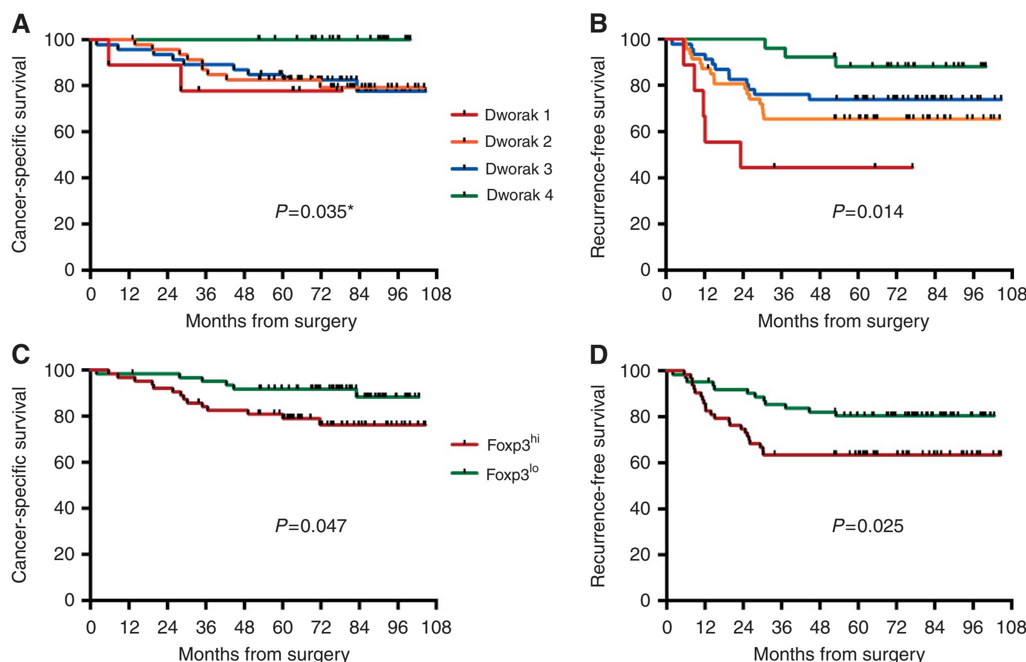

Figure 3. Survival by Dworak grade and stromal Foxp3$^+$ cell density. Kaplan–Meier estimates for cancer-specific and recurrence-free survival by (**A** and **B**) Dworak grade and (**C** and **D**) stromal Foxp3$^+$ cell density. Groups compared using the log-rank test. *Test for trend.

dendritic cell activation and CD8-driven antitumour immunity (Apetoh *et al*, 2007; Gupta *et al*, 2012; Liang *et al*, 2013; Gerber *et al*, 2014). Similar to many other chemotherapy drugs, 5-FU induces HMGB1 release from tumour cells, one of the major hallmarks of immunogenic cell death (Frey *et al*, 2012), and may preferentially deplete Tregs over other T-cell subsets (Maeda *et al*, 2011). If tumour regression following CRT in rectal cancer is, at least in part, due to CRT-induced antitumour immunity, it is rational that Tregs may play an antagonistic role.

The density of tumour-infiltrating T cells, and more specifically, CD8$^+$ T cells, is a strong indicator of improved survival in colorectal cancer (Pages *et al*, 2009; Nosho *et al*, 2010; Mlecnik *et al*, 2011), and in rectal cancer biopsies, CD8 T-cell density predicts the likelihood of tumour regression following CRT (Yasuda *et al*, 2011; Anitei *et al*, 2014). Therefore, the association between low stromal CD3$^+$ (and CD4$^+$) cell density and pCR found in the current study seems counterintuitive. It is possible that a higher density of lymphocytes in the local tumour microenvironment after CRT in poor responders simply correlates with residual tumour burden. However, only Foxp3$^+$ cell density was independently associated with treatment response in this study, indicating that the presence of Tregs is specifically important.

Given the strong association between tumour regression and stromal Foxp3$^+$ Treg density observed, it is perhaps surprising that we found no association between tumour regression and Treg density in the tumour cores. Selection of truly representative target areas is a challenge common to all studies using TMAs, and can be particularly difficult when using tissue from pretreated patients with significant variation in treatment response. Although target sites were carefully selected by an experienced specialist gastro-intestinal pathologist, we cannot exclude the possibility that the lack of correlation between Treg density in the stromal and tumour cores (for Dworak grade 1–3 patients) may be a function of target site selection. However, ours is not the first study to find T-cell subsets in the stromal compartment to be most strongly associated with outcome (Tougeron *et al*, 2013; Vayrynen *et al*, 2013; Mei *et al*, 2014), and importantly, the strongest association observed in the current study was between low stromal Treg density and pCR, the patient group for whom tumour cores were not available.

Our data support the hypothesis that response to CRT in rectal cancer is partially immune-mediated and inhibited by the presence of Tregs. It is possible that Tregs are selectively depleted in responding patients during CRT, providing a window of opportunity for the initiation of an effective antitumour immune response. Alternatively, fewer Tregs in the local tumour environment before treatment may allow for effective CRT-induced antitumour immunity. Which of these scenarios is most likely cannot be deduced from our data. We plan to answer this question by evaluating Treg density in pre-CRT biopsies in conjunction with subsequent surgical specimens.

If Tregs do limit tumour regression following CRT in rectal cancer, response rates may potentially be improved by combining current neoadjuvant regimens with an immune-modulating therapy. Although there is as yet no drug available that selectively depletes Tregs in humans effectively, ‘checkpoint blockade’, an approach that aims to stimulate an effective antitumour immune response through blocking inhibitory signals in T cells, has shown major promise in other solid cancers (Postow *et al*, 2015) and may be useful in this setting. It is of particular interest in the context of the current study that the anticytotoxic T lymphocyte-associated protein 4 (CTLA-4) monoclonal antibody, ipilimumab, exerts its immune-stimulating effect, not only through blocking inhibitory signals in effector T cells, but also through inhibiting Treg function (Peggs *et al*, 2009; Qureshi *et al*, 2011). However, given the important role played by Tregs in maintaining gut homeostasis (Barnes and Powrie, 2009), the timing and mode of delivery of any immune-modulating agent will likely be critical. Indeed, in a murine model of colitis-associated colon cancer (CAC), transient depletion of Tregs during the acute inflammation phase leads to uncontrolled inflammation and death, whereas depletion during the late phase results in enhanced CD8$^+$ T cell-driven antitumour immunity and reduced tumour formation (Pastille *et al*, 2014). Clinical trials in this field will therefore require careful design.

In summary, we show that low stromal Foxp3$^+$ Treg density post CRT is associated with pCR and improved long-term outcome in locally advanced rectal cancer. Our data suggest that Tregs in the local microenvironment may inhibit the response of rectal

Table 3. Recurrence-free survival according to stromal Foxp3$^+$ cell density and clinicopathological factors

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Foxp3						
High	1.00					
Low	0.46	0.23–0.92	0.0288			
Dworak grade						
1–4[a]	0.55	0.37–0.82	0.0031	0.61	0.39–0.95	0.0300
Age (years)						
<65	1.00					
⩾65	0.83	0.43–1.59	0.5669			
Sex						
Male	1.00					
Female	0.68	0.31–1.50	0.3414			
Distance from anal verge						
<7 cm	1.00					
⩾7 cm	0.81	0.42–1.56	0.5329			
Pretreatment clinical T stage						
cT4	1.00		0.0554			
cT3	0.39	0.17–0.90				
cT2	0.18	0.02–1.44				
Pretreatment clinical N stage						
cN1–2	1.00					
cN0	1.35	0.65–2.83	0.4226			
Pretreatment clinical M stage						
cM1–2	1.00			1.00		
cM0	0.20	0.09–0.44	<0.0001	0.28	0.12–0.66	0.0038
Interval between CRT and surgery						
⩽7 Weeks	1.00					
>7 Weeks	0.92	0.48–1.77	0.8101			
Post-treatment pathologic T stage						
ypT3–4	1.00		0.0006			
ypTis/ypT1/ypT2	0.26	0.11–0.64				
ypT0	0.18	0.06–0.60				
Post-treatment pathologic N stage						
ypN1–2	1.00					
ypN0	0.45	0.23–0.87	0.0181			
Resection margin status						
R1–2	1.00					
R0	0.21	0.07–0.60	0.0035			
Lymphovascular invasion						
Yes	1.00					
No	1.62	0.39–6.73	0.5091			
Perineural invasion						
Yes	1.00			1.00		
No	0.28	0.14–0.57	0.0004	0.41	0.19–0.90	0.0252
Extramural venous invasion						
Yes	1.00					
No	0.25	0.10–0.61	0.0021			

Abbreviations: CI = confidence interval; CRT = chemoradiotherapy; Foxp3 = forkhead box P3; HR = hazard ratio; Tis = carcinoma *in situ*. Variables included in the multivariate analysis: stromal Foxp3, Dworak, cT, cM, ypT, ypN, R status, perineural invasion and extramural venous invasion.
[a]Trend analysis.

carcinomas to neoadjuvant CRT and may represent a therapeutic target in rectal cancer.

ACKNOWLEDGEMENTS

This study was funded by the Cancer Council Western Australia (ID 1048279), the Raine Medical Research Foundation, the St John of God Foundation, the Colorectal Surgical Society of Australia and New Zealand (CSSANZ), the Tonkinson Foundation for Colorectal Cancer Research and the McCusker Charitable Foundation. We thank Dr Tim Threlfall of the Western Australian Cancer Registry and Cheryl Penter of St John of God Subiaco Hospital for assistance with the data collection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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