ONCOIMMUNOLOGY
2023, VOL. 12, NO. 1, 2238506
https://doi.org/10.1080/2162402X.2023.2238506

Taylor & Francis
Taylor & Francis Group

ORIGINAL RESEARCH

OPEN ACCESS Check for updates

CD11c$^+$ and IRF8$^+$ cell densities in rectal cancer biopsies predict outcomes of neoadjuvant chemoradiotherapy

Benita C. Y. Tse[a], Sarah Bergamin[b], Pascal Steffen[a], George Hruby[b], Nick Pavlakis[c,d], Stephen J. Clarke[c,d], Justin Evans[e], Alexander Engel[d,e], Andrew Kneebone[b,d], and Mark P. Molloy[a]

[a]Bowel Cancer and Biomarker Laboratory, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Camperdown, Australia; [b]Department of Radiation Oncology, Royal North Shore Hospital, Sydney, Australia; [c]Department of Medical Oncology, Royal North Shore Hospital, Sydney, Australia; [d]Sydney Medical School, Faculty of Medicine and Health, University of Sydney, Camperdown, Australia; [e]Colorectal Surgical Unit, Royal North Shore Hospital, Sydney, Australia

ABSTRACT

Approximately 20% of locally advanced rectal cancer (LARC) patients treated preoperatively with chemoradiotherapy (CRT) achieve pathologically confirmed complete regression. However, there are no clinically implemented biomarkers measurable in biopsies that are predictive of tumor regression. Here, we conducted multiplexed immunophenotyping of rectal cancer diagnostic biopsies from 16 LARC patients treated preoperatively with CRT. We identified that patients with greater tumor regression had higher tumor infiltration of pan-T cells and IRF8$^+$HLA-DR$^+$ cells prior to CRT. High IRF8$^+$HLA-DR$^+$ cell density was further associated with prolonged disease-specific survival with 83% survival at 5 y compared to 28% in patients with low infiltration. Contrastingly, low CD11c$^+$ myeloid cell infiltration prior to CRT was a putative biomarker associated with longer 3- and 5-y disease-free survival. The results demonstrate the potential use of rectal cancer diagnostic biopsies to measure IRF8$^+$ HLA-DR$^+$ cells as predictors of CRT-induced tumor regression and CD11c$^+$ myeloid cells as predictors of LARC patient survival.

ARTICLE HISTORY
Received 10 August 2022
Revised 9 July 2023
Accepted 16 July 2023

KEYWORDS
Dendritic cell; immune cell; immunophenotype; neoadjuvant chemoradiotherapy; rectal cancer

Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer worldwide, accounting for 10% of all cancer diagnoses and the second leading cause of cancer death[1]. Rectal cancer alone accounted for 39% of colorectal cancer incidences in 2020[1]. Despite the incidence of CRC decreasing in patients aged 50 y or older, the incidence of CRC and rectal cancer especially continues to rise in young adults[2].

Patients diagnosed with locally advanced rectal cancer (LARC) (T3–4, presence of extramural venous invasion, and/or node positive without distant metastasis) are recommended preoperative chemoradiotherapy (CRT) prior to surgical resection to downstage the tumor and minimize the risk of locoregional recurrence. A subset (approximately 15–30%) of these patients achieve pathologically confirmed complete tumor regression[3,4]. These patients have improved disease-free survival and overall survival compared with patients without complete tumor regression[3,4]. A meta-analysis of 867 patients found that there was no significant difference in disease-free survival, overall survival, or cancer-specific mortality between patients with clinical complete response managed conservatively by watch-and-wait and those with pathological complete tumor regression post-resection[5]. This suggests that rectum preservation is a possibility in patients with complete clinical response.

Immune cells are emerging as possible predictors of response to preoperative CRT. Not only are immune cells critical in the anti-tumor response[6], a low density of cytotoxic T cells and helper T cells prior to preoperative CRT was associated with poor tumor regression[7]. Indeed, an immunoscore established using CD3 and CD8 T cell staining in LARC biopsies prior to CRT demonstrated immunoscore high patients to be at lower risk of relapse and death[8]. Furthermore, the immunoscore in combination with imaging and physical examination could identify patients with complete clinical CRT response and thus candidates for watch-and-wait. Immunoscore high patients in watch-and-wait did not experience relapse[8].

The evidence thus far shows an important role for T cells in predicting patient response to preoperative CRT. The contribution of myeloid cells in LARC biopsies as predictors of tumor regression is less well studied. Some studies have associated CRT tumor regression with increased CD20$^+$ B cell tumor infiltration[9] and reduced CD163$^+$ macrophage infiltration[10].

To expand our knowledge of tumor immune cell phenotypes and their associations with CRT outcomes in LARC, we used a multiparametric approach to quantify T cells, B cells, and multiple myeloid cell subsets for their association with tumor regression following highly protocoled CRT used in a clinical trial setting.

CONTACT Mark P. Molloy m.molloy@sydney.edu.au Bowel Cancer and Biomarker Laboratory, School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Camperdown, 2006 Australia
Supplemental data for this article can be accessed online at https://doi.org/10.1080/2162402X.2023.2238506

Materials and methods

Patient cohort and preoperative chemotherapy

This study was approved by the Northern Sydney Local Health District HREC (HREC/14/HAWKE/173). All participants provided written informed consent. From February 2015, 26 LARC patients deemed suitable for preoperative CRT were enrolled on the prospective Predicting RadIoTherapy ReSponse of Rectal Cancer with MRI and PET (PRISM) trial (ClinicalTrials.gov Identifier: NCT02233374). Eligibility criteria included age greater than 18 y, T3/4 or node positive biopsy proven rectal adenocarcinoma suitable for preoperative CRT and surgical resection, able to undergo ^{18}F-FDG PET/CT and MRI imaging, and gross disease visible on MRI. Eligible patients required an Eastern Cooperative Group performance status of 0–2. Patients were excluded if they had previously received radiotherapy to the pelvis. A subset of 16 patients with available diagnostic biopsies obtained prior to preoperative CRT were included in the current study.

Patients received 45 Gy in 25 fractions to the posterior pelvis and 50 Gy in 25 fractions to the gross tumor, lymph nodes, and associated mesorectal fascia. Treatment was delivered over 5 weeks, with nine fractions per fortnight. Capecitabine was administered orally at 1.5 g twice daily Monday to Friday during radiotherapy. Fifteen patients underwent a total mesorectal excision 8–10 weeks after the completion of CRT. A single patient opted not to undergo surgical resection being deemed to have a clinical complete response by medical imaging and this was confirmed by biopsy. Patient treatment and study timeline are summarized in Supplementary Figure S1.

Pathologic tumor response evaluation

Pathology results were synoptically recorded as per the Royal College of Pathology Australia colorectal cancer structured reporting protocol (2nd and 3rd edition). Tumor stage (AJCC 6th edition)[11], histological grade, depth of invasion, margin status, lymph node involvement, microsatellite instability and BRAF (V600E) mutation status were reported. Pathologic tumor response was assessed using the AJCC Tumor Regression Grade (TRG) scoring[12] where 0 = complete response with no viable cancer cells, 1 = moderate response with single cells or rare small groups of cancer cells, 2 = minimal response with residual cancer with evident tumor regression, and 3 = poor response with extensive residual cancer with no evident tumor regression. The single patient with clinical complete response was included as TRG 0 for the purposes of this study.

Multiplexed immunohistochemistry staining

First, 4 µm formalin-fixed paraffin-embedded sections of tumor biopsies obtained prior to preoperative CRT were deparaffinized and antigen retrieved for 20 min in a microwave using either 10 mM sodium citrate and 0.05% (v/v) tween 20 (pH 6) or 10 mM tris base with 1 mM EDTA and 0.05% (v/v) tween 20 (pH 9). The slides were cooled to room temperature, then blocked first using 3% hydrogen peroxide for 30 min, followed by protein block (Akoya, Marlborough, USA) for 10 min. Slides were stained using the Opal Kit (Akoya) with reagents and dilutions as described in Supplementary Table S1. In brief, slides were mounted in a Sequenza staining rack (Thermo Fisher Scientific, Waltham, USA) and incubated with the primary antibody for 35 min at room temperature. Slides were then washed and incubated with undiluted Opal polymer horseradish peroxidase (HRP), undiluted MACH3 antibody polymer HRP (Biocare Medical, Pacheco, USA), or 2 µg/mL donkey anti-sheep IgG conjugated to HRP (A16041, Thermo Fisher Scientific, Waltham, USA) for 10 min. After washing, the slides were incubated with the opal fluorophore using tyramide signal amplification for 10 min. In order to multiplex the antibodies, the slides were antigen retrieved prior to restarting the process with incubation of the next antibody. Following the last antibody stain, slides were antigen retrieved, stained with 5 µg/mL DAPI for 10 min and mounted using Prolong Diamond antifade (Thermo Fisher Scientific). The slides were imaged on the Mantra microscope (Akoya) with a minimum of three fields of views and images analyzed on inForm software (Akoya). An image analysis algorithm that included spectral unmixing, cell segmentation, and immune cell phenotyping was applied in a batch manner to ensure consistent analysis. For T cell subset analysis, segmented single cell data obtained from inForm were converted into fcs files using the R package, Spectre[13]. The cell subsets were then analyzed and quantified using FlowJo (v10.8).

Statistical analysis

Statistical analysis was conducted using Graphpad Prism (9.3.1). Patient characteristics were analyzed using Chi-square tests for qualitative variables and Student's t-test for quantitative variables. For comparisons between TRG 0 and 1 versus TRG 2 and 3, normality was assessed using D'Agostino and Pearson test. Student's t-test was used if the groups were normally distributed; otherwise, the Mann-Witney test was used. P-value <0.05 was considered significant. Predictive ability of immune cells was assessed using receiver operating characteristic (ROC) curves. Assessment of patient survival was conducted using Kaplan-Meier curves with Log-rank Mantel-Cox test. A p-value <0.05 was considered significant.

Results

Patient characteristics

This study included 15 males and one female with a median age at diagnosis of 61 (interquartile range of 52–69) (Table 1). Nine patients (56.3%) had low rectal cancer (0–5 cm from anal verge), five patients (31.3%) had mid rectal cancer (5–10 cm), and two patients (12.5%) had high rectal cancer (10–15 cm). Histologically, 13 (81.3%) of these tumors were moderately differentiated whilst two (12.5%) were well differentiated and one (16.7%) was of high grade. After preoperative CRT and subsequent resection, histopathological assessment revealed three patients (25%) were tumor regression grade (TRG) 0, four patients (25%) were TRG 1, six patients (37.5%) were TRG

Table 1. Patient characteristics.

Variable	Total patients ($n = 16$)	TRG 0 and 1 patients ($n = 8$)	TRG 2 and 3 patients ($n = 8$)	p-Value
Gender				
Male	15	7	8	*ns*
Female	1	1	0	
Age at diagnosis, y				
Mean (interquartile range)	61 (54–69)	61 (52–71)	62.5 (52–69)	*ns*
Preoperation				
Tumour location				
Low	9	6	3	*ns*
Mid	5	2	3	
High	2	0	2	
Histological grading				
Low grade				*ns*
Well differentiated	2	1	1	
Moderately differentiated	13	7	6	
grade	1	0	1	
Clinical stage				
cT2	3	1	2	*ns*
cT3	12	7	5	
cT4	1	0	1	
cN0	4	1	3	*ns*
cN1	8	5	3	
cN2	3	2	1	
cM0	15	8	7	*ns*
cM1	1	0	1	
Pathological response				
TRG 0	4	4	0	**
TRG 1	4	4	0	
TRG 2	6	0	6	
TRG 3	2	0	2	
Post-operation#				
Pathologic stage				
ypT0	3	3	0	*
ypTis	1	1	0	
ypT1	1	1	0	
ypT2	4	2	2	
ypT3	6	0	6	
ypN0	12	6	6	*ns*
ypN1	3	1	2	
ypM0	15	7	8	
EMVI: absent	10	4	6	*
EMVI: present	2	0	2	
EMVI: N/A	4	4	0	
MMR: proficient	11	4	7	*ns*
MMR: deficient	2	1	1	
MMR: N/A	3	3	0	
BRAF (V600E): wild type	13	5	8	*ns*
BRAF (V600E): mutated	0	0	0	
BRAF (V600E): N/A	3	3	0	

[#]One patient had clinical complete response.
* $p \le 0.05$; ** $p \le 0.01$.

2, and two patients (12.5%) were TRG 3. One patient achieved clinical complete response confirmed by biopsy and was included in the TRG 0 group for the purpose of this study. Analysis of TRG 0 and 1 patients versus TRG 2 and 3 patients showed no statistical difference in gender, age, tumor location and histological grade prior to CRT, or mismatch repair (MMR) and BRAF status assessed post-resection.

TRG 0 and 1 patients had higher tumor infiltration of IRF8$^+$ cells and CD3$^+$ T cells at diagnosis.

To determine if specific immune cell subsets in pre-CRT biopsies may be predictive of patient response, we conducted a 7-color multiplex immunohistochemistry stain of LARC biopsies (Figure 1a). Using the markers CD3, CD8, CD11c, CD68, and CD20, we derived that the total number of immune cells detectable using this panel was significantly fewer in the TRG 2 and 3 patients compared to TRG 0 and 1 patients (Figure 1b). Indeed, the same trend was observed when total detected immune cells were calculated as a proportion of all cells (DAPI$^+$), suggesting an association between tumor immune infiltration and tumor regression following CRT treatment.

To interrogate which immune cell subsets were decreased in TRG 2 and 3 patient biopsies, we classified immune cells into T cells (CD3$^+$), B cells (CD20$^+$HLA-DR$^+$), CD68$^+$ myeloid cells (CD3$^-$CD20$^-$CD11c$^-$CD68$^+$HLA-DR$^+$), and CD11c$^+$ myeloid cells (CD3$^-$CD20$^-$CD11c$^+$HLA-DR$^+$). There was no significant difference in the infiltration of B cells, CD68$^+$ myeloid cells, or CD11c$^+$ myeloid cells between TRG 0 and 1 patients and TRG 2 and 3 patients (Figure 1c). We also observed in our staining a CD3$^-$CD20$^-$CD11c$^-$CD68$^-$HLA-DR$^+$ subset which we

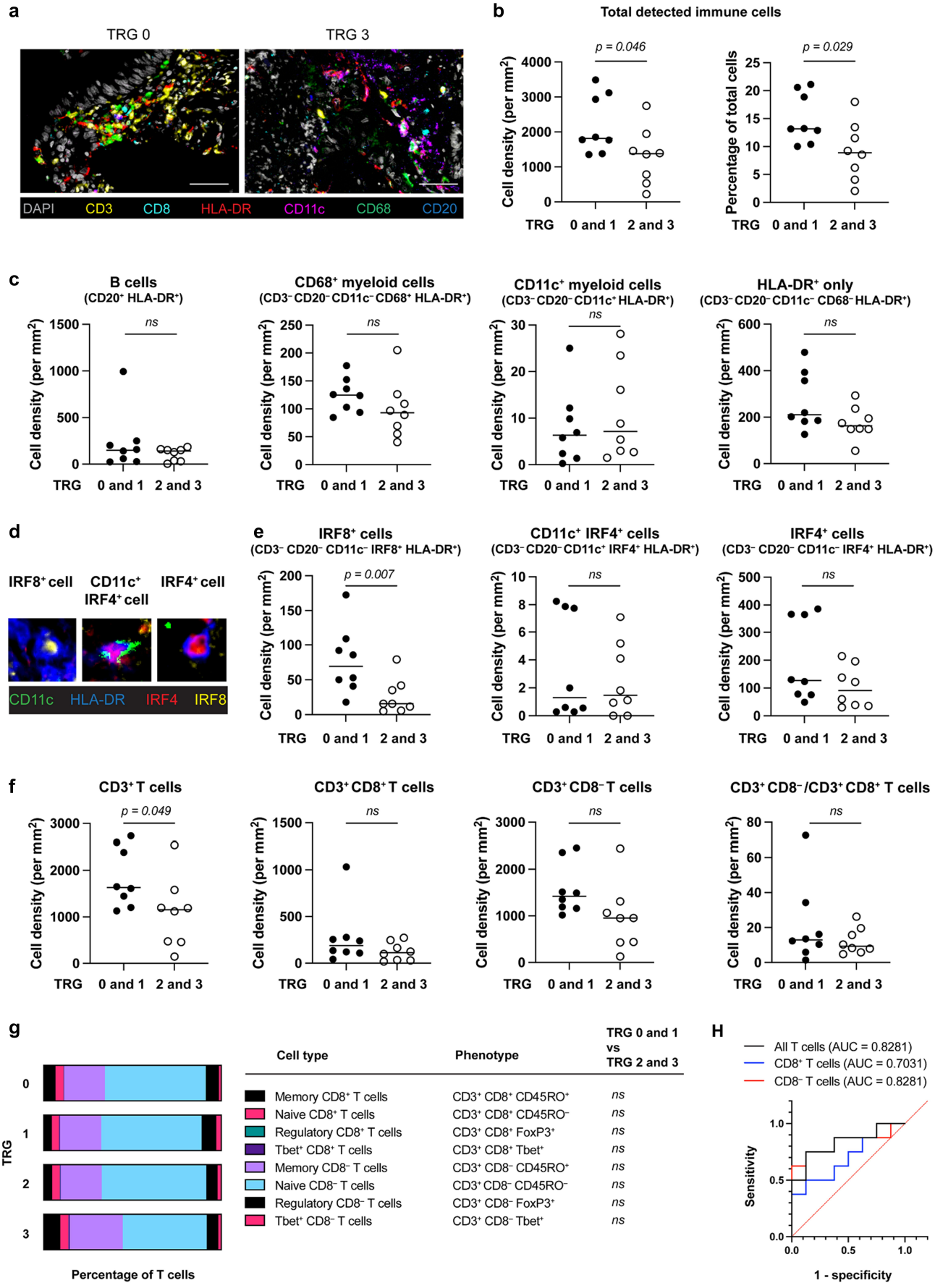

Figure 1. Pre-CRT biopsies of tumor-regression grade 0 and 1 patients show higher infiltration of IRF8$^+$ cells and CD3$^+$ T cells. Rectal tumor biopsies obtained prior to preoperative CRT were stained for immune cells using multiplex immunohistochemistry and compared against tumor regression grade (TRG) post-CRT. (a) Representative images of multiplex IHC panel containing CD3, CD8, CD20, CD68, CD11c, and HLA-DR. Scale bar represents 50 µm. (b) Comparison of total detected immune cells in biopsies as cell density and percentage of total cells. (c) Density of B cells, CD68$^+$ myeloid cells, CD11c$^+$ myeloid cells, and HLA-DR$^+$ only cells analyzed

termed HLA-DR$^+$-only cells. This subset was also unchanged between TRG 0 and 1 patients and TRG 2 and 3 patients.

Conventional markers CD68 and CD11c can be expressed on multiple myeloid populations, including macrophages and dendritic cells (DCs)[14], and cannot be used solely to distinguish between human DC subsets which have different functions in anti-tumor immunity[15]. Therefore, we conducted additional staining using IRF4 and IRF8 transcription factors to further phenotype the CD3$^-$CD20$^-$ population (Figure 1d). Conventional DC type 1 (cDC1) cells, conventional DC type 2 (cDC2), and plasmacytoid DC (pDC) cells are CD11c^{low}IRF4$^-$IRF8$^+$HLA-DR$^+$, CD11c$^+$IRF4$^+$IRF8$^-$ HLA-DR$^+$, CD11c$^-$IRF4$^-$IRF8$^+$ HLA-DR$^+$, respectively, and are negative for T cell marker CD3, B cell marker CD20, natural killer cell marker CD56, and monocyte marker CD14[14]. Using 7-color multiplex IHC, we identified an IRF8$^+$ cell subset (CD3$^-$CD20$^-$CD11c$^-$IRF8$^+$HLA-DR$^+$), a CD11c$^+$IRF4$^+$ cell subset (CD3$^-$CD20$^-$CD11c$^+$IRF4$^+$HLA-DR$^+$), and an IRF4$^+$ cell subset (CD3$^-$CD20$^-$CD11c$^-$IRF4$^+$HLA-DR$^+$) (Figure 1d). TRG 0 and 1 patients had significantly higher density of IRF8$^+$ cells in their rectal cancer biopsy compared to TRG 2 and 3 patients (Figure 1e). No difference was observed for CD11c$^+$IRF4$^+$ cells and IRF4$^+$ cells.

The density of T cells and CD8$^+$ cytotoxic T cells, known as the immunoscore, has been used in combination with neoadjuvant CRT and medical imaging to identify rectal cancer patients who respond to neoadjuvant CRT[8]. We interrogated T cell density using multiplex IHC and observed that TRG 0 and 1 patients had significantly greater T cell density (CD3$^+$) compared to TRG 2 and 3 patients (Figure 1f). The decrease in T cell density in TRG 2 and 3 patients appear to be responsible for majority of the decrease seen in total detected immune cells (Figure 1b). Separation into CD3$^+$CD8$^-$ T cells and CD3$^+$CD8$^+$ T cells showed a similar though non-significant trend in CD3$^+$CD8$^-$ T cells but not the CD3$^+$CD8$^+$ T cell subset as previously described[8]. Other literatures have reported CD4/CD8 ratio as prognostic markers in colorectal cancer and other cancers[16]. Here, no difference was observed in CD3$^+$CD8$^-$/CD3$^+$CD8$^+$ cells between TRG 0 and 1 patients and TRG 2 and 3 patients.

Since many subsets of T cells exist, we further phenotyped T cells to assess if specific subsets are responsible for the decrease seen in TRG 2 and 3 patient biopsies. However, the proportions of naïve (CD45RO$^-$), memory (CD45RO$^+$), regulatory (FoxP3$^+$), and Tbet-expressing CD3$^+$CD8$^+$ and CD3$^+$CD8$^-$ T cells as a percentage of total T cells were consistent across all TRG (Figure 1g). Comparison of these T cell subsets between TRG 0 and 1 patients and TRG 2 and 3 patients also revealed no significant changes, indicating that good CRT response is associated with a nonspecific accumulation in T cells. Indeed, receiver-operating characteristic (ROC) analysis showed that all CD3$^+$ T cells and CD3$^+$CD8$^-$ T cells had a greater area under the curve than cytotoxic T cells and had the highest predictability of TRG 0–1 (Figure 2c). Together, patients with improved CRT response had greater tumor infiltration of IRF8$^+$ cells and CD3$^+$ T cells prior to commencing CRT.

Low infiltration of CD11c$^+$ cells and high infiltration of IRF8$^+$ cells are associated with improved survival

We next assessed the association of immune subsets in the diagnostic biopsy with patient survival. Strong CRT-induced regression patients (TRG 0 and TRG 1) trended for increased 3 and 5 y disease-free survival (DFS) compared to TRG 2 and TRG 3 patients, although this did not meet statistical significance ($p = 0.27$) (Figure 2a). No significant difference in disease-specific survival (DSS) based on TRG was observed. Stratification of patients into those with higher than median infiltration of T cells and those with lower than median did not show differences in DFS or DSS (Figure 2b).

In contrast, patients with lower than median infiltration of CD11c$^+$ myeloid cells had significantly greater 3 y ($p = 0.038$) and 5 y DFS ($p = 0.038$) compared to patients with higher than median infiltration of CD11c$^+$ myeloid cells, where 88% of these patients were disease-free after 5 y (Figure 2c). While a trend for improved DSS for patients with lower than median infiltration of CD11c$^+$ cells was also seen, this did not reach significance. Separation of patients into those higher than median or lower than median infiltration of CD11c$^+$IRF4$^+$ cells showed no significant differences in survival (Figure 2d), demonstrating that the IRF4$^+$ subset of CD11c$^+$ cells was not solely responsible for the improved survival seen in Figure 2c. Finally, stratification of patients into higher than median or lower than median density of IRF8$^+$ cells showed a significant improvement in 5 y DSS for patients with high density ($p = 0.034$, Figure 2e) with 83% patients alive at 5 y. A trend for improved DFS was also observed in patients with high IRF8$^+$ cell density but this did not reach statistical significance ($p = 0.18$). Together this suggests that low infiltration of CD11c$^+$ myeloid cells and a high density of IRF8$^+$ cells in rectal tumors are associated with improved patient survival.

Discussion

Rectum preservation following neoadjuvant CRT has been proposed as a possibility in patients with complete clinical regression. Previous studies have highlighted the value of T cells as a predictor of tumor regression[7,8]. Here, we showed that patients with TRG 0 and 1 have higher total T cell infiltration in the diagnostic biopsy, but this was not associated with improved survival in this small cohort. Rather, we made the novel observation that patients with low CD11c$^+$ myeloid cell density have significantly beneficial long-term (>3 y) DFS and that high density of IRF8$^+$ cells is associated with both better CRT response and 5 y DSS.

against TRG. (d) Representative images of multiplex IHC panel containing CD3, CD20, CD11c, HLA-DR, IRF4, and IRF8. Left image shows representative IRF8$^+$ cell. Middle image shows representative CD11c$^+$IRF4$^+$ cell. Right image shows representative IRF4$^+$ cell. (e) Density of IRF8$^+$ cells, CD11c$^+$IRF4$^+$ cells, and IRF4$^+$ cells analyzed against TRG. (f) Density of CD3$^+$ T cells, CD3$^+$CD8$^+$ T cells, and CD3$^+$CD8$^-$T cells, and proportion of CD3$^+$CD8$^-$ to CD3$^+$CD8$^+$ T cells, analyzed against TRG. (g) Proportion of T cell subsets as percentage of CD3$^+$ T cells. Statistics conducted using Student's t-test or Mann-Witney test. (H) ROC analysis of total T cell, cytotoxic T cell and helper T cell density. All analyses comparing TRG 0 and 1 versus TRG 2 and 3 were conducted using Student's t-test or Mann-Witney test.

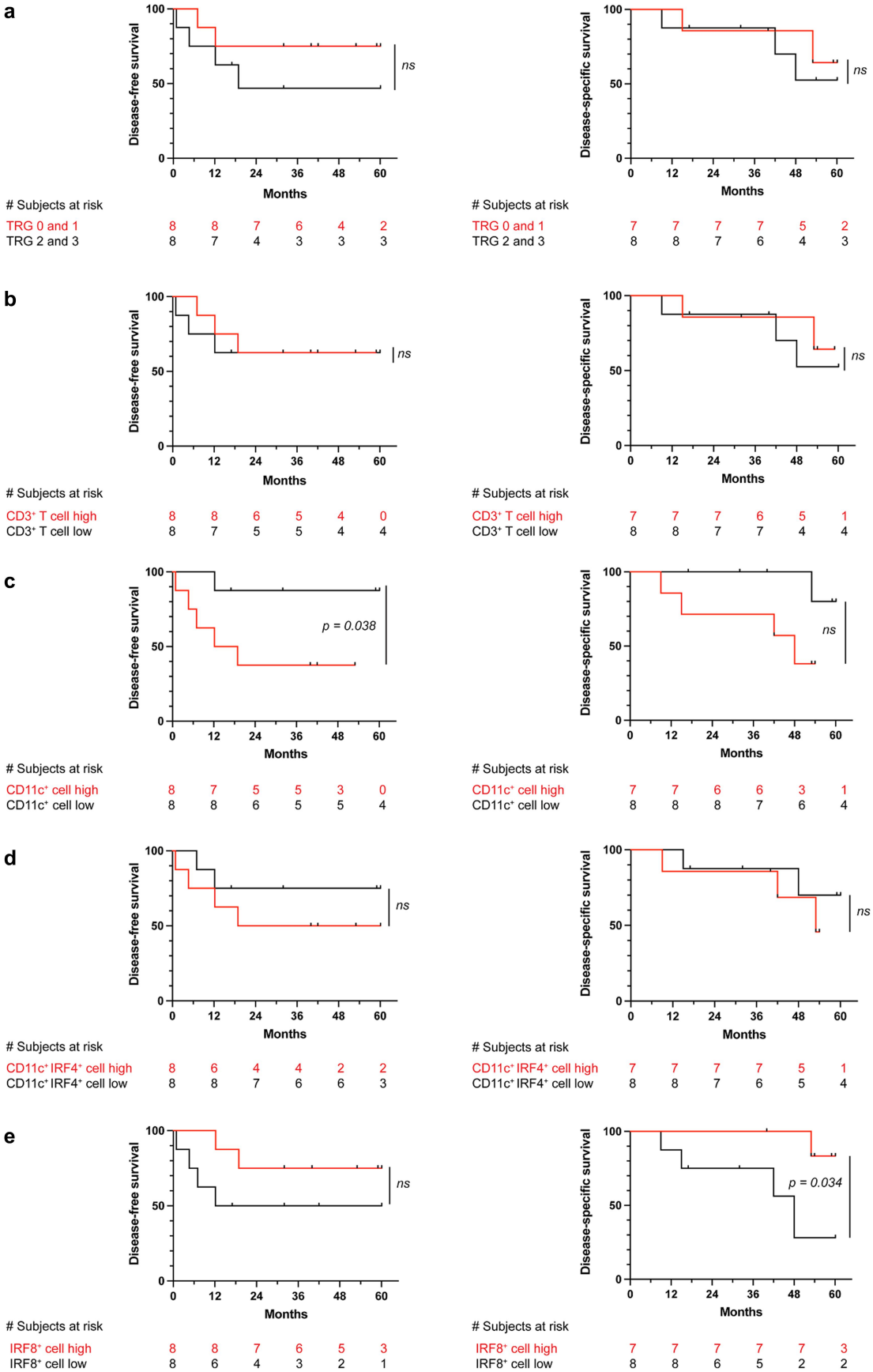

Figure 2. Pre-CRT biopsies containing low $CD11c^+$ myeloid cell density and high $IRF8^+$ cell density associated with improved survival outcomes. Patient disease-free survival and disease-specific survival from date of surgery were analyzed for (a) TRG, (b) $CD3^+$ T cell density, (C) $CD11c^+$ myeloid cell ($CD3^-CD20^-CD11c^+HLA\text{-}DR^+$) density, (d) $CD11c^+IRF4^+$ cell ($CD3^-CD20^-CD11c^+IRF4^+HLA\text{-}DR^+$) density, and (e) $IRF8^+$ cell ($CD3^-CD20^-CD11c^-IRF8^+HLA\text{-}DR^+$) density. Cell density was defined as number of immune cells per mm^2 of tissue. Patients were stratified into high or low immune cell density using the median. Statistics conducted using the log-rank, Mantel-Cox test.

The findings in this study support the notion that high tumor T cell density is associated with tumor regression[8,17]. However, the subset of T cells responsible for this response is unclear. Whilst some studies have noted $CD8^+$ densities to be associated with TRG[7,18,19], others have indicated $CD4^+$ densities to be important in tumor regression[7,17] and tumor shrinkage rate as determined through MRI[17]. It is critical to note that majority of past studies stained for CD4 and CD8 independently with or without CD3 in their immunohistochemistry analysis. Our study, which utilized multiplexed immunohistochemistry to co-stain CD3 and CD8, enabled us to clearly delineate helper T and $CD8^+$ T cells. This allowed our observation that $CD3^+CD8^-$ population was largely responsible for the T cell association with CRT response. This finding is consistent with Miyakita et al. whose analysis, using CD3, CD4, and CD8 immunohistochemical staining, identified that high density of $CD4^+$ cells in biopsies obtained 7 d prior to CRT treatment was significantly associated with tumor regression[17].

Our attempts to further immunophenotype T cells into memory or naïve T cells and Tbet or FoxP3- expressing T cells yielded no associations of these subsets with patient response to CRT. In fact, comparisons across pre-treatment tumor biopsies of subsequent TRG 0–3 patients showed similar distributions of T cell subsets as a proportion of total T cells. In contrast, Kitagawa et al. showed that high intratumoural $CD8^+$ and PD-1^+ cell density was independently associated with tumor regression and improved recurrence-free survival[18]. Park et al. also noted that TRG 0 patients had higher proportion of PD-$L1^+$ tumor cells, which could be used to predict response[20]. Attempts to investigate FoxP3 have conflicting results with some suggesting high density of $FoxP3^+$ cells significantly associated with tumor regression and tumor shrinkage rate[17]. Others, however, are consistent with our findings whereby FoxP3 did not predict TRG and was not associated with long-term survival[18,21]. Studies by these groups agree though that low $FoxP3^+$ cell density post-CRT predict improved tumor regression, tumor shrinkage rates, and recurrence-free survival[17,22]. Whilst our study adhered to a rigorous CRT protocol, the above study cohorts showed variations in CRT dose and schedule and were conducted in mostly cT3 patients.

Here, we showed for the first time that low $CD11c^+$ myeloid cell density and high $IRF8^+$ cell density within the tumor prior to neoadjuvant CRT are associated with improved survival in LARC patients. We confirmed that these populations were not lymphocytes as they were negative for T and B cell markers, CD3 and CD20. Both populations were also HLA-DR^+, suggesting that they were subsets of dendritic cells (DCs) and/or macrophages. Our multiplex IHC protocol allowed for a maximum of seven markers, which did not allow for inclusion of additional markers such as CD14 or CD88 to better distinguish between DCs and macrophages and markers such as CD45 and cytokeratin to conclusively confirm the origin of the $IRF8^+$ expressing cells.

We attempted to validate our findings using RNA sequencing data from a larger rectal cancer study[23]. From this cohort, 97 patients had transcriptomic data from pre-treatment biopsies and were treated with a variety of neoadjuvant chemotherapy protocols. We identified 19 patients with RNA sequencing data who received neoadjuvant CRT in the form of capecitabine or 5FU, best matching our protocol. Comparison of AJCC TRG 0 and 1 ($n = 10$) patients versus TRG 2 and 3 ($n = 9$) patients showed no significant difference in CD11c (*ITGAX*), *IRF4*, or *IRF8* expression, contrasting our finding that $IRF8^+$ cells were significantly lower in TRG 2 and 3 patients. This difference may be due to bulk RNA sequencing in this study compared to IHC protein expression used at a single cell level by us. Comparison of activated DC, immature DC, macrophage, and T cell infiltration inferred from single-sample gene set enrichment analysis (ssGSEA) scores as defined using the established CRC immune signatures[24] also showed no significant difference between TRG 0 and 1 versus TRG 2 and 3, and no association between these cells and survival for the 19 patients. It is essential to note that CD11c (*ITGAX*), *IRF4*, and *IRF8* were not used in the CRC immune signatures to define the DC subsets. DCs were also not subdivided into cDC1 and cDC2, giving limited DC phenotype information, in contrast to our findings. Therefore, future studies with a larger cohort investigating rectal cancer at the single cell level will be important for validating our observations.

Dendritic cells are important in initiating immune responses by sampling and presenting antigens to naïve T cells in lymph nodes and regulating T cell responses in tissue through cell-to-cell contact and cytokine production[25]. Previous studies have shown that CRC patients with high infiltration of $S100^+$ DCs have improved survival rates compared to those with low infiltration, although correcting for tumor stage abrogated this association[26]. The reason for the association between low CD11c myeloid cell density and high $IRF8^+$ density and beneficial survival in rectal cancer patients receiving neoadjuvant CRT was not investigated in our study. Others have noted that rectal tumor DC functions (defined by cytokine secretion and marker expression) are altered following CRT, including the upregulation of co-stimulatory marker CD80 but also the immune-suppressive marker PD-L1[27]. Future research would examine whether rectal tumors with low CD11c myeloid density also have low PD-L1 expression leading as a contributor to improved anti-tumor immunity and prolonged survival.

While single nucleotide polymorphism in IRF8 gene has been associated with rectal cancer risk[28], very little is known about the involvement of $IRF8^+$ immune cells in CRC. Previous literature noted that pDC infiltration, assessed by BDCA-2 (CD303) expression, is significantly increased following CRT but its association with survival is unknown[29]. Here, the $IRF8^+$ cell population, which was $CD11c^-$ and thus included pDCs, is linked to prolonged DSS. However, the $IRF8^+$ population could also include cDC1s, $IRF8^+$ macrophages, and potentially $IRF8^+$ tumor cells. Future studies should include BDCA-2, CD14, and CD45 to specifically identify the pDC population and validate these findings in a larger cohort. This may require techniques such as imaging mass cytometry or cyclic immunofluorescence which allow for greater degree of antibody multiplexing.

High IRF8 expression in the context of other solid cancers has also been associated with beneficial effects. In grade 2 and 3 estrogen-receptor negative breast cancers, IRF8 protein expression can predict response to trastuzumab treatment and

patients with high IRF8 expression have prolonged recurrence-free survival[30]. Similarly, analysis of TCGA data for renal cell carcinoma showed that patients with high IRF8 expression had improved DFS[31]. Patients with high IRF8 in metastatic sites also had prolonged overall survival. These studies, however, did not directly demonstrate that immune cell populations were responsible for the elevated IRF8 expression. Here, we show that high IRF8$^+$ cell density is associated with prolonged DSS in LARC. Of interest, 10 out of the 16 patients had an inverse relationship between IRF8$^+$ cell and CD11c$^+$ cell densities (i.e. high IRF8$^+$ and low CD11c$^+$ cell density or low IRF8$^+$ and high CD11c$^+$ cell density). This suggests that patients with a combined high IRF8$^+$ and low CD11c$^+$ cell densities may achieve the greatest survival outcomes. Future studies with larger cohorts are required to confirm the association of myeloid cell densities and patient survival and will be able to dissect the relationship between these myeloid cell populations in greater detail.

The association between CD11c$^+$ myeloid cell density and DFS may have prognostic value for selecting watch-and-wait patients. The watch-and-wait management strategy offers rectum preservation in patients with a clinical complete response following CRT[5]. However, intense follow-up schedules are required due to an increased risk of local recurrence in 15–30% of watch-and-wait patients after a median of 9 months[5,32]. The majority of watch-and-wait local recurrences were mucosal and salvageable in 94% of patients[32]. There are no current biomarkers to predict local recurrence in watch-and-wait patients. In this study, the association of CD11c$^+$ myeloid cell density with recurrence was shown but we did not have watch-and-wait patients to assess the prognostic value in this cohort. Future investigations should assess the efficacy of this CD11c$^+$ population as a predictive biomarker of recurrence in watch-and-wait patients to help stratify high-risk patients for follow-up.

In conclusion, this study highlighted the predictive ability of infiltrating pan-T cells and IRF8$^+$ cells as predictors of tumor regression and DSS, respectively, following neoadjuvant CRT in LARC patients. Tumor CD11c$^+$ myeloid cells were identified as a potential predictive biomarker for recurrence following CRT and resection of rectal cancer. IRF8$^+$ cell density may also act as a potential biomarker of LARC patient survival. These molecular markers may be used in the future in combination with other correlates of TRG, such as pre-treatment carcinoembryonic antigen, mucin status, interval between end of radiation and surgery, distance from anal verge, and imaging modalities, to establish a multiparametric prediction of response.

Abbreviations

Chemoradiotherapy	CRT
Colorectal cancer	CRC
Horseradish peroxidase	HRP
Locally advanced rectal cancer	LARC
Receiver operating characteristic	ROC
Tumour regression grade	TRG

Acknowledgments

We thank David Nevell and Geoff Yuet Mun Wong for their assistance with this study. Aspects of this research were conducted with the Australian Centre for Microscopy and Microanalysis.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

MPM acknowledges funding support from the à Beckett Cancer Research Trust and Bowel Cancer Research Foundation Australia. PS is recipient of Cancer Institute NSW early career fellowship (2019/ECF011)..

ORCID

Mark P. Molloy http://orcid.org/0000-0003-4679-5868

Data availability statement

The data that support the findings of this study are available from the corresponding author, MPM, upon reasonable request.

Author contributions

Conceptualization: BT, PS, SB, AE, AK, MPM
Formal Analysis: BT
Investigation: BT, PS
Methodology: BT, SB, GH, NP, SJC, JE, AE, MPM
Resources: SB, GH, NP, SJC, JE, AE, AK, MPM
Supervision: AK, MPM
Writing original draft: BT
Writing review and editing: SB, AE, AK, MPM

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA A Cancer J Clin. 2021;71(3):209–249. doi:10.3322/caac.21660.
2. Vuik FE, Nieuwenburg SA, Bardou M, Lansdorp-Vogelaar I, Dinis-Ribeiro M, Bento MJ, Zadnik V, Pellisé M, Esteban L, Kaminski MF, et al. Increasing incidence of colorectal cancer in young adults in Europe over the last 25 years. Gut. 2019;68(10):1820–1826. doi:10.1136/gutjnl-2018-317592.
3. Wan T, Zhang XF, Liang C, Liao CW, Li JY, Zhou YM. The prognostic value of a pathologic complete response after neoadjuvant therapy for digestive cancer: systematic review and meta-analysis of 21 studies. Ann Surg Oncol. 2019;26(5):1412–1420. doi:10.1245/s10434-018-07147-0.
4. Jalilian M, Davis S, Mohebbi M, Sugamaran B, Porter IW, Bell S, Warrier SK, Wale R. Pathologic response to neoadjuvant treatment in locally advanced rectal cancer and impact on outcome. J Gastrointest Oncol. 2016;7(4):603–608. doi:10.21037/jgo.2016.05.03.
5. Dossa F, Chesney TR, Acuna SA, Baxter NN. A watch-and-wait approach for locally advanced rectal cancer after a clinical complete response following neoadjuvant chemoradiation: a systematic review and meta-analysis. Lancet Gastroenterol Hepatol. 2017;2(7):501–513. doi:10.1016/S2468-1253(17)30074-2.
6. Matsushita H, Vesely MD, Koboldt DC, Rickert CG, Uppaluri R, Magrini VJ, Arthur CD, White JM, Chen Y-S, Shea LK, et al. Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. Nature. 2012;482(7385):400–404. doi:10.1038/nature10755.

7. Yasuda K, Nirei T, Sunami E, Nagawa H, Kitayama J. Density of CD4(+) and CD8(+) T lymphocytes in biopsy samples can be a predictor of pathological response to chemoradiotherapy (CRT) for rectal cancer. Radiat Oncol. 2011;6(1):49. doi:10.1186/1748-717X-6-49.
8. El Sissy C, Kirilovsky A, Van den Eynde M, Musina AM, Anitei MG, Romero A, Marliot F, Junca A, Doyen J, Mlecnik B, et al. A diagnostic biopsy-adapted immunoscore predicts response to neoadjuvant treatment and selects patients with rectal cancer eligible for a watch-and-wait strategy. Clin Cancer Res. 2020;26 (19):5198–5207.
9. Sendoya JM, Iseas S, Coraglio M, Golubicki M, Robbio J, Salanova R, Kujaruk M, Mikolaitis V, Rizzolo M, Ruiz G, et al. Pre-existing tumoral B cell infiltration and impaired genome maintenance correlate with response to chemoradiotherapy in locally advanced rectal cancer. Cancers. 2020;12(8):2227. doi:10.3390/cancers12082227.
10. Yang Y, Tian W, Su L, Li P, Gong X, Shi L, Zhang Q, Zhao B, Zhao H. Tumor-infiltrating cytotoxic T cells and tumor-associated macrophages correlate with the outcomes of neoadjuvant chemoradiotherapy for locally advanced rectal cancer. Front Oncol. 2021;11:743540. doi:10.3389/fonc.2021.743540.
11. Cancer AJCo. AJCC Cancer Staging Manual. 6 ed. F. L. Greene DLP, I. D. Fleming, A. G. Fritz, C. M. Balch, D. G. Haller, & M. Morrow, editor. New York, NY: Springer; 2002.
12. Cancer AJCo. AJCC Cancer Staging Handbook. 7 ed. Stephen B. Edge DRB, Carolyn C. Compton, April G. Fritz, Frederick L. Greene, Andrew Trotti, editors. New York, NY: Springer; 2010.
13. Ashhurst TM, Marsh-Wakefield F, Putri GH, Spiteri AG, Shinko D, Read MN, Smith AL, King NJC. Integration, exploration, and analysis of high-dimensional single-cell cytometry data using Spectre. Cytometry A. 2022;101(3):237–253. doi:10.1002/cyto.a.24350.
14. Mair F, Liechti T. Comprehensive phenotyping of human dendritic cells and monocytes. Cytometry A. 2021;99(3):231–242. doi:10.1002/cyto.a.24269.
15. Plesca I, Müller L, Böttcher JP, Medyouf H, Wehner R, Schmitz M. Tumor-associated human dendritic cell subsets: phenotype, functional orientation, and clinical relevance. Eur J Immunol. 2022;52 (11):1750–1758. doi:10.1002/eji.202149487.
16. Diederichsen AC, Hjelmborg J, Christensen PB, Zeuthen J, Fenger C. Prognostic value of the CD4+/CD8+ ratio of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells. Cancer Immunol Immunother. 2003;52 (7):423–428. doi:10.1007/s00262-003-0388-5.
17. Miyakita H, Sadahiro S, Suzuki T, Chan LF, Ogimi T, Okada K, Yamamoto S, Kajiwara H. Tumor-infiltrating lymphocytes in biopsy specimens obtained 7 days after starting chemoradiotherapy for rectal cancer are predictors of the response to chemoradiotherapy. Oncology. 2020;98(12):869–875. doi:10.1159/000508922.
18. Kitagawa Y, Akiyoshi T, Yamamoto N, Mukai T, Hiyoshi Y, Yamaguchi T, Nagasaki T, Fukunaga Y, Hirota T, Noda T, et al. Tumor-infiltrating PD-1+ immune cell density is associated with response to neoadjuvant chemoradiotherapy in rectal cancer. Clin Colorectal Cancer. 2022;21(1):e1–e11. doi:10.1016/j.clcc.2022.01.004.
19. Shinto E, Hase K, Hashiguchi Y, Sekizawa A, Ueno H, Shikina A, Kajiwara Y, Kobayashi H, Ishiguro M, Yamamoto J. CD8+ and FOXP3+ tumor-infiltrating T cells before and after chemoradiotherapy for rectal cancer. Ann Surg Oncol. 2014;21(Suppl S3): S414–421. doi:10.1245/s10434-014-3584-y.
20. Park IJ, An S, Kim SY, Lim HM, Hong SM, Kim MJ, Kim YJ, Yu CS. Prediction of radio-responsiveness with immune-profiling in patients with rectal cancer. Oncotarget. 2017;8 (45):79793–79802. doi:10.18632/oncotarget.19558.
21. McCoy MJ, Hemmings C, Anyaegbu CC, Austin SJ, Lee-Pullen TF, Miller TJ, Bulsara MK, Zeps N, Nowak AK, Lake RA, et al. Tumour-infiltrating regulatory T cell density before neoadjuvant chemoradiotherapy for rectal cancer does not predict treatment response. Oncotarget. 2017;8(12):19803–19813. doi:10.18632/oncotarget.15048.
22. McCoy MJ, Hemmings C, Miller TJ, Austin SJ, Bulsara MK, Zeps N, Nowak AK, Lake RA, Platell CF. Low stromal Foxp3+ regulatory T-cell density is associated with complete response to neoadjuvant chemoradiotherapy in rectal cancer. Br J Cancer. 2015;113(12):1677–1686. doi:10.1038/bjc.2015.427.
23. Chatila WK, Kim JK, Walch H, Marco MR, Chen CT, Wu F, Omer DM, Khalil DN, Ganesh K, Qu X, et al. Genomic and transcriptomic determinants of response to neoadjuvant therapy in rectal cancer. Nat Med. 2022;28(8):1646–1655. doi:10.1038/s41591-022-01930-z.
24. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, Angell H, Fredriksen T, Lafontaine L, Berger A, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity. 2013;39 (4):782–795. doi:10.1016/j.immuni.2013.10.003.
25. Wculek SK, Cueto FJ, Mujal AM, Melero I, Krummel MF, Sancho D. Dendritic cells in cancer immunology and immunotherapy. Nat Rev Immunol. 2020;20(1):7–24. doi:10.1038/s41577-019-0210-z.
26. Nagorsen D, Voigt S, Berg E, Stein H, Thiel E, Loddenkemper C. Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. J Transl Med. 2007;5(1):62. doi:10.1186/1479-5876-5-62.
27. Heeran AB, Dunne MR, Morrissey ME, Buckley CE, Clarke N, Cannon A, Donlon NE, Nugent TS, Durand M, Dunne C, et al. The protein secretome is altered in rectal cancer tissue compared to normal rectal tissue, and alterations in the secretome induce enhanced innate immune responses. Cancers. 2021;13(3):571. doi:10.3390/cancers13030571.
28. Slattery ML, Lundgreen A, Bondurant KL, Wolff RK. Interferon-signaling pathway: associations with colon and rectal cancer risk and subsequent survival. Carcinogenesis. 2011;32(11):1660–1667. doi:10.1093/carcin/bgr189.
29. Wagner F, Hölig U, Wilczkowski F, Plesca I, Sommer U, Wehner R, Kießler M, Jarosch A, Flecke K, Arsova M, et al. Neoadjuvant radiochemotherapy significantly alters the phenotype of plasmacytoid dendritic cells and 6-Sulfo LacNAc(+) monocytes in rectal cancer. Front Immunol. 2019;10:602. doi:10.3389/fimmu.2019.00602.
30. Gatti G, Betts C, Rocha D, Nicola M, Grupe V, Ditada C, Nuñez NG, Roselli E, Araya P, Dutto J, et al. High IRF8 expression correlates with CD8 T cell infiltration and is a predictive biomarker of therapy response in ER-negative breast cancer. Breast Cancer Res. 2021;23(1):40. doi:10.1186/s13058-021-01418-7.
31. Muhitch JB, Hoffend NC, Azabdaftari G, Miller A, Bshara W, Morrison CD, Schwaab T, Abrams SI. Tumor-associated macrophage expression of interferon regulatory Factor-8 (IRF8) is a predictor of progression and patient survival in renal cell carcinoma. J Immunother Cancer. 2019;7(1):155. doi:10.1186/s40425-019-0630-0.
32. van der Sande ME, Figueiredo N, Beets GL Management and outcome of local regrowths in a watch-and-wait prospective cohort for complete responses in rectal cancer. Ann Surg. 2021;274(6):e1056–e62.