



Neoadjuvant Botensilimab/Balstilimab for Localized Mismatch Repair–Proficient and –Deficient Colon Cancer: Results of the NEST Phase II Clinical Trial

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ABSTRACT

Purpose: Effective immunotherapy for mismatch repair–proficient (pMMR) colorectal cancer is lacking. We examined the safety and efficacy of the novel next-generation immune activator/Fc-enhanced cytotoxic T lymphocyte–associated protein 4 (CTLA-4) inhibitor botensilimab (BOT) plus programmed cell death protein 1 (PD-1) inhibitor balstilimab (BAL) in the neoadjuvant setting for patients with resectable colorectal cancer.

Patients and Methods: Patients 18 years of age or older with nonmetastatic colorectal cancer awaiting surgical resection were eligible. BOT/BAL was administered followed by surgical resection. In cohort A, patients received BOT 75 mg on day 1 and BAL 240 mg on days 1 and 15; in cohorts B/C, patients received two additional BAL doses (on days 29 and 43). The primary study objectives were safety, feasibility (based on surgery delay), and pathologic response. Exploratory analyses examined changes in the tumor microenvironment.

Results: Twenty-four eligible patients [26 tumors, $n = 22$ pMMR; $n = 4$ mismatch repair deficient (dMMR)] were enrolled

(two patients had synchronous primary tumors). Neoadjuvant BOT/BAL was safe and did not delay planned surgery in any patient. The major pathologic response rate was 41% [95% confidence interval (CI), 21%–64%] for pMMR and 100% (95% CI, 40%–100%) for dMMR colorectal cancer. BOT/BAL was associated with significant antitumor effects in the tumor microenvironment, with an increase in the density and proportion of CD8⁺ T cells, a reduction in tumor-infiltrating FOXP3⁺ regulatory T cells, and evidence of an increased immune cell–cell interaction in responding patients.

Conclusions: These findings demonstrate safety, feasibility, and encouraging pathologic responses for BOT/BAL in both nonmetastatic pMMR and dMMR colorectal cancer. Tumor microenvironment remodeling suggests a robust antitumor immune response induced by immunotherapy. These data support the continued development of BOT/BAL in colorectal cancer.

Introduction

Colorectal cancer is the most commonly diagnosed gastrointestinal cancer and a leading cause of cancer-related death in the United States, with approximately 154,270 new cases diagnosed and 52,900 deaths in 2025 (1). Multidrug chemotherapy regimens in

combination with targeted therapy remain the standard of care for most patients with metastatic colorectal cancer (2, 3). With the inclusion of targeted therapies and novel agents, the median overall survival (OS) for metastatic disease has improved from about 12 months to about 23 months over the past two decades (4–7). However, there have been few advances in the available treatment options for locally advanced colorectal cancer since the introduction of FOLFOX therapy in the adjuvant (8) or preoperative (neoadjuvant) setting (9, 10). Although immune checkpoint blockade has revolutionized the treatment of many solid tumors, the efficacy of traditional inhibitors of programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte–associated protein 4 (CTLA-4) in colorectal cancer has been limited to cancers that are mismatch repair deficient (dMMR)/microsatellite instability high, comprising only 5% to 15% of all colorectal cancer cases (11, 12).

Next-generation immunotherapy agents have demonstrated modest activity in mismatch repair–proficient (pMMR) colorectal cancer. Botensilimab (BOT) is a second-generation Fc-enhanced anti–CTLA-4 antibody that strengthens the immune synapses between antigen-presenting cells and cytotoxic CD8⁺ T cells in addition to downregulating immunosuppressive regulatory T cells (Treg; ref. 13). In a phase Ib trial, the combination of BOT and balstilimab (BAL), an anti–PD-1 antibody (13, 14), showed an encouraging objective response rate (ORR) of 17% with a median progression-free survival (PFS) of 3.5 months in patients with refractory pMMR metastatic colorectal cancer (15). A subsequent randomized phase II

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Translational Relevance

Immunotherapy has limited efficacy in metastatic mismatch repair-proficient (pMMR) colorectal cancer; however, when used in the neoadjuvant setting, while the primary tumor and regional lymph nodes remain intact, there is greater potential for efficacy. We present the results from the phase II Novel Exploratory Study to Test novel immunotherapy combinations for resectable colon cancer (NEST) clinical trial which evaluated neoadjuvant botensilimab [BOT; Fc-enhanced anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4)] plus balstilimab [BAL; anti-programmed cell death protein 1 (PD-1)] in resectable pMMR and mismatch repair-deficient colorectal cancer. Treatment was feasible and associated with meaningful pathologic responses in pMMR colorectal cancer, exceeding the efficacy in metastatic pMMR colorectal cancer. Treatment-associated remodeling of the tumor-immune microenvironment was observed in tumors that demonstrated a response to BOT/BAL therapy, including increased CD8⁺ T cells, intratumoral FOXP3⁺ regulatory T-cell depletion, and immune cell spatial reorganization. Responses were accompanied by circulating tumor DNA clearance and ongoing disease-free survival at follow-up. These data support further development of immunotherapy in pMMR colorectal cancer and provides clinical evidence of an antitumor immune response.

trial (NCT05608044) confirmed the efficacy of BOT/BAL in patients with refractory pMMR metastatic colorectal cancer without liver metastases, demonstrating an ORR of 19% and disease control rate of 55% (16).

Neoadjuvant immunotherapy, administered while the primary tumor and regional lymph nodes remain intact, enhances T-cell activation by leveraging abundant tumor antigens and a less developed resistance profile compared with the metastatic setting (17, 18). Neoadjuvant immunotherapy is characterized by robust antitumor T cell responses potentially due to improved reactivation of tumor-infiltrating PD-1⁺ cytotoxic T cells and improved priming of naïve T cells in tumor-draining lymph nodes (17, 19, 20). This emerging treatment paradigm has already demonstrated superior efficacy across multiple solid tumors, including melanoma (21), breast cancer (22), non-small cell lung cancer (23, 24), and dMMR colorectal cancer (25–27). In colorectal cancer, the phase II NICHE trial evaluated neoadjuvant combined checkpoint blockade with ipilimumab and nivolumab in patients with resectable colorectal cancer (28). The treatment was well tolerated, and all patients underwent surgery within the predefined 6-week period. In 30 patients with nonmetastatic pMMR colorectal cancer, the major pathologic response rate was 20% ($n = 6$), including three (10%) patients with a complete pathologic response at the time of resection (29).

We developed the phase II Novel Exploratory Study to Test novel immunotherapy combinations for resectable colon cancer (NEST; NCT05571293) based on the hypothesis that dual checkpoint blockade with BOT/BAL may demonstrate improved activity in the neoadjuvant treatment of localized colorectal cancer. Herein, we present the initial findings of neoadjuvant BOT/BAL in resectable colorectal cancer. Our objectives were to examine the safety, feasibility, and preliminary efficacy of neoadjuvant therapy and to examine correlates for immune activation and pathologic response.

Patients and Methods

Study design and treatment

NEST (NCT05571293, registered in October 2022) was a single-center, nonrandomized, open-label, phase II exploratory study, conducted at Weill Cornell Medicine/NewYork-Presbyterian Hospital, New York, USA. Cohort A (NEST-1) evaluated BOT 75 mg administered intravenously on day 1 and BAL 240 mg administered intravenously on days 1 and 15 (–2 to +5 days), prior to planned resection 1 to 6 weeks after the second BAL treatment. This initial exploratory cohort enrolled 12 patients from March 14, 2023, to September 5, 2023, and included three patients with dMMR and nine patients with pMMR colorectal cancer. Of the nine pMMR patients, two patients were subsequently considered inevaluable: NEST1-07 had a rectal tumor and did not undergo resection of the primary tumor, and NEST1-12 had an occult lung metastasis that was subsequently documented (Supplementary Fig. S1; CONSORT diagram).

After preliminary results from NEST-1 revealed encouraging clinical activity and safety, the study was amended (NEST-2) to include cohort B (pMMR, 12 patients) and cohort C (dMMR, 12 patients) and opened to enrollment in February 2024. Cohorts B and C evaluated BOT 75 mg administered intravenously on day 1 and BAL 240 mg administered intravenously on days 1, 15, 29, and 43 (–2 to +5 days), followed by resection 1 to 6 weeks after the fourth planned dose of BAL (Supplementary Fig. S2). Cohort B (pMMR) completed enrollment with 13 patients instead of a planned 12, due to simultaneous consent of subject 12, and cohort C (dMMR) enrolled 1 patient. The study was closed to further enrollment at that time, as the primary focus for further development was on pMMR colorectal cancer. Patients underwent standard oncologic surgical resection combined with Enhanced Recovery After Surgery protocol (30). All surgical complications were tracked for 30 days postoperatively. Following surgical resection, patients were evaluated for toxicity for up to 6 months following resection and then followed for 8 weeks by phone or in-person evaluation for a final adverse event review.

The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice guidelines. The protocol was approved by the institutional review boards of each participating site. All patients provided written informed consent prior to enrollment.

Patients

For all cohorts, patients 18 years of age or older with nonmetastatic, histologically proven adenocarcinoma of the colon awaiting surgical resection were eligible. Patients with rectal cancer were eligible if radiotherapy was not planned and up-front resection was recommended after multidisciplinary review. Patients were required to have adequate end-organ function, with an absolute neutrophil count of $\geq 1.5 \times 10^9/L$, platelet count $\geq 100 \times 10^9/L$, hemoglobin ≥ 8 g/dL, aspartate aminotransferase and alanine aminotransferase $\leq 2.5 \times$ upper limit of normal (ULN), total bilirubin $\leq 1.5 \times$ ULN, and calculated creatinine clearance ≥ 40 mL/minute. Patients with metastatic cancer, those who had received prior immune checkpoint inhibitors or other systemic therapy, or with contraindications to immunotherapy (i.e., history of autoimmune disease requiring steroids) were ineligible. Patients with active cardiovascular disease, such as stroke or myocardial infarction within 6 months of enrollment, unstable angina, congestive heart failure, or serious uncontrolled cardiac arrhythmia requiring medication that may prevent surgery were

excluded. Pregnancy was exclusionary, and adequate birth control was required. Full eligibility criteria are provided in the protocol (Supplementary Materials).

Tumor mismatch repair (MMR) status was assessed by immunohistochemical (IHC) analysis of MLH1, MSH2, MSH6, and PMS2 proteins using biopsy samples. Loss of one or more proteins within the tumor cells indicated dMMR. Cohort A eligibility included both pMMR ($n = 9$) and dMMR ($n = 3$); cohort B, pMMR ($n = 13$); and cohort C, dMMR ($n = 1$).

Objectives and endpoints

The primary objectives were to assess the safety, feasibility, and efficacy (antitumor effects) of the BOT/BAL combination. Safety was assessed by adverse events (AE) and serious AEs (SAE) graded by the Common Terminology Criteria for Adverse Events (CTCAE) v5.0 up to 90 days following the last study drug dose. Any AEs/SAEs that led to a delay in surgery >6 weeks from the last treatment were recorded. Feasibility was assessed as follows: If ≥ 2 of the first six patients (or ≥ 4 of the full planned 12) had treatment-related surgery delays of greater than 12 weeks (cohort A) or 16 weeks (cohorts B or C) from treatment initiation, then the BOT/BAL combination/dosage would not be considered safe or feasible in the given population. Interim safety analyses monitored for stopping criteria, which included determination of nonfeasibility, or if $\geq 33\%$ of patients experienced grade ≥ 3 treatment-related AEs (TRAE).

Efficacy in cohorts A and B was measured by pathologic overall response (pOR) rate, which included pathologic complete response (pCR), major pathologic response (MPR), and partial response (PR). pCR was defined as no viable invasive cancer in the tumor bed or adjacent lymph nodes (tumor regression score = 0). Resected specimens with residual *in situ* disease but without an invasive component were considered pCR (26). MPR was defined as $\geq 90\%$ pathologic response ($\leq 10\%$ of residual viable tumor cells, tumor regression score = 1 or 2), according to the Mandard score (31). PR was defined as ≥ 50 – 90% tumor regression. No response was defined as $\leq 10\%$ tumor regression. In cohort C, for patients who elected not to have surgery, antitumor effects were assessed with composite rate of clinical complete response (CR; using clinical, radiographic, endoscopic, and/or other assessments), or MPR at 6 months. Histologic evaluation of pretreatment biopsies and posttherapy resected specimens (residual tumor or tumor bed) was used to assess pathologic response categories, as well as for qualitative tissue analyses.

The secondary endpoint in all cohorts included changes in minimal residual disease by circulating tumor DNA (ctDNA) before and after surgical resection. Pathologic response in combined pMMR tumors (cohort A pMMR, cohort B) and dMMR tumors (cohort A dMMR, cohort C) was also assessed as part of a pre-specified secondary analysis. Exploratory correlative analysis included assessment of immune cell composition and biomarkers in the tumor microenvironment.

Pathologic response criteria

Resected tumors were examined in their entirety, and regression of resected tumors was assessed by estimating the percentage of residual viable tumor of the macroscopically identifiable tumor bed, as identified on routine hematoxylin and eosin (H&E) staining. Regression was classified using the Mandard tumor regression grading system (31). MPR was defined as $\leq 10\%$ of residual viable tumor cells (or $\geq 90\%$ response), corresponding to Mandard tumor regression grade 1 (CR) or 2 (near-CR). PR was defined as at least 50% tumor regression. However, considering the lack of consensus

on the definition of PR after immunotherapy, tumors with >50% and <90% residual viable tumor would be labeled accordingly as “10% to 50% tumor regression,” as per the NICHE study (28) (See Supplementary Table S1).

Histologic evaluation

Pretreatment biopsies and posttherapy resected specimens (residual tumor or tumor bed) were histologically assessed using formalin-fixed, paraffin-embedded (FFPE) tissue sections as part of the routine clinical workflow with H&E-stained slides. Cases were centrally reviewed by an expert gastrointestinal (GI) and molecular pathologist. Histologic response was evaluated by estimation of the percentage of residual viable tumors of the macroscopically identifiable tumor bed. Tumors with prominent Crohn disease–like lymphoid reaction (32), characterized by peritumoral lymphocytic aggregates found at the advancing edge of the tumor or in the tumor bed and resembling tertiary lymphoid structures (TLS), were identified. Prominence is defined as being present diffusely throughout the tumor (>50% of the tissue sampled) and containing at least five TLSs/4 $\times$ field. Select slides were also reviewed at a GI pathology consensus conference with up to five expert GI pathologists to help determine the percent of viable tumor cells in tumor beds.

ctDNA assessment

Changes in ctDNA were assessed using the clinical Signatera tumor-informed platform. Summary statistics including mean, standard deviation, median, and range were provided. As this was a pilot study, the analyses are descriptive in nature, with means, medians, ranges, and/or proportions (%) where appropriate.

Immunofluorescence of the tumor microenvironment

Multiparametric *in situ* fluorescence imaging to examine the tumor microenvironment was performed using the Lunaphore COMET instrument (33, 34). A subset of pre- and posttreatment samples was examined to capture pMMR and dMMR tumors and evaluate tumors with and without responses (6 baseline biopsies and 11 resection specimens). Samples were prioritized based on availability and variability of pathologic response and MMR status. Specifically, we tried to ensure that we had at least two matched baseline and resection samples that had an MPR and no response, as well as at least two samples with dMMR disease. Samples were stained with a multiplex immuno-oncology panel with antibodies selected from validated clones in the Clinical Laboratory Improvement Amendments–certified IHC laboratory at Weill Cornell Medicine/New York-Presbyterian Hospital (see Supplementary Fig. S4A). Panel development was performed as previously described (34); normal human lymph node and tonsil tissues were used as controls, given adequate representation of positively staining cells for all evaluated markers. Human tonsil tissue was included as a positive control on all study slides. Tonsil and corresponding colon FFPE blocks were sectioned at 5 μ m thickness and mounted in close proximity on the same Leica charged slides. Prior to COMET processing, slides were manually dewaxed and subjected to antigen retrieval (pH 9, 20 minutes) using the Leica BOND RX system. To minimize tissue autofluorescence, a standard photobleaching protocol employing hydrogen peroxide and sodium hydroxide was applied. Slides were then washed with PBS and stored in solution until instrument initialization and reagent preparation were ready. Up to four slides were processed per run using the fully automated, sequential multiplex protocol based on Lunaphore's pressurized

microfluidic technology. Each staining cycle comprised six steps: (i) primary antibody incubation (mouse or rabbit anti-human antibodies); (ii) secondary antibody incubation with Goat Anti-Mouse Alexa Fluor Plus 555 (Thermo Fisher Scientific, cat. #A32727, 1:100) and Goat Anti-Rabbit Alexa Fluor Plus 647 (Thermo Fisher Scientific, cat. #A32733, 1:200), followed by 4',6-diamidino-2-phenylindole (DAPI) counterstaining (Thermo Fisher Scientific, cat. #62248, 1:1,000); (iii) imaging through the DAPI, tetramethylrhodamine (TRITC), and cyanine-5 (CY5) channels; (iv) washing; (v) elution; and (vi) quenching.

COMET analysis and quality control

Immunofluorescence intensity values were extracted using HALO (35). Mean nuclear area intensity values were used for protein markers FOXP3, PCNA, PAX5, and MKI67, and mean cytoplasmic intensity values were used for the remaining markers. We defined three regions of interest (ROI): (i) tumor in the baseline biopsy sample, (ii) tumor, and (iii) tumor bed in posttreatment resection samples. After obtaining per-cell intensities for each ROI, we excluded 2% of cells with smallest size and 2% of lowest DAPI signal from further analysis to reduce artifacts. We generated a binary expression matrix as described by Sanz-Moreno 2024 (33). In brief, probe intensities within each ROI were *z* score-normalized and fitted onto a density function (using *gaussian_filter1d* from *scipy.ndimage*). The lowest peak value of the density was assumed to represent the background and to be normally distributed (*find_peaks* from *scipy.signal*), and cells with probe intensities above 6σ from this fitted background distribution were classified as positive for that probe.

We identified 16 cell types from a combination of 17 markers according to predefined marker scheme (see Supplementary Fig. S4A). Cells with conflicting positive markers were labeled as “unknown.” We then focused on immune cell subtypes by excluding vimentin, podoplanin, and smooth muscle actin (SMA) probes. To identify differences in cellular densities between conditions, counts of cell types were normalized to the total area of each ROI [(cell_type_count/ROI_area) * 1,000], and statistical differences were evaluated using the paired *t* test for each cell type between different conditions.

Immune cell analyses focused on (i) differences in the tumor-immune infiltrate between the baseline biopsy sample and matched resection specimens, (ii) differences between responders and non-responders in the resection specimen, and (iii) differences between pMMR and dMMR within the responders. We also evaluated proportions of immune cell types as a fraction of total immune cells. To further describe the tumor-immune microenvironment, cell type-combined niches were identified (36), and neighborhood enrichment analysis was performed.

Cell type-combined niches within our samples were identified following the approach by Schürch and colleagues (36). For each annotated cell, across all samples, we define a neighborhood region consisting of the 10 nearest neighboring cells (including the center cell) based on the Euclidean distance between spatial positions. These regions were then clustered using *k*-means (*k* = 10) based on the composition of 16 cell types identified in our dataset. In addition, to focus on the immediate cellular environment of our cell types of interest, we calculated the proportion of neighboring immune cell types located within a 50-micron radius of each cell. These proportions were computed separately for each sample and then averaged across samples within each condition. To assess changes in the composition of neighboring cells between conditions, we performed statistical comparisons using the *t* test.

Sample size and statistical methods

For cohort A (NEST-1), enrollment was limited to no more than 25% of patients with dMMR tumors (*n* = 3). Based on previous studies, the null hypothesis for NEST-1 was that the probability of achieving a pathologic response (e.g., PR or better) to treatment is ≤5% versus the alternative *H*_a pathologic response rate ≥35% requires a total sample size of 12 patients (including 3 dMMR). Cohort B (NEST-2) was restricted to pMMR patients. Based on previous studies, the null hypothesis was that the probability of achieving pOR to treatment is ≤5% versus the alternative ≥35% requires a total sample size of 12 patients with 90% power at a one-sided significance level of 0.10. Cohort C (NEST-2) was restricted to dMMR patients. The null hypothesis was that the probability of achieving pOR to treatment is ≤80% versus the alternative 100% requires a total sample size of 15 patients with 90% power at a one-sided significance level of 0.10.

Primary endpoints were summarized using counts and proportions. Safety analyses were planned for all enrolled patients and efficacy for treated patients with pathologic response assessment. Exact binomial confidence intervals (CI) were provided for response rates. Survival probabilities were estimated using the Kaplan–Meier method. Correlation between response and *KRAS/BRAF* mutational status was tested using the Fisher exact test. Correlation between response and the presence of Crohn disease-like reaction and TLS was tested using the χ^2 test. Additional details are provided in the protocol and statistical analysis plan (Supplementary Materials).

Important protocol amendments

NEST-1 (cohort A) enrolled 12 patients from March 17, 2023, to September 5, 2023, and demonstrated encouraging safety and feasibility results. This supported the study amendment (NEST-2), which was activated in February 2024 to enroll cohort B (pMMR) and cohort C (dMMR). Enrollment to the amended study (NEST-2) started from February 14, 2024, to May 3, 2024.

Results

Patients

From March 17, 2023, to May 3, 2024, 26 patients consented, and 24 were eligible for the study and received BOT/BAL (Supplementary Fig. S1). In NEST-1, cohort A enrolled 10 eligible patients (*n* = 7 pMMR; *n* = 3 dMMR). In NEST-2, cohort B enrolled 13 patients (all pMMR) and cohort C enrolled 1 patient (dMMR). In cohort B, two patients (NEST2-07 and NEST2-08) each had synchronous primary tumors. Therefore, the safety population included 24 patients, and 26 tumors were evaluated for efficacy. As shown in Table 1, the median age was 64 years (range, 35–75), with an even distribution of males (*n* = 13, 54%) and females (*n* = 11, 46%). Half of the study population was White, and the majority (*n* = 21, 88%) were non-Hispanic. Fourteen (58%) patients had radiologically locally advanced tumors (T3+), and 42% (*n* = 10) had radiographically discernable lymph nodes. The representativeness of the study population is shown in Supplementary Table S2.

At the data cutoff of March 31, 2026, the median follow-up duration was 32.2 and 23.5 months, respectively, for cohort A and cohorts B/C. The study was closed after the completion of enrollment to cohort B and enrollment of one patient to cohort C, due to an administrative reason (PMK left the institution).

Safety and feasibility

Neoadjuvant BOT/BAL was safe with no delays to planned surgery in any patient. The median time to surgery from start of

Table 1. Patient demographics.

Patient characteristic	Total (N = 24)	Cohort A (n = 10)	Cohort B (n = 13)	Cohort C (n = 1)	dMMR/MSI-H (n = 4)
Age, years, median (Q1, Q3)	64 (46, 73)	57 (35, 69)	68 (59, 75)	71	61 (43, 72)
Male:female	13:11	5:5	8:6	1 male	3:1
Race					
White	12	5	6	1	3
Asian	4	1	3	0	0
Black	4	2	2	0	0
Other	4	2	2	0	1
Ethnicity					
Hispanic	1	1	0	0	0
Non-Hispanic	21	9	11	1	4
Other	2	0	2	0	0
Tumor location					
Left sided	14	5	9	0	3
Right sided	12	5	6	1	1
Clinical T stage					
T1/T2	10	1	9	1	1
T3+	14	9	4	0	3
Clinical N stage					
N+	10	9	1	0	3
Nx (or N0)	16	1	14	1	1

Abbreviations: N, node; T, tumor.

treatment was 29 days (range, 21–37) for cohort A and 55 days (range, 21–104) for cohorts B and C. All patients underwent minimal invasive resection (laparoscopic or robotic), with one conversion to open surgery. Among all cohorts, the median hospitalization length of stay was 2 days (range, 1–14).

TRAEs are provided in Supplementary Table S3. No grade 4 toxicities occurred. In cohort A, one patient had grade 2 diarrhea/colitis that resolved with TNF α inhibition (e.g., infliximab). We also observed five patients with a common symptom pattern of fever, chills, and fatigue, noted within 2 weeks of BOT/BAL administration. These symptoms were readily managed by proactive supportive care, which included nonsteroidal anti-inflammatory medication (e.g., naproxen) or acetaminophen as an antipyretic, and rarely, a short course of steroids. Four of five patients with these symptoms were able to receive their second dose of BAL without delay; the fifth patient had fever accompanied by grade 3 fatigue. In total, 2 of 10 patients in cohort A required high-dose steroids, and one patient required TNF α inhibition.

In cohorts B and C, five patients had immune-mediated toxicity: colitis/diarrhea [$n = 5$; grade 2 ($n = 2$) and grade 3 ($n = 3$)], grade 2 myositis/myocarditis ($n = 1$) requiring the use of high-dose steroids and TNF α inhibitor (infliximab) therapy (Supplementary Table S3). Of the 14 patients enrolled in cohorts B and C, 9 received all planned treatment (BOT/BAL on day 1 followed by three doses of BAL), 3 patients received 3 BAL doses [treatment held because of colitis ($n = 3$)], and 2 patients received 2 BAL doses [treatment held because of colitis ($n = 1$) and myositis ($n = 1$)]. Among all 24 patients (all cohorts), 2 (8%) patients had a Clavien–Dindo classification grade 1 complication. No deaths or study discontinuations were observed.

Efficacy: pathologic response

The pOR rates were 70% ($n = 7/10$ patients) in cohort A overall and 57% ($n = 4/7$) for pMMR patients. In cohort B, the pOR rate was 62% ($n = 8/13$; all pMMR). **Figure 1A** shows a representative example of clinical response following treatment in a pMMR patient.

The pathologic response for each tumor is shown in **Fig. 1B**. In cohort A, 2 of 7 (29%; 95% CI, 4%–71%) pMMR tumors demonstrated MPR, and in cohort B 7 of 15 (47%; 95% CI, 21%–73%) pMMR tumors demonstrated MPR. In a prespecified secondary analysis, we examined all pMMR tumors from cohorts A and B combined ($n = 22$ tumors). For all pMMR tumors, the pathologic response rate (>50% tumoral response) was 59% (13/22; 95% CI, 36%–79%), the MPR rate was 41% (9/22; 95% CI, 21%–64%), and the pCR rate was 32% (7/22; 95% CI, 14%–55%). Molecular annotation was available in all 22 pMMR tumors (Supplementary Table S4). Although there were no associations between tumor mutational burden or PD-L1 combined positive score and treatment response, tumors with *KRAS* G12D ($n = 2$) or an activating *BRAF* mutation ($n = 1$, *BRAF* V600R) had no pathologic response (0%–10% response). In contrast, only 3 of 19 (14%) tumors with other *KRAS*/*BRAF* mutations ($n = 2/7$), or that with *KRAS* and *BRAF* wild-type ($n = 1/12$), had no pathologic response ($P = 0.048$; Supplementary Table S5).

For dMMR tumors ($n = 4$ overall), the MPR rate was 100% (95% CI, 40%–100%). Two had pCR, and one each showed 99% and 98% treatment effect (each having 1–3 small clusters of cancer cells identified within mucin pools).

As of the data cutoff, no patients had colorectal cancer recurrence, as shown in the PFS Kaplan–Meier curve (**Fig. 1C**). ctDNA was examined in 15 patients using the commercial tumor-informed Signatera assay. Nine had ctDNA collected at baseline (prior to study initiation), and all were positive (range, 0.06–54.65 mean tumor molecules (MTM)/mL). Of these patients, we collected a presurgical sample (within 30 days of surgery) in 8 patients, and 7 (88%) patients had clearance of their ctDNA (Supplementary Table S6). As shown in **Fig. 1D**, ctDNA remained undetectable following resection in all patients.

Histologic analysis of tumor response

Qualitative review of baseline H&E-stained tissue showed no discernible histologic differences in the immune infiltrate between

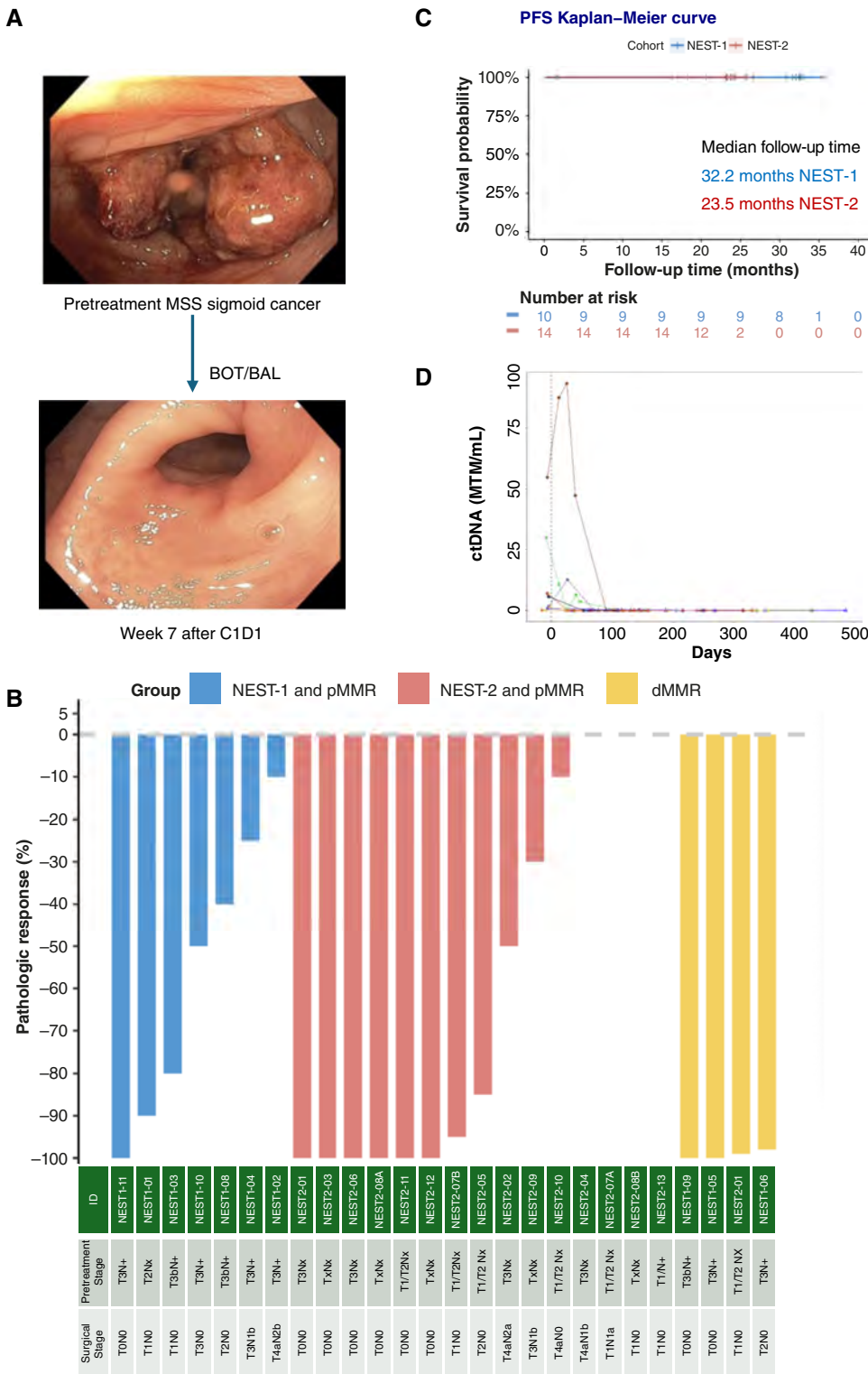


Figure 1. Efficacy of neoadjuvant BOT/BAL in patients with localized colon cancer. **A**, Patient with clinical response. CID1, cycle 1 day 1. **B**, Pathologic tumor response for all $n = 26$ tumors, shown as cohort A/NEST-1 pMMR ($n = 7$), cohort B/NEST-2 pMMR ($n = 15$), and dMMR ($n = 3$ cohort A; $n = 1$ cohort C). N, node; T, tumor. **C**, Kaplan–Meier curve for DFS for cohort A (NEST-1) and cohorts B/C (NEST-2). **D**, Clinical ctDNA for patients ($n = 15$) prior to and following initiation of therapy. MTM, mean tumor molecules

responding and nonresponding tumors. However, the posttreatment tumor bed in cases achieving MPR demonstrated several distinctive histologic features. These tumor beds were characterized by dense mixed inflammatory infiltrates, composed largely of

lymphocytes, plasma cells, and histiocytes, accompanied by mucosal-based granulation tissue with neovascularization, acellular mucin pools, and submucosal fibrosis. Eosinophils and neutrophils were also variably present (Supplementary Fig. S3).

As expected, three of four dMMR tumors exhibited prominent Crohn disease–like reaction surrounding the tumor bed indicative of a robust antitumor immune response. However, this reaction was also present in 73% of pMMR cases with >50% pathologic response after treatment, compared with only 13% without pathologic response, revealing a significant association between the development of prominent Crohn disease–like reaction and MPR following BOT/BAL ($P = 0.028$; Supplementary Table S7). In all dMMR and pMMR tumors with MPR, large acellular mucin pools were striking, with a subset containing sparse clusters of residual tumor cells. In pMMR tumors with PR (50%–90% pathologic regression), inflammatory infiltrates were dense and qualitatively similar to those observed in MPR cases but were most prominent at the tumor–stromal interface (periphery of viable tumor) and deep within the wall of the colon. In cases with a PR, the tumor bed had residual viable tumor that tended to be superficially oriented within the center of the tumor bed, a pattern distinctive from the haphazard arrangement typically seen following neoadjuvant chemotherapy. These residual tumors had viable tumor glands that frequently showed evidence of ongoing immune-mediated destruction with disrupted crypts, incomplete lumens, and frequent luminal microabscesses with necrosis.

Analysis of the tumor-immune microenvironment

Using the multiplex imaging COMET platform, we studied the composition and spatial organization of the tumor-immune microenvironment. We identified 286,935 total immune cells within the ROIs (tumor, peritumoral area, and tumor bed) across all samples (Supplementary Fig. S4A and S4B). Our analysis revealed notable differences in the character and organization of the immune infiltrate between responding and nonresponding tumors at the time of resection. Tumors achieving MPR tended to demonstrate a robust, organized immune infiltrate with co-localization of multiple cell types, a pattern consistent with coordinated antitumor immunity (Fig. 2A and B). In contrast, tumors with minimal to no pathologic response exhibited spatially isolated immune infiltrates with a greater proportion of FOXP3⁺ Tregs (Fig. 2C and D). We quantified these differences across defined ROIs to characterize how BOT/BAL immunotherapy modulates the tumor-immune microenvironment in

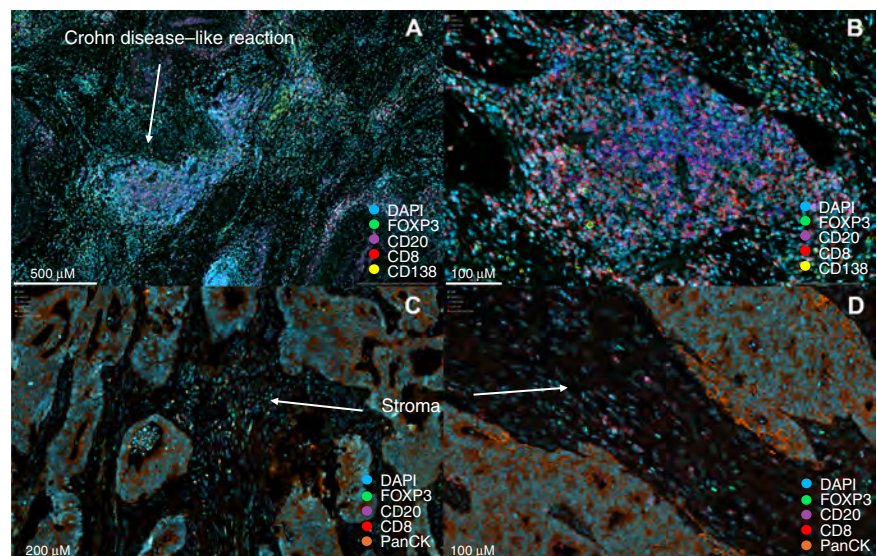
both responding and nonresponding tumors and to identify potential differences between pMMR and dMMR responses.

Analysis of baseline biopsies and matched tumor resection specimens suggests treatment-induced remodeling of the tumor-immune landscape. Following BOT/BAL, we observed a numerical decrease in the density of both CD3⁺FOXP3⁺ and CD3⁺CD4⁺FOXP3⁺ Tregs, concurrent with a 13-fold increase in CD8⁺ T-cell density (Fig. 3A). This shift in the density of infiltrating immune cell subsets from baseline to resection was also accompanied by changes in the proportion of immune cell populations at the time of resection, with CD8⁺ T cells increasing from 5% of the immune infiltrate at baseline to 30% in the resection specimen ($P = 0.077$; Supplementary Fig. S5A). This resulted in a 21-fold increase in the CD8⁺/Treg ratio from baseline (CD8⁺/Treg, 0.286) to resection (CD8⁺/Treg, 6.20), consistent with an activated antitumor immune response (Supplementary Fig. S5B). These changes in the immune infiltration were similar when limiting the analysis to pMMR colorectal cancer (Supplement Figs. S11–S14).

Notably, we observed an increased immune infiltrate in the resection specimens of responders compared with nonresponders (Fig. 3B). The infiltrating immune cell density in nonresponding tumors was 0.52 immune cells/ μm^2 , whereas in responding tumors it was 3.1 immune cells/ μm^2 ($P = 0.0048$), largely due to changes in the density and proportion of effector immune cells. Specifically, CD8⁺ T-cell density was 19-fold higher in responding versus nonresponding tumors ($P = 0.022$), whereas B-cell density was 59-fold higher ($P = 0.07$). These differences in immune cell density were associated with compositional shifts in effector immune cells. Specifically, CD8⁺ T cells and B cells each comprised 34% of the immune infiltrate in responding tumors, compared with 15% and 6%, respectively, in nonresponding tumors ($P = 0.11$ for CD8⁺ T cells and $P = 0.018$ for B cells; Supplementary Fig. S5C). Although the density of Tregs is paradoxically higher in responding tumors ($P = 0.039$), they comprise a smaller portion of the total immune infiltrate in responding tumors compared with nonresponding tumors (15% vs. 31%, respectively). This would indicate that there is proportionally an even greater increase in the infiltration of CD8⁺ T cells in responding tumors. The net result is a notable difference in CD8⁺ T-cell/Treg ratio (0.52 in nonresponse and 5.27 in response; Supplementary Fig. S5D), consistent with the activation of an antitumor

Figure 2.

Multiplex immunofluorescence images of a tumor bed in a responding patient (A low power; B high power) and the tumor of a non-responding patient (C low power; D high power). Pathologic response is associated with a dense and diverse immune infiltrate, consistent with the activation of antitumor immunity following BOT/BAL.



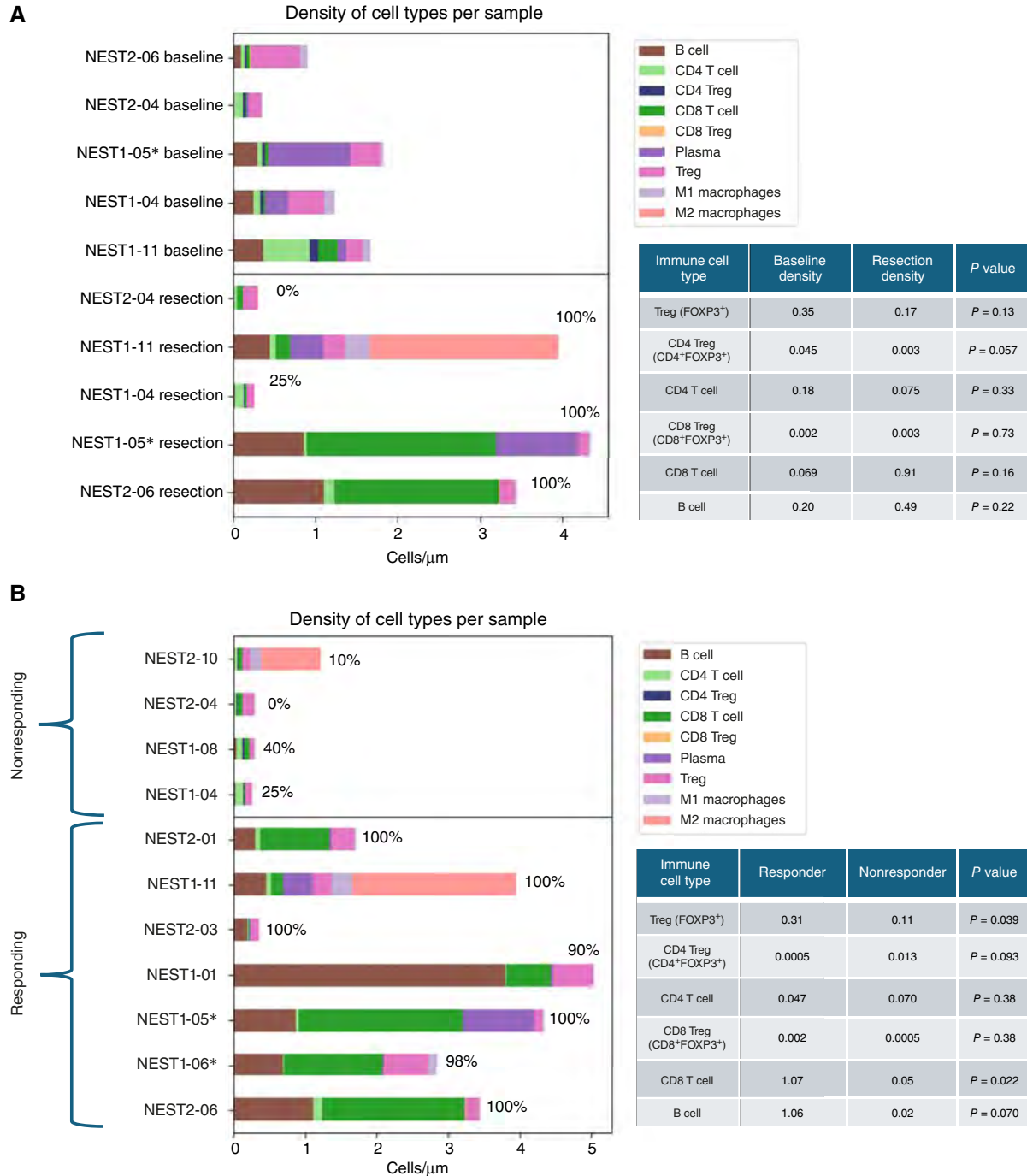


Figure 3. Density of immune infiltrate (**A**) in matched baseline vs. resection paired specimens and in the (**B**) resection specimens of responders and nonresponders. **A**, The density of Tregs is numerically reduced in the resection specimen compared with baseline, whereas CD8 T-cell density is increased. **B**, The density of immune cells in responders is significantly higher than that in nonresponders (3.1 cells/ μm vs. 0.52 cells/ μm ; $P = 0.0048$), dominated by an infiltrate of B cells and CD8⁺ T cells. Pathologic treatment effect (0%-100%) is indicated. In **A** and **B**: *, dMMR colorectal cancer.

immune response in tumors that have pathologic evidence of response following BOT/BAL.

We observed no significant difference in the immune cell infiltrate in the baseline biopsy samples of responders versus

nonresponders (Supplementary Fig. S6), possibly suggesting that preexisting inflammation was not a major driver of BOT/BAL response. However, analysis of matched baseline and resection specimens confirmed that the CD8⁺ T-cell/Treg ratio improved in both

responders and nonresponders following BOT/BAL therapy (Supplementary Fig. S7), but numerically to a greater extent in responders (Fig. 4).

This difference in the immune cell composition in responders versus nonresponders was accompanied by notable differences in the spatial distances between immune cells (Supplementary Fig. S8). Specifically, CD8⁺ T cells and B cells were significantly closer to plasma cells in responders versus nonresponders, whereas Tregs were further away from CD4⁺ T cells in responders versus nonresponders. These differences in distances between key regulatory and effector immune cells suggest potential differences in local immune cell microenvironments in tumors that demonstrated a response to BOT/BAL versus nonresponse. The differences in distances between key immune cell mediators between responders and nonresponders were similar in pMMR tumors, suggesting a similar immune microenvironment in responders, independent of MMR status (Supplementary Fig S14).

Cellular neighborhood enrichment analysis confirmed spatial reorganization of the immune microenvironment following BOT/BAL. We identified 10 distinct cellular neighborhoods, or niches, defined by the spatial proximity of different cell types within 50 mm radius in pathologic ROIs (Supplementary Fig. S9A). Responding tumors were characterized by immune-enriched communities, particularly niche 3 (B-cell enriched) and niche 6 (CD8⁺ T-cell enriched), which were almost exclusively observed in responders, consistent with the TLSs observed histologically (Supplementary Fig. S9B). In contrast, nonresponding tumors were enriched by niches 0, 4, and 8, characterized by epithelial cells, stroma, and an immune cluster with elevated macrophages (M1 and M2) and CD4⁺ Tregs, respectively. Quantitative analysis of the local cellular environment of CD8⁺ T cells, Tregs, and B cells further illustrated this spatial reorganization (Fig. 5).

At baseline, these key immune cells were primarily surrounded by stromal cells, tumor epithelium, and lymphatic endothelium across all

patients. Following therapy, this pattern persisted in nonresponding tumors, where immune cells remained isolated within stromal and epithelial compartments. However, in responding tumor beds, the majority of cells within the niche surrounding CD8⁺ T cells, Tregs, and B cells were other immune cells creating the organized, immune-dense microenvironment (Figs. 2 and 5).

Although both dMMR and pMMR tumors demonstrated robust immune infiltration following BOT/BAL in responding cases, we observed quantitative differences in immune composition in these two colorectal cancer subtypes (Supplementary Fig. S10A). dMMR tumors exhibited a numerically higher proportion of CD8⁺ T cells (51% vs. 27% in pMMR responders; $P = 0.13$), consistent with the inherently more immunogenic nature of dMMR tumors. Despite this difference, both dMMR and pMMR responders showed prominent B-cell and plasma-cell enrichment in their local microenvironment (Supplementary Fig. S10B), suggesting that organized humoral immunity contributes to therapeutic response across these molecular subtypes.

Discussion

Neoadjuvant immunotherapy for solid tumor malignancies is an emerging paradigm based on a strong scientific rationale (17, 19) and has demonstrated compelling efficacy across several malignancies [reviewed by Awada and colleagues (37); refs. 21–24]. In colorectal cancer, most of the available data are from patients with dMMR. For example, neoadjuvant immunotherapy has been established in patients with dMMR rectal cancer with a remarkable pathologic and clinical response rate and excellent OS (27). For dMMR colon cancer, immunotherapy is also now established as the standard of care for adjuvant therapy for locally advanced disease (38), and emerging evidence strongly supports its use in the neoadjuvant setting as well (39). However, the data for the use of neoadjuvant immunotherapy for pMMR colon cancer are limited (29).

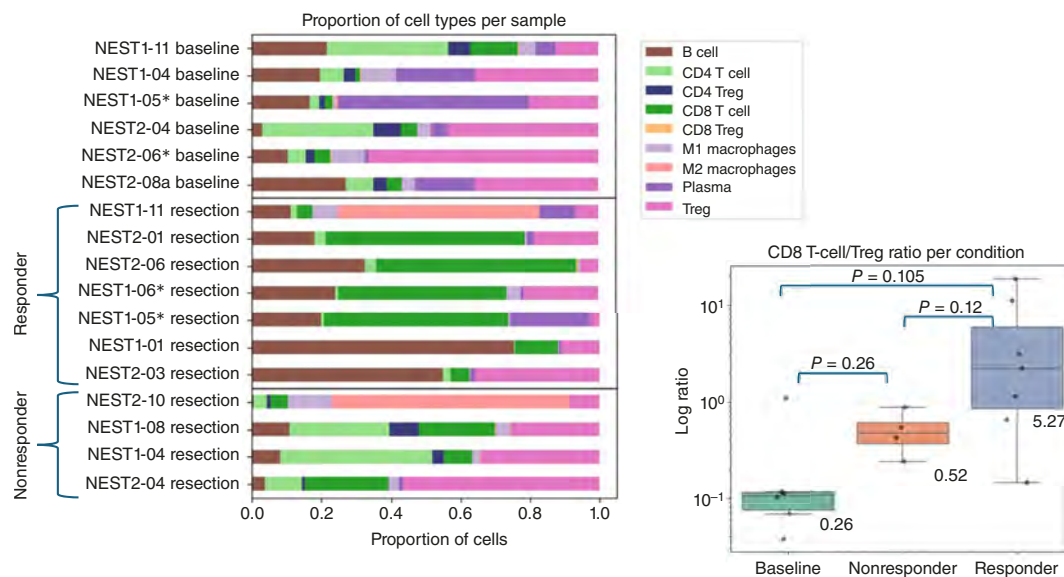
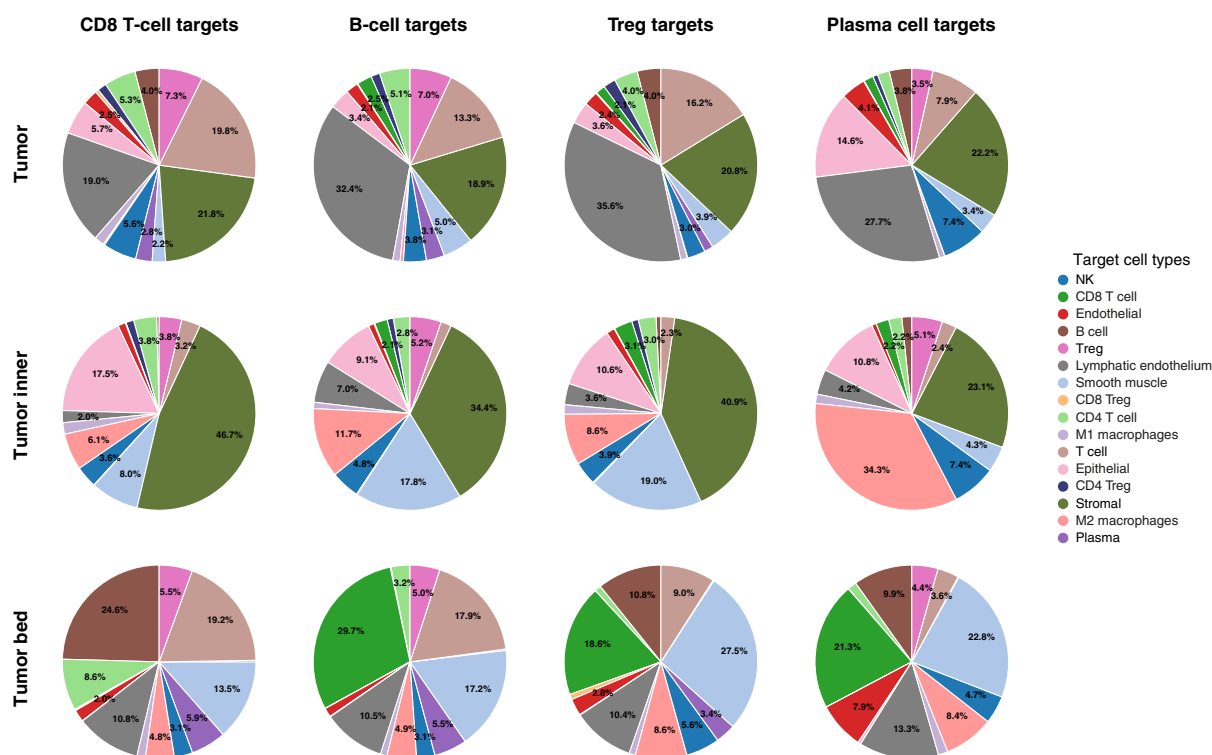


Figure 4.

Composition of the immune infiltrate from baseline to resection, in both responding and nonresponding tumors. The proportion of Tregs decreased following BOT/BAL therapy, whereas CD8⁺ T cells increased. This results in a numerical increase in the CD8⁺ T-cell/Treg ratio, more in the responders than in the nonresponders. Log-rank P value comparing the CD8⁺ T-cell/Treg ratio per condition. *, dMMR colorectal cancer.

**Figure 5.**

The immune cell community is distinct in responding ($n = 7$) vs. nonresponding ($n = 4$) tumors. The pie chart identifies the prevalence of cells within 50 microns of the cell type of interest. At baseline, the microenvironment of immune cells of interest (CD8⁺ T cells, Tregs, and B cells) is predominantly composed of lymphatic endothelium, stromal cells, and undefined T cells. Conversely, these immune cells are notably infrequently found co-localizing with other B cells, CD4⁺ T cells, or CD8⁺ T cells. In nonresponding patients, the microenvironment of the immune cells of interest is largely composed of stromal and smooth muscle cells, accompanied by a notable increase in M2 macrophages. We also observe that the local microenvironment of plasma cells is mostly comprised of M2 macrophages. In contrast, responding patients are characterized by a markedly different immune cell microenvironment composition, with relative enrichment for B cells, T cells (CD4⁺, CD8⁺, and other undefined subtypes), and Tregs (accounting for >50% of cells within these localized regions).

Here, we report the initial safety and efficacy of BOT/BAL as a novel, next-generation, dual checkpoint inhibition combination for locoregional colon cancer. In patients with dMMR colorectal cancer, this combination achieved an MPR in 4 of 4 cases, consistent with the known activity of checkpoint blockade in dMMR colorectal cancer. However, in patients with pMMR colorectal cancer, we observed an MPR rate of 41% ($n = 9/22$), including 32% ($n = 7/22$) of patients whose tumors achieved a pCR. We also noted that 7 of 8 patients who were ctDNA positive at baseline had evidence of ctDNA clearance prior to resection, further supporting the activation of antitumor immunity with this novel checkpoint combination.

The phase II NICHE-1 trial initially examined the dual checkpoint combination of nivolumab and ipilimumab in a cohort of 31 patients with pMMR colon cancer, with half of the patients randomly assigned to also receive celecoxib (28, 29). Similar to the NICHE-1 experience, in both NEST cohorts (NEST-1 and NEST-2), neoadjuvant BOT/BAL was safe and tolerable, with no patient experiencing a delay in proceeding to surgery. In NICHE-1, the median time from study initiation to surgery was 33 days, similar to our experience in NEST-1 (29 days). In NICHE-1, in a similarly staged population of pMMR colon cancer (e.g., 51% locally advanced), nivolumab/ipilimumab demonstrated modest antitumor activity, with an MPR of 19% ($n = 6/31$; ref. 29). In NEST, with the combination of BOT/BAL, the MPR was 41%, with 32% of patients

experiencing a pCR. Both NEST and NICHE studies demonstrate remarkable activity of dual checkpoint blockade in the neoadjuvant treatment setting for pMMR colorectal cancer. In our study, with 24 to 33 months of follow-up, our disease-free survival (DFS) rate is 100%. In NICHE-2, with longer follow-up, the 3-year DFS rate is 93% (29). Alternatively, with neoadjuvant chemotherapy in the FOXTROT study, the recurrence rate at 2 years was 16.9% (10).

The MPR rate in NEST-2 is numerically higher (47%) compared with NEST-1 (27%). We note that the study was not designed to compare the two treatment schedules. More patients experienced immune-mediated AEs in NEST-2 ($n = 5/14$ vs. $2/10$ in NEST-1), and 5 of 14 patients did not receive the fourth scheduled BAL dose. Although this toxicity suggests that more than two doses of immunotherapy in the neoadjuvant setting may not be feasible, it remains unclear whether NEST-2 indeed results in a greater pathologic response, and if so whether it is because of the additional BAL doses or the increased time to surgery, which would allow for more time for the immune system to attack the tumor.

Our initial correlative analysis of NEST reveals notable changes in the immune density, composition, and immune cell niche from baseline to resection in responding tumors. We identified depletion of the Treg population in the resected specimen when compared with the matching baseline biopsy in all tumors but more profound Treg depletion in tumors that responded to BOT/BAL. This is consistent with

the mechanism of action of BOT (13), indicating the activation of antitumor immunity, and is similar to NICHE-1 in which there was evidence of immune activation in both responding and nonresponding cases. However, in tumors that achieved an MPR, we observed co-localization of immune cells, in particular CD8⁺ T cells and B cells, supporting the concept of the development of an organized antitumor immune response and immune activation in response to BOT/BAL. Alternatively, in nonresponding tumors, although we saw evidence of Treg depletion and an increase in CD8⁺ T cells, we found no evidence that they co-localized or were organized to generate an antitumor immune response. Furthermore, although there was a numerically greater infiltration of CD8⁺ T cells in dMMR tumors compared with pMMR tumors with major pathologic response, resulting in a larger CD8⁺ T-cell/Treg ratio, we found similar patterns of immune cell microenvironments, suggesting similar immune activation with BOT/BAL therapy in both dMMR and pMMR tumors.

Our observation that activating mutations of the RAS/RAF pathway may be associated with less activity with BOT/BAL is limited by the small sample size. In non-small cell lung cancer, *KRAS* G12D is associated with resistance to immunotherapy, and an isogenic lung cancer model showed that *KRAS* G12D is associated with specific reduction in CD8⁺ T-cell infiltration and reduced PD-L1 expression (40). *KRAS* G12D mutations are associated with a significant reduction in IFN α and IFN γ signaling in colorectal cancer and reduced T-cell infiltrate (41), both of which would predict resistance to immunotherapy, consistent with our observations. Alternatively, in dMMR metastatic colorectal cancer, *BRAF* V600E mutation does not predict resistance to immunotherapy in a large retrospective analysis (42). These preliminary data will need further evaluation and validation.

This study has several limitations. The sample size of both NEST-1 and NEST-2 is small, and the follow-up period of the study is also relatively short; thus, the durable efficacy of the combination of BOT/BAL is unknown. Furthermore, the study was underpowered to determine the optimal duration of neoadjuvant therapy versus the number of immunotherapy treatments prior to surgical resection. However, these data highlight critical knowledge gaps in advancing checkpoint blockade for neoadjuvant colorectal cancer treatment: (i) Does therapy duration affect local and systemic outcomes? (ii) Are there biomarkers predictive of resistance and fundamentally, (iii) why is immune activation effective in the localized setting but diminished once metastases develop?

Despite these limitations, the NEST study provides preliminary evidence of the efficacy and safety of BOT/BAL in the neoadjuvant treatment setting. The compelling pathologic response, coupled with the DFS and evidence of ctDNA clearance prior to resection with BOT/BAL therapy, as well as the reorganization of the local immune infiltrate suggesting activation of a robust antitumor immune response, suggests the continued development of BOT/BAL in the neoadjuvant treatment setting for pMMR colorectal cancer.

Data Availability

Data supporting the findings of this study are available at: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE337193>. Additional deidentified patient and correlative data are available upon reasonable request to the corresponding author (MAS).

Code Availability

The code for correlative analyses can be found at: https://github.com/abcwcm/shah_NEST2025.

Authors' Disclosures

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Authors' Contributions

M.A. Shah: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, investigation, methodology, writing—original draft, project administration, writing—review and editing. **E. Hisson:** Resources, data curation, formal analysis, methodology, writing—original draft, writing—review and editing. **M.D. Jafari:** Resources, formal analysis, supervision, investigation, writing—original draft, writing—review and editing. **P.M. Kasi:** Conceptualization, data curation, funding acquisition, investigation. **M. Astorkia:** Formal analysis, methodology, writing—original draft, writing—review and editing. **S. Khan:** Data curation, writing—review and editing. **C. Owens:** Resources, data curation, supervision, writing—review and editing. **Z. Chen:** Formal analysis, validation, methodology. **H. Yeo:** Resources, data curation, supervision, investigation, writing—review and editing. **F. Socciarelli:** Data curation, formal analysis, investigation, methodology. **S. Sarkar:** Resources, supervision, investigation. **A. Nguyen:** Investigation, project administration, writing—review and editing. **D. Siolas:** Data curation, supervision, investigation, writing—review and editing. **A. Ocean:** Resources, supervision, investigation, writing—review and editing. **K. Garrett:** Resources, investigation, writing—review and editing. **L. Lowenfeld:** Data curation, investigation, writing—review and editing. **A. Pigazzi:** Resources, supervision, investigation. **S. Patel:** Resources, data curation, formal analysis, supervision, methodology, writing—review and editing. **D. Betel:** Resources, data curation, formal analysis, supervision, methodology, writing—original draft, writing—review and editing. **M. Hidalgo:** Conceptualization, resources, supervision, investigation, writing—original draft, writing—review and editing.

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Note

Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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