Potential immune priming of the tumor microenvironment with FOLFOX chemotherapy in locally advanced rectal cancer

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ABSTRACT
Strategies to enhance tumor immunogenicity may expand the role of immunotherapy beyond the mismatch repair-deficient subtype. In this pilot study, biopsies were performed at baseline and after four cycles of FOLFOX in eight patients receiving neoadjuvant chemotherapy for stage II/III locally advanced rectal cancer. Immunostaining was performed for T cell subsets (CD3+, CD8+, CD45RO+); macrophages (CD163+); T regulatory cells (FOXP3+); and expression of MHC class I, PD-1 and PD-L1. Changes in cell number or intensity were quantified and correlated with treatment response. Pretreatment patterns of immune infiltrates were mixed and did not correlate with treatment response. Posttreatment increases in T cell infiltrates (CD3+, CD8+ and CD45RO+) and MHC-I expression were observed in five patients. CD163+ cells increased in four patients. FOXP3+ cell numbers increased in two patients, decreased in two other patients and remained unchanged in three patients. PD-1 scores increased in seven patients, and PD-L1 scores increased in four patients. Changes in tumor T cell responses did not correlate with treatment response. Changes in FOXP3+ cells were associated with treatment response in some patients: two patients with increases in FOXP3+ cells had poor responses, whereas the patient with the greatest reduction in FOXP3+ cells had a complete response. The patient with a complete clinical response had a much higher increase in MHC-I expression than other patients. These results suggest that chemotherapy can increase immune activity in the tumor microenvironment and could potentially be utilized to prime immune responses prior to immunomodulatory treatments.

Introduction
Immunotherapies have become routine in treatment of various cancers, such as melanoma, where checkpoint blockade of CTLA-4 and PD-1 T-cell inhibitory molecules helps lengthen progression-free survival and overall survival.1 T-cell regulatory (Treg) receptor PD-1 and its ligand PD-L1 are expressed in most cancers,2 and together they can dampen the antitumor immune response. Tumors thought to be susceptible to anti-PD-1 include those with high mutational load and neoantigen burden3 or with evidence of an immune-active tumor microenvironment (CD3/CD8+ infiltrates and MHC class I expression).4,5 Intratumoral PD-1/PD-L1 expression and presence of T cells within the tumor microenvironment are thought to represent the most useful indicator that anti-PD-1 therapy may be effective.

To date, the role of immunotherapy in colorectal cancer (CRC) has been limited to the mismatch repair-deficient (dMMR) subtype. dMMR tumors are strongly immunogenic, with high numbers of tumor-infiltrating lymphocytes thought to be responding to neoantigens brought about by the high mutational burden.6 Compared with mismatch repair-proficient (pMMR) CRC, dMMR tumors also express higher levels of PD-1/PD-L1.7,8 Phase II trials have shown that anti-PD-1/PD-L1 agents help lengthen progression-free and overall survival in patients with stage IV dMMR CRC.9,10 However, since dMMR tumors account for only 25%, 12% and 4% of stage II, III and IV CRCs, respectively, immunotherapy is a treatment option in only a small subset of CRCs.

The majority of pMMR CRCs exhibit low numbers of T cells in the tumor microenvironment, along with low levels of PD-1/PD-L1 expression; therefore, checkpoint blockade immunotherapy is not thought to be effective.11 Strategies aimed at enhancing immunogenicity in pMMMR CRC may allow broader use of immunotherapy. Along this line of reasoning, conventional cytotoxic chemotherapy and radiotherapy (RT) are thought to result in immunogenic cell death, with T cell infiltrates responding to release of antigen from damaged cells.12 Such treatments may synergize with immunotherapies, enhancing treatment response. Trials are currently under way employing combinations of immunotherapies with immune-priming strategies in gastroesophageal, head and neck, and lung cancers (NCT02872116, NCT02764593 and NCT02944396 on www.ClinicalTrials.gov).13 Observational data suggest that RT enhances T cell infiltration in locally advanced rectal cancer, and preclinical models suggest that cytotoxic chemotherapy may have similar effects.14-18 However, the effect of cytotoxic chemotherapy on intratumoral immune responses in patients with CRC has not previously been studied. In this prospective pilot study, we investigated whether...
conventional cytotoxic chemotherapy can induce immune priming in pMMR locally advanced rectal cancer.

**Results**

Matched biopsies taken before and after four cycles of induction FOLFOX chemotherapy (folinic acid, fluorouracil and oxaliplatin) were collected in eight patients: six men and two women, ages 33–78 years. The clinical and pathological characteristics of the cohort are listed in Table 1. All eight patients had clinical stage III (node-positive) disease; two had cT4 tumors, five had cT3 tumors, and one had a cT2 tumor. The distance of the tumor from the anal verge was ≤5 cm in three patients, 5–10 cm in three other patients and >10 cm in two patients.

Pretreatment MMR status was available for seven patients; all seven had retained expression of MMR proteins. The number of days between the index biopsy and completion of four cycles of FOLFOX ranged from 61 to 127 days (mean 82 days). By the completion of neoadjuvant treatment, six patients had downstaging of T stage and all eight patients had downstaging of N stage. Two patients had a poor clinical response, five patients had an incomplete response, and one patient had a complete response, based on digital exam, endoscopy and MRI.

The data on pre- and posttreatment tumor immune responses are shown in Table 2 and Fig. 1. Pretreatment patterns of immune infiltrates were mixed and did not appear to correlate with responses to treatment. CD3+ and CD8+ infiltrates increased in five patients, CD45RO+ infiltrates increased in four patients, and CD163+ cells increased in four patients. The MHC-I histoscore increased in five patients, the PD-1 score increased in seven patients, and the PD-L1 score increased in four patients. FOXP3+ infiltrates increased in two patients, decreased in two other patients and remained unchanged in three patients (pretreatment FOXP3+ data was not available for one of the eight patients). The two patients with increases in FOXP3+ cells had poor responses to treatment, whereas the patient with the greatest reduction in FOXP3+ cells had a complete clinical response (Fig. 2). The patient with a complete response had a much higher increase in MHC-I expression within the tumor than the other patients.

### Table 1. Clinical and pathological characteristics of the study cohort.

| Patient | Age (yr) | Sex | Initial MRI Stage | Distance from Anal Verge (cm) | Days between Biopsies | Posttreatment Stage | Downstaging | T | N | Complete Response |
|---------|----------|-----|------------------|-------------------------------|-----------------------|---------------------|-------------|---|---|-------------------|
| 1       | 63       | M   | cT4b N1          | 7.6                           | 82                    | ypT1 N0             | Yes         | Yes| No            |
| 2       | 69       | M   | cT3 c N1         | 5.0                           | 62                    | ypT2 N0             | Yes         | Yes| No            |
| 3       | 67       | F   | cT4b N1          | 3.2                           | 81                    | ypT2 N0             | Yes         | Yes| No            |
| 4       | 75       | M   | cT2 N1           | 6.5                           | 89                    | ycT0 N0             | Yes         | Yes| Yes          |
| 5       | 34       | M   | cT3 c N2         | 13.0                          | 78                    | ypT3 N1             | No          | Yes| No            |
| 6       | 78       | M   | cT3b N1          | 2.8                           | 127                   | ypT2 N0             | Yes         | Yes| No            |
| 7       | 48       | F   | cT3b N2          | 5.8                           | 61                    | ypT3 N1             | No          | Yes| No            |
| 8       | 33       | M   | cT3b N1          | 12.0                          | 74                    | ypT2 N0             | Yes         | Yes| No            |

*aAll patients had received four cycles of FOLFOX by the time of the second biopsy.

### Table 2. Pre- and posttreatment immune responses.*

| Biopsy | Cells/HPF |
|--------|-----------|
|        | CD3+      | CD8+      | CD45RO+    | FOXP3+     | CD163+     | MHC-I Histoscore | PD-1 Score | PD-L1 Score |
| Patient 1 |          |           |            |            |            |                  |            |            |
| Pre     | 23        | 22        | 20         | 10         | 22         | 220              | 1          | 2+          |
| Post    | 5         | 5         | 7          | 6          | 10         | 280              | 2+         | 1+          |
| Patient 2 |          |           |            |            |            |                  |            |            |
| Pre     | 13        | 6         | 17         | 8          | 10         | 175              | 1+         | 0           |
| Post    | 21        | 18        | 29         | 7          | 16         | 270              | 1+         | 1+          |
| Patient 3 |          |           |            |            |            |                  |            |            |
| Pre     | 14        | 12        | 22         | 10         | 10         | 200              | 0          | 1+          |
| Post    | 19        | 17        | 26         | 12         | 15         | 230              | 1+         | 0           |
| Patient 4 |          |           |            |            |            |                  |            |            |
| Pre     | 15        | 8         | 16         | 17         | 15         | 45               | 0          | 0           |
| Post    | 25        | 14        | 23         | 5          | 14         | 210              | 1+         | 1+          |
| Patient 5 |          |           |            |            |            |                  |            |            |
| Pre     | 9         | 3         | 15         | 5          | 11         | 180              | 0          | 0           |
| Post    | 14        | 9         | 22         | 8          | 16         | 230              | 1+         | 1+          |
| Patient 6 |          |           |            |            |            |                  |            |            |
| Pre     | 21        | 15        | 22         | NA         | 15         | 210              | 1+         | 1+          |
| Post    | 22        | 7         | 23         | 5          | 14         | 125              | 2+         | 0           |
| Patient 7 |          |           |            |            |            |                  |            |            |
| Pre     | 20        | 8         | 15         | 8          | 12         | 220              | 0          | 0           |
| Post    | 10        | 9         | 20         | 14         | 14         | 210              | 1+         | 0           |
| Patient 8 |          |           |            |            |            |                  |            |            |
| Pre     | 8         | 5         | 17         | 5          | 9          | 155              | 0          | 0           |
| Post    | 22        | 12        | 15         | 4          | 14         | 405              | 1+         | 1+          |

*Increases of >25% are boldfaced. Decreases of >25% are italicized. HPF, high-power field; NA, not available.*
Discussion

The findings of our pilot study suggest that FOLFOX chemotherapy is capable of inducing changes in the immune contexture within the tumor microenvironment, such as increases in T cells (CD3+, CD8+ and CD45RO+) and in MHC-I and PD-1 expression. These changes are similar to those observed in preclinical models after chemoradiotherapy (CRT) secondary to immunogenic cell death with antigen release, increased MHC-I and cross-presentation to T cells.15-19 In rectal cancer patients treated with CRT, higher numbers of CD4+/CD8+cells in resected specimens than in pretreatment biopsies have been reported.14,20 CRT also induces systemic immune changes, including reductions in circulating Tregs and myeloid-derived suppressor cells.21 This immunomodulation may account for the reported abscopal effects on distant metastases after RT to the primary tumor, potentially mediated by cross-priming of T cells following tumor cell death.12,18,19

Our results are consistent with previous reports of association between changes in Tregs and treatment response. We observed that tumors in which FOXP3+ Tregs increased had a poor treatment response. The role of Tregs in CRC is not clear,
but they are known to harbor immunosuppressive activity, dampening T cell responses.\textsuperscript{22} Low numbers of FOXP3+ Tregs in posttreatment resection specimens have been reported to be associated with greater treatment response after CRT.\textsuperscript{23} No strong relationship between pretreatment Tregs and treatment response has been observed, suggesting that the type of immune response generated by RT is more important.\textsuperscript{24}

The patient with a complete clinical response had the largest increase in MHC-I expression. High MHC-I expression reflects increased antigen presentation and is considered a measure of
an immune-active microenvironment; it may therefore indicate ongoing immunogenic cell death in response to FOLFOX. Further work is required to test this hypothesis and to determine whether MHC-I has potential as an early biomarker of response during chemotherapy treatment.

We found no appreciable relationship between T cell changes and treatment response, even for tumors with a 200% increase in T cell numbers. Further work is required to determine whether the effects of these T cell responses are mitigated by the presence of inhibitory immune checkpoints (e.g., PD-1 and CTLA-4) or other T cell suppressors (myeloid-derived suppressor cells or Tregs).

No biomarkers of response to immunotherapies (e.g., anti-CTLA-4 and anti-PD-1/ PDL1) are available, but pretreatment evidence of an immune-active tumor microenvironment (e.g., high T cells and MHC-I expression) is considered important. Where such features are absent, strategies to prime the microenvironment in order to induce immune responses are sought. FOLFOX may be capable of achieving these desirable effects in a proportion of patients. Sequencing of chemotherapy and RT

Figure 3. Representative micrographs of immunostaining for antibody expression in rectal cancer biopsies. Scale bars: 500 μm for FOXP3+, 1 mm for all others.
to optimize immunological sequelae is likely to be critical to successful utilization of immunotherapy and requires further investigation. The relative ease with which tumor biopsies were collected from outpatients in our study indicates that rectal tumors are ideal for studying the scheduling of priming treatments.

In summary, the results of our pilot study indicate that FOLFOX chemotherapy in patients with locally advanced rectal cancer was associated with increases in T cell infiltrates, MHC-I expression and PD-1 expression. It is likely that the immunogenicity generated in response to chemotherapy plays a role in treatment response, and evidence of this role may be more apparent in a larger study. Our findings suggest that neoadjuvant chemotherapy can potentially be utilized to prime immune responses prior to immunomodulatory treatments. These observations provide support for ongoing work investigating the role of immune stimulation by conventional treatments in rectal cancer and for future trials aimed at evaluating combinations of chemotherapy, RT, and immunotherapy.

**Patients and methods**

**Patients**

The study population consisted of nonconsecutive patients seen over a 4-month period with locally advanced rectal cancer clinically staged cT3/4 or N+ (American Joint Committee on Cancer stage II and III) who received neoadjuvant therapy prior to planned rectal resection. As per National Comprehensive Cancer Network guidelines, the patients received eight cycles of FOLFOX chemotherapy with approval from the CINET Oncology Network guidelines. The patients received eight cycles of induction FOLFOX prior to CRT. With approval from the institutional review board, we prospectively evaluated the immune contexture of tumor biopsies taken at baseline and after four cycles of FOLFOX chemotherapy. All patients were considered to have pMMR tumors based on pretreatment biopsy immunohistochemistry (no loss of MLH1, PMS2, MSH2 or MSH6 protein expression).

**Samples**

Immunohistochemistry was performed on matched samples (biopsy at initial diagnosis and after four cycles of FOLFOX) to grade infiltrates of T cells (CD3+, CD8+, CD45RO+), Tregs (FOXP3+) and macrophages (CD163+) (Fig. 3). We also evaluated MHC-I (as a measure of antigen presentation) and PD-1/PDL-1 expression. Sections of 4-μm thickness were obtained from paraffin-embedded biopsy samples. After deparaffinization and rehydration, sections were incubated with primary monoclonal antibodies recognizing CD3 (LN10, 1:100; Leica Biosystems), CD8 (SP57, ready to use; Ventana), CD45RO (UCHL1, 1:200; Dako), FOXP3 (236 A/E7, 1:500; Abcam), CD163 (MRQ-26, ready to use; Cell Marque), MHC-I (A4; Thermo Fisher Scientific), PD-1 (NAT105, ready to use; Cell Marque), or PD-L1 (E1L3 N, 1:250; Cell Signaling Technology). We used the staining platforms BenchMark Ultra from Roche (CD3 and CD163) and BenchMark Ultra from Ventana (CD8) and the detection systems OptiView from Ventana (CD3, CD8, CD163 and PD-1) and Bond Polymer Refine from Leica Biosystems (CD45RO, FOXP3, MHC-I and PD-L1).

**Immune response**

Immune cells expressing CD3+, CD8+, CD45RO+, FOXP3+ or CD163+ were quantified in biopsy specimens using a cell counting approach. For each specimen, three high-power fields containing both tumor stroma and epithelium were selected at random. Cell counts were averaged over the three fields. MHC-I expression was assigned a modified histoscore obtained by multiplying the percentage of cells expressing MHC-I by the intensity of staining, which was graded on a scale of 0 to 3. Histoscore values therefore ranged from 0 to 900. PD-1 and PD-L1 were assessed using a previously described method. Briefly, tumor cells staining for PD-L1 and tumor-infiltrating lymphocytes expressing PD-1 were graded on a scale from 0 to 2 (0, no staining; 1+, faint or weak staining; 2+, moderate or strong staining). Increases or decreases of >25% in absolute cell counts or antibody staining intensity, in addition to any point change in the PD-1/PD-L1 scores (0–2) in posttreatment samples, were categorized as changes in the immune response. C.R. scored all the slides, with dual scoring for three patients by J.S. to ensure consistency.

**Staging and clinical response**

Pretreatment staging was based on MRI data. Posttreatment staging was based on MRI/CT data in patients with a complete clinical response or on resection specimen pathology in patients undergoing surgery. A downward posttreatment change in T or N stage (e.g., T4 to T3 or N1 to N0) was categorized as downstaging. Complete clinical response was defined as absence of clinical, endoscopic or radiographic evidence of tumor at completion of neoadjuvant therapy. At Memorial Sloan Kettering, such patients are offered the option of nonoperative, watch-and-wait management. A reduction of <50% in tumor bulk was categorized as poor response, and a reduction of 50–99% was categorized as a good but incomplete response.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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