Effect of Neoadjuvant Chemoradiation on Tumor-infiltrating/associated Lymphocytes in Locally Advanced Rectal Cancers

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Abstract. *Background: Lymphocytes and natural killer cells (NK) appear to be important in colorectal cancer. Their role in chemoradiotherapy for rectal cancers is unclear. We evaluated T-lymphocytes (CD3), sub-groups CD4 and CD8, and NK cells (CD56+CD57) in normal and rectal tumor tissues pre- and post-chemoradiotherapy, and investigated their relationship to tumor regression grade, disease-free survival and pathological stage. Materials and Methods: Tissue microarrays from colonoscopic biopsies, resection specimens and normal tissues, from 52 patients, were immunostained. Results: NK cell counts were significantly lower in tumor samples compared to normal tissues (p=0.007). T-lymphocyte counts were higher in post-treatment compared to pre-treatment samples (p=0.025), specifically in the CD8 subgroup after long-course treatment. The results suggested an association between post-treatment CD8 and NK cell counts with higher tumor regression. No associations were found with regard to stage or disease-free survival. Conclusion: NK cell counts were significantly reduced in rectal cancers compared to normal tissues, while total T-lymphocyte counts increased post-chemoradiotherapy. Both appeared important in tumor regression.*

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The immune system is increasingly recognised to play an important role in cancer (1-3). Antigens present on tumor cell surfaces, in combination with the breakdown products of stromal extracellular matrix, can elicit a host immune response through recognition of these epitopes by natural killer (NK) and T-lymphocytes.

Tumor-infiltrating/associated lymphocytes (TIL) have been shown to be a good prognostic marker in colorectal cancer (4-6). Increasingly, studies are focusing on T-cell subsets to better reflect their individual functional roles (7-10). The majority of studies have included both colon and rectal cancers. However, rectal cancers undergo a different treatment algorithm in the curative setting that may, in addition to their biological differences to colon cancers, confound interpretation of these correlative studies. T-lymphocytes do appear to play a role in rectal tumour control (11, 12); however, it is unclear as to how they are affected by neoadjuvant therapy.

NK cells have also been shown to have prognostic and predictive roles in colorectal cancer. NK activity has been found to be lower in tumor compared to normal tissue, lower in higher stage disease, correlate with survival and be affected detrimentally by chemoradiotherapy (13, 14).

In this context, we sought to further understand the role of the immunological microenvironment in patients with locally advanced rectal cancer treated with neoadjuvant radiotherapy with or without chemotherapy and to clarify their relationship to clinical outcomes.

Patients and Methods

Institutional ethics approval for this study was obtained from the Human Research Ethics Committee, South Western Sydney Local Health District, and conforms to the provisions of the Declaration of Helsinki in 1995. The institutional review board waived the need for written informed consent from the participants as the project was deemed to be in the low-risk category. Information was de-identified prior to analysis.

We aimed to compare CD3, CD4, CD8 and CD56+CD57 in normal rectal tissue and in tumor tissues sampled pre- and post-neoadjuvant radiotherapy with or without chemotherapy. We investigated the association of pre- and post-treatment findings with early tumor response as assessed histologically by the extent of tumour regression (TRG), as well as late tumor response measured clinically by disease-free survival (DFS). We also examined their relationship to the pathological stage.

A sample of 52 locally advanced rectal cancer patients with adequate clinical follow-up was randomly selected retrospectively from the colorectal and pathology databases at Royal Prince Alfred Hospital for the period 1998 to 2007. Locally advanced rectal cancer was defined as T3-4 or node-positive disease, with no evidence of distant disease, as staged clinically by digital rectal examination and imaging with computed tomography (CT) and magnetic resonance imaging (MRI). Biopsy confirmation of adenocarcinoma was obtained on endoscopic biopsy. Staging was based on the American Joint Committee on Cancer (AJCC) tumor-node-metastases (TNM) system. Pre-operative treatment consisted of either short-course radiotherapy or long-course radiotherapy. Short-course radiotherapy consisted of 25 Gy in 5 fractions, 5 Gy per fraction, over 5 days, followed by surgical resection one week later. Long-course chemoradiotherapy consisted of 45 to 50.4 Gy, 1.8 Gy per fraction, over 5 to 6 weeks, with concurrent infusional 5-fluorouracil (5-FU; 225 mg/m^2/day), followed by surgical resection 4 to 6 weeks later. Surgery consisted of total mesorectal excision, with anterior or abdominoperineal resection. Variables of interest included age, gender and pathological stage of tumor. Outcomes of interest were histological tumour regression in the resected bowel (5 tier Mandard grading, ranging from 1=no residual tumour to 5=no evidence of tumor regression (15)) and DFS. DFS was defined as time to first recurrence. Follow-up consisted of regular clinic visits, colonoscopy, blood tests and imaging at the discretion of the treating specialist.

For each patient, donor blocks of paraffin-embedded tissue from pre- and post-chemoradiotherapy rectal cancer, as well as the adjacent colorectal mucosa in the resected bowel were retrieved from the Anatomical Pathology Department of Royal Prince Alfred Hospital. The corresponding 156 hematoxylin and eosin sections were reviewed to localise the most representative areas of tumour and normal colorectal mucosa in the donor blocks, from which 1mm diameter tissue cores were obtained and transferred into the pre-drilled wells in the recipient tissue microarray (TMA) block using the Beecher© Manual tissue arrayer-1 (Beecher Instruments Inc., Sun Prairie, WI, USA). The recipient blocks were produced using the standard 37×24×5 mm mould. Shortly after their construction, the TMA blocks were heated for 5 min in a 60˚C oven to seal the gaps between tissue cores and surrounding paraffin. Tissue cores of the TMA were stained for CD3, CD4, CD8 and CD56+CD57 (Figures 1A and B). The immunostained sections were examined by manual counting of cells in each TMA dot, including both the cells amongst the tumour or normal epithelium and within the immediate stroma. The observer was blinded to clinical outcomes.

Paired *t*-tests were used to analyse differences in CD counts between pre-treatment, post-treatment and normal tissue. Analyses were also stratified by duration of radiotherapy. Logistic and ordinal regression was used to correlate CD counts with TRG (divided into 1-2 and 3-5) and stage respectively. This was adjusted for duration of radiotherapy. Cox regression was used to analyze DFS. Data analysis was generated using the SAS Enterprise Guide software (Version 5.1 for Windows, copyright© 2012 SAS Institute Inc.,Cary, NC, USA).

Results

The characteristics of the patient population are listed in Table I. The median age was 63 years, with 65% males and 35% females. The median follow-up was 3.2 years with median DFS of 2.8 years. The median counts for CD3, CD4, CD8 and CD56+57 are illustrated in Figure 1, with highest CD3, CD4 and CD8 counts evident in the post-treatment samples (Figures 2A-C) while in contrast, NK counts were lowest in the post-treatment samples (Figure 2D).

The difference in tumor NK cell counts pre- and post-treatment did not reach statistical significance (p=0.306, Table II). NK cell counts were significantly lower in rectal tumor samples compared to normal tissue (p=0.007, Table II). The higher T-lymphocyte count in post-treatment compared to pre-treatment samples was of borderline significance (p=0.025), whereas the change in CD8 (p=0.07) and CD4 (p=0.44) counts did not reach significance (Table II). However, when stratified by radiotherapy duration, CD8 count demonstrated a trend toward an increase in post-treatment rectal tumor samples in the long-course treatment group (p=0.04) (Figure 3A). No differences were found in the short-course radiotherapy group (Figure 3B).

Post-treatment CD8 was associated with higher tumor regression with an odds ratio (OR) of 1.01 (p=0.048) (Figure 4A). Similarly, post-treatment NK cell counts were associated with higher tumour regression (OR 1.04, p=0.038) (Figure 4B). We did not obtain any valid OR estimates when attempting to adjust for duration of radiotherapy due to the small sample size. No associations were found between the CD groups with pathological stage or DFS. The survival curves for CD8 and NK post-treatment counts with DFS are illustrated in Figure 5.

Discussion

Our study found that NK cell counts were significantly reduced in rectal cancer compared to normal tissues, which is consistent with other studies. We also found an association with NK count with tumour regression, albeit not with survival. What is equally found in literature and our study is the association of the presence of NK cells with a better

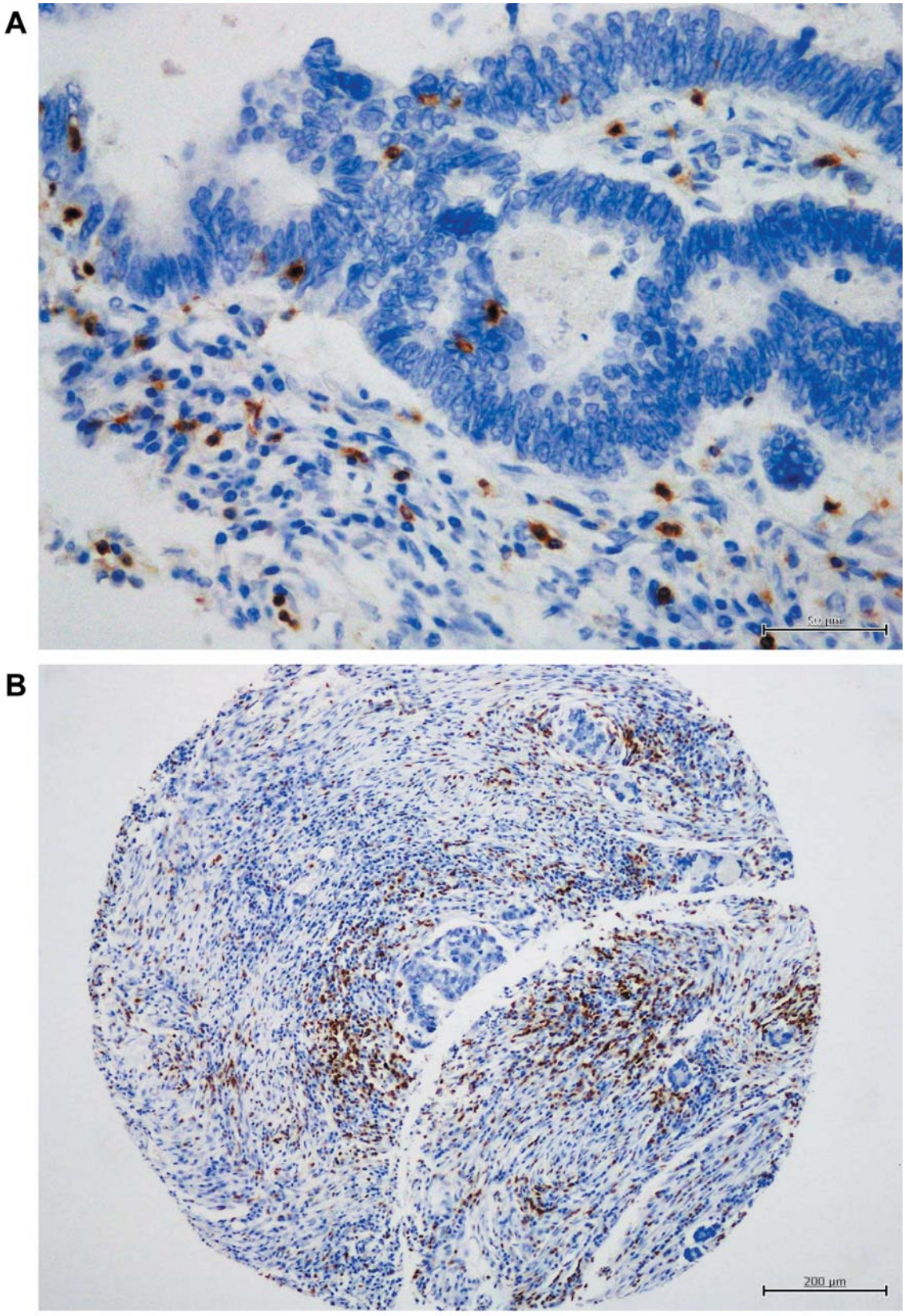

Figure 1. *Immunostaining for CD3 at different magnifications. A. CD3 staining ×400 magnification; B: CD3 staining ×100 magnification.*

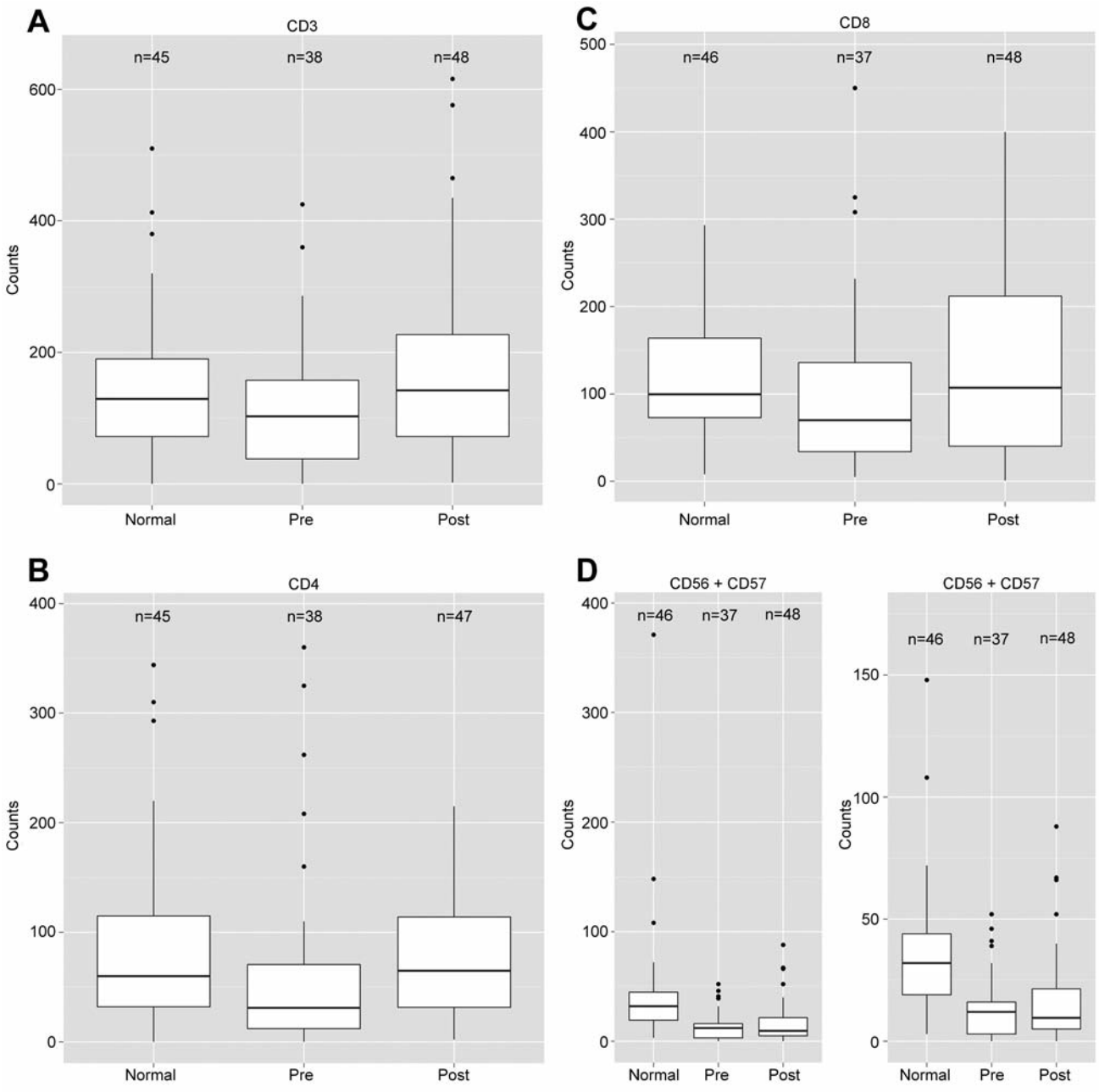

Figure 2. *Box and whisker plots for CD counts in normal, pre-treatment and post-treatment tissue. N refers to number of samples. A. CD3; B: CD4; C: CD8; D: CD56+57, the right panel excludes the outlier to better visualise the box plots.*

outcome. The role of NK cell activity in tumor tissue and its possible influence in rectal cancer control was previously studied by a Japanese group, that showed that mean NK activity was lower in tumor tissue compared to normal and lower in higher stage disease (13). Higher NK cell activity also correlated with 5-year metastasis-free rates, while NK activity fell after neoadjuvant chemoradiotherapy. In another study of 42 patients who had undergone resection for colon cancer, NK activating assays in tumor tissues had lower killing rates compared to normal controls (14), suggesting some impairment associated with the presence of tumor. Extensive intra-tumoral infiltration of NK cells was found to be associated with improved outcomes in another study of colorectal cancers, especially in stage III disease (16). More recently, 196 patients with stages II and III colorectal cancer were evaluated for immunohistochemical staining of CD3,

Table I. *Characteristics of the study population.*

Median age (years)	63
Gender (%)	male 65%, female 35%
Radiotherapy (%)	short-course 37%, long-course 63%
Pathological stage (%)	I=29%, II=38%, III=31%, no residual tumour=2%
Tumour regression grade (%)	1=2%, 2=15%, 3=38%, 4=37%, 5=7%
Median disease-free survival (years)	2.8
Median follow-up (years)	3.2

Note: Percentages may not add up to 100% due to rounding.

CD4, CD8, CD57 and CD68 (10). Lower CD57 and CD68 were significantly associated with worse DFS and overall survival. Decreased staining was found in samples obtained from tumor Centres compared to normal tissues, whereas pre-operative radiotherapy was found to increase expression of CD57 and CD68. The effect of neoadjuvant therapy on NK cells is unclear, with conflicting reports. We found the lowest median NK counts in the post-treatment samples, in contrast to the other CD groups; however, this small difference was not statistically significant.

These studies suggest an association of lower NK counts in the presence of colorectal cancer but, within the tumor tissue, a higher NK cell count was associated with improved survival. Studies in different tumor types support the same conclusion, for example in squamous cell lung cancer (17). In gastric adenocarcinomas, high NK infiltration using CD57 immunohistostaining correlated with earlier stage disease and improved survival (18). It is unclear whether NK cells represent an epiphenomenon or a conditioner of a pro-tumor microenvironment. There is, however, evidence that the absence of NK or its activity is thought to be present early on in the adenoma-carcinoma-metastasis sequence, as supported by a study quantifying infiltrating NK and T-cells, along with the chemokine and cytokine environment, in colorectal cancer samples, adenomas, adjacent normal mucosa and liver metastases (19). Cells with the NK-cell-specific receptor NKp46 were scarce in colorectal cancer and adenoma samples, despite high chemokine levels. Moreover, a prospective cohort study of 3,625 patients showed that a low NK serum cytotoxicity activity, measured by the ^{51}Cr-release assay, correlated with a higher risk of cancer incidence. The relative risk of cancer incidence in patients with high NK cytotoxicity activity was 0.63 (20).

Our findings demonstrate that CD3 lymphocyte counts are increased by chemoradiotherapy. Our data also suggest that the CD8 subset is likely responsible for this increase, which is evident after long-course chemoradiotherapy. This CD8 rise also appears to correlate with tumor regression. This is consistent with studies that have demonstrated CD4 and CD8 T-cells to be significantly correlated with histological grade after chemoradiotherapy, and CD8 with complete response after treatment (11). The immunoscore initiative taskforce is currently seeking to incorporate host immune response scoring into the TNM staging system, based on the belief that tumour progression is not a cell-autonomous process (21, 22). This score is based on CD3 and CD8. A recent study in rectal cancer utilizing this immunoscore has demonstrated that high scores of CD3 and CD8 in non-treated surgical samples were associated with superior DFS and overall survival (12). They also found that CD3 and CD8 counts were higher in biopsy samples from a separate group of 55 patients who responded to neoadjuvant treatment.

Table II. *Correlation of CD counts in normal (n), pre- and post-treatment samples.*

Pairs	Paired t-test p-Value	Mean difference	Number of pairs analysed
CD3n – CD3pre	0.731	9.0	35
CD4n – CD4pre	0.844	3.4	35
CD8n – CD8pre	0.484	14.6	36
CD56+57n – CD56+57pre	0.007	29.3	36
CD3post – CD3pre	0.025	63.7	36
CD4post – CD4pre	0.439	13.2	36
CD8post – CD8pre	0.070	44.1	35
CD56+57post – CD56+57 pre	0.306	4.8	35

In another study of 179 patients with locally advanced rectal cancer, patients were stratified into pathological complete responders (CR) (n=15) and non-complete responders (n=164) (23). In the CR group, pre-operative ratio of peripheral lymphocytes in white cell counts was higher and the ratio of neutrophils lower. Halama *et al*. reported in a study of 22 patients with metastatic colorectal cancer that the location and type of TIL in the primary tumor were associated with chemotherapy response in colorectal cancers (24). Another study showed regulatory (FoxP3+) T-cell infiltrates to be a predictive factor for response to chemotherapy or chemoimmunotherapy in advanced colon cancer patients (25). The role of TIL in radiotherapy was shown in 1979 by Stone *et al*. (26) who found that an intact

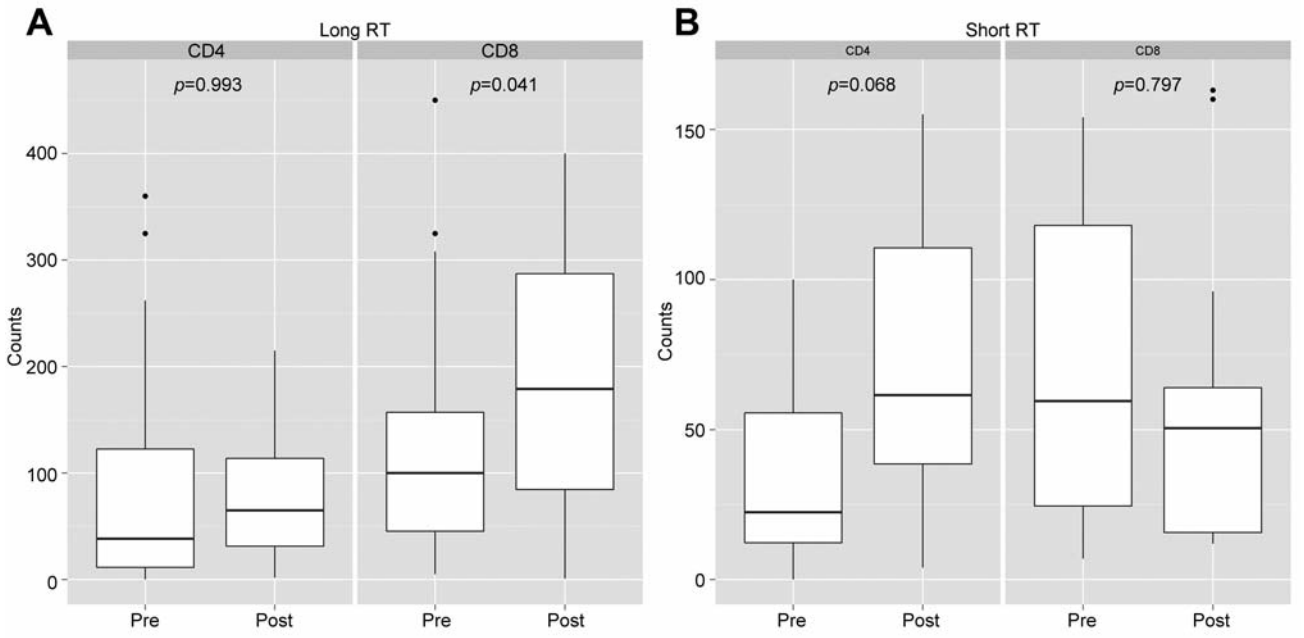

Figure 3. *Box and whisker plots for pre- and post-treatment CD4 and CD8 counts stratified by duration of radiotherapy (RT). A. Long course radiotherapy; B: short course radiotherapy.*

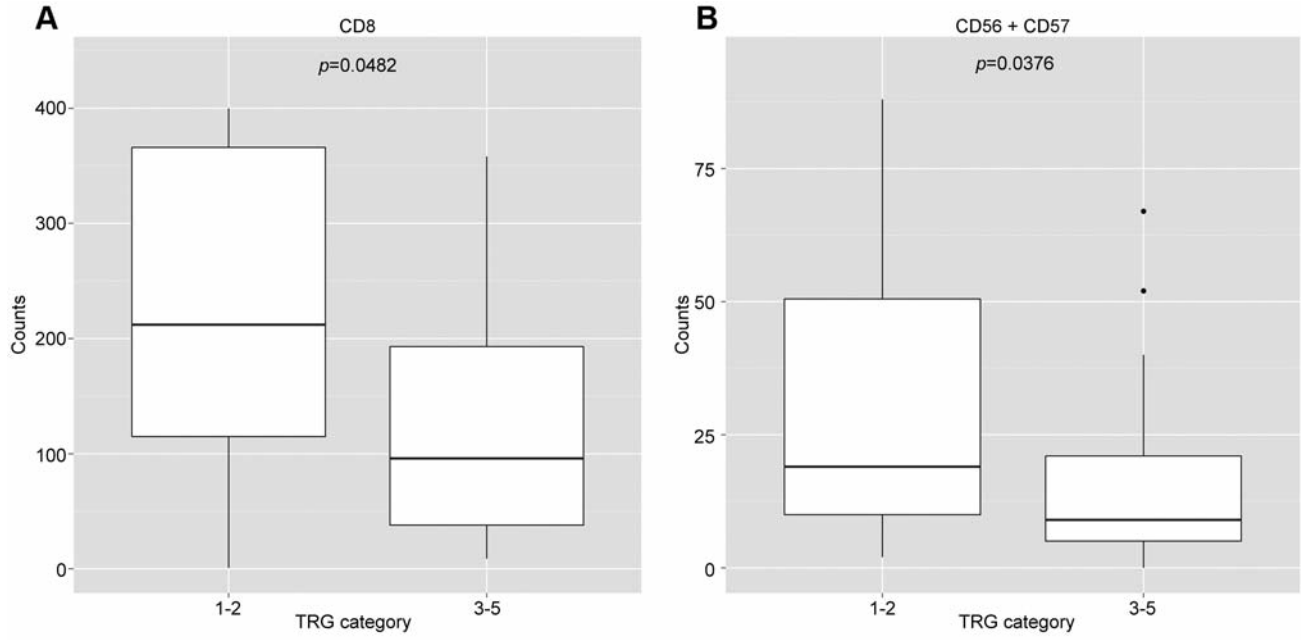

Figure 4. *Box and whisker plot for post-treatment CD8 and CD56+57 counts and correlation with tumour regression grade (TRG, categorised into TRG 1-2 and 3-5). p-values are obtained using logistic regression.*
A. CD8; B: CD56+57.

host T-cell immune response was required for optimal responses to radiation therapy. In essence, these studies suggest that TIL may be a predictive marker of treatment response in colorectal cancer, consistent with our findings.

We showed that the increase in CD8 count post-treatment was evident with long-course radiation. This is consistent with long-course treatment being known to result in a higher rate of pathological complete response or complete tumor

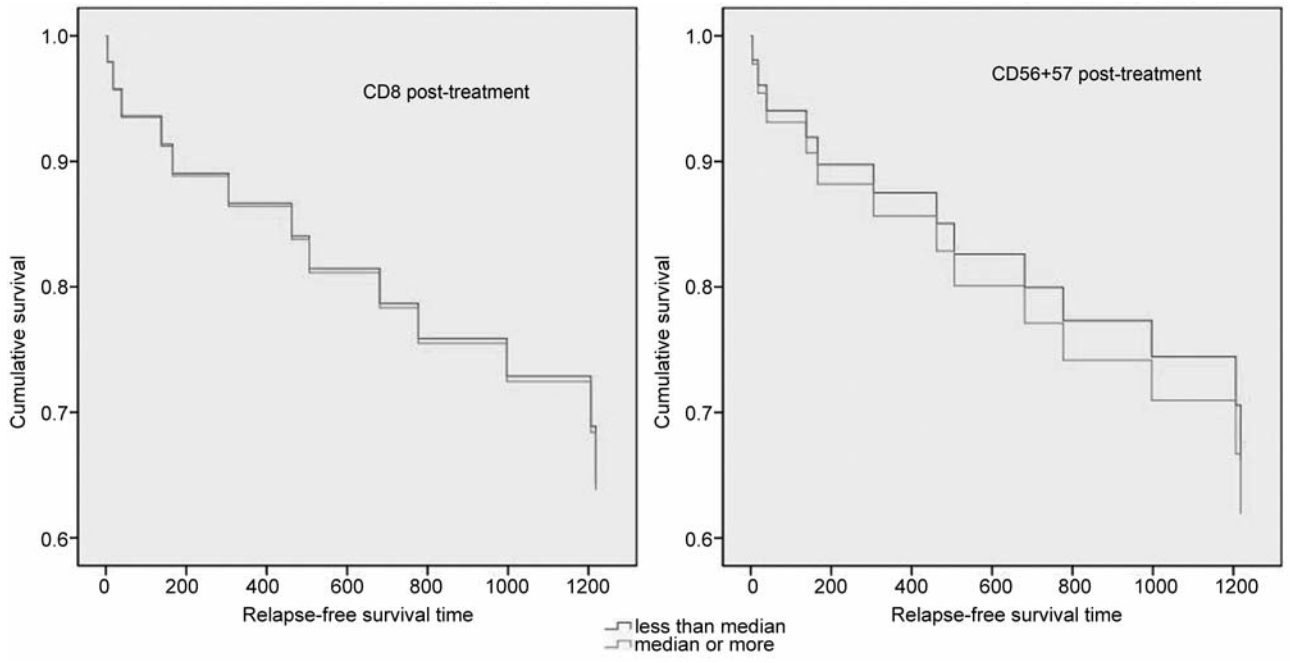

Figure 5. *Survival curves for CD8 (A) and CD56+57 (B) post-treatment counts, stratified by their medians, and disease-free survival (in days).*

regression (27). It is tempting to hypothesize that CD8 may be one of the drivers of treatment response. Nonetheless, the effect of neoadjuvant chemoradiotherapy on TIL in the literature remains unclear. Some studies have demonstrated the pro-inflammatory effects of radiotherapy (28); however, other studies have shown no differences in these immune cells post-chemoradiotherapy (29). Several studies on serum lymphocyte counts have demonstrated a decline with treatment; however, this cannot be extrapolated to tissue. In the aforementioned study of 179 patients with locally advanced rectal cancer (23), serum lymphocyte counts decreased post-radiotherapy, while neutrophil counts remained stable in patients with a complete pathological response. A small controlled study of 15 patients with advanced rectal cancer undergoing neoadjuvant chemoradiotherapy sought to measure serum levels of interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α) and lymphocyte counts (total T-lymphocytes, T-helper cells, NK cells and B-lymphocytes) (30). There was an absence of the expected post-operative increase in proinflammatory cytokines, whereas lymphocyte, monocyte and granulocyte counts were decreased post-therapy.

It is possible that many studies, as our own, have been hampered by small numbers of patients and, therefore, reduced statistical power. Furthermore, while the prognostic value of TIL in colorectal cancer has been proposed, different methodologies make it difficult to directly compare studies. In a study of 546 patients with non-metastatic treatment-naïve colorectal cancer, inclusive of 281 rectal cancers, TIL were prognostic for recurrence (5) when classified as either low-grade (Klintrup system scores 0 and 1) or high-grade (scores 2 and 3) with multivariate analysis revealing age, nodal status and TIL grade to be independent prognostic factors for overall survival. In another study of 843 treatment-naïve colorectal cancers inclusive of 23% rectal cancers, lymphocytic reaction was found on multivariate analysis to be associated with improved cancer-specific survival and overall survival (6). The lymphocytic reaction was scored on four components including TIL, the other three components being Crohn's-like reaction, peritumoral reaction and intratumoral periglandular reaction. This association was independent of the lymph node count, microsatellite instability and CpG island methylation. This finding is important as the lymphocytic reaction has been shown to be associated with lymph node recovery and microsatellite-instability, with the latter being associated with CpG island methylator phenotype.

T-lymphocyte subsets have also been studied, as they may better reflect functional characteristics of the immune system in response to cancer. In functional gene cluster analyses of CD4 helper T-cell markers Th1, Th2, Th17 and Treg in resectable colorectal cancers, patients with Th17 expression had a poorer prognosis. Conversely, high expression of Th1 cluster had a longer disease-free survival (8). Validation studies with TMA confirmed these findings. Galon *et al*. (9) constructed TMA from the tumor centre and invasive margin

of colorectal cancers and assessed immunostaining for total T-lymphocytes (CD3), CD8 and CD45RO (memory T cells). They also confirmed the prognostic value and also showed that combined analysis with two sampling sites improved detection as compared to single-region analysis. Another study of 768 colorectal cancer cases found a positive correlation between $CD45RO^+$ cells and survival (4). This remained significant after taking into account MSI-high and tumor LINE-1 methylation level, which were independent predictors of $CD45RO^+$-cell density. In a study of 93 patients, CD8 and CD57 infiltration in the advancing tumour margin correlated with improved disease-free survival and were associated with MSI-high tumors (31). On the other hand, peritumoral inflammation was assessed histologically in 168 patients who underwent neoadjuvant long course chemoradiotherapy and was not found to be a prognostic factor in 5-year DFS and overall survival (32).

Given these multitude of challenges, the immunoscore initiative seeks to standardize scoring and reach assay harmonisation (21, 22). The valid assessment of immune function in the cancer microenvironment is challenging but the use of more sophisticated techniques such as microarrays, as in our study, may help standardize immunohistochemical methods, as it enables multiple samples to be compared concurrently and minimizes batch variability, while cDNA microarrays allow screening of large numbers of genes. These static investigations could also be combined with functional tests such as NK and T-cell function and reactivity in order to better-understand the role of lymphocytes in the control of rectal cancer.

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