Clinical Cancer Research

Intratumoral Adaptive Immunosuppression and Type 17 Immunity in Mismatch Repair Proficient Colorectal Tumors

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Abstract

Purpose: Approximately 10% of patients with mismatch repair–proficient (MMRp) colorectal cancer showed clinical benefit to anti-PD-1 monotherapy (NCT01876511). We sought to identify biomarkers that delineate patients with immunoreactive colorectal cancer and to explore new combinatorial immunotherapy strategies that can impact MMRp colorectal cancer.

Experimental Design: We compared the expression of 44 selected immune-related genes in the primary colon tumor of 19 patients with metastatic colorectal cancer (mCRC) who responded ($n = 13$) versus those who did not ($n = 6$) to anti-PD-1 therapy (NCT01876511). We define a 10 gene–based immune signature that could distinguish responder from nonresponder. Resected colon specimens ($n = 14$) were used to validate the association of the predicted status (responder and nonresponder) with the immune-related gene expression, the phenotype, and the function of tumor-infiltrating lymphocytes freshly isolated from the same tumors.

Results: Although both $IL17^{Low}$ and $IL17^{High}$ immunoreactive MMRp colorectal cancers are associated with intratumor correlates of adaptive immunosuppression (CD8/IFNγ and PD-L1/IDO1 colocalization), only $IL17^{Low}$ MMRp tumors (3/14) have a tumor immune microenvironment (T*i*ME) that resembles the T*i*ME in primary colon tumors of patients with mCRC responsive to anti-PD-1 treatment.

Conclusions: The detection of a preexisting antitumor immune response in MMRp colorectal cancer (immunoreactive MMRp colorectal cancer) is not sufficient to predict a clinical benefit to T-cell checkpoint inhibitors. Intratumoral IL17-mediated signaling may preclude responses to immunotherapy. Drugs targeting the IL17 signaling pathway are available in clinic, and their combination with T-cell checkpoint inhibitors could improve colorectal cancer immunotherapy.

See related commentary by Willis et al., p. 5185

Introduction

Therapeutic antibodies inhibiting immune checkpoints, cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), and programmed cell death protein 1 (PD-1), have provided remarkable durable clinical benefit in treating cancer across histologies (1). However, the impact of these targeted therapies on colorectal cancer remains limited (2). In our clinical trial NCT01876511 using the anti-PD-1 drug pembrolizumab to treat patients with mismatch repair (MMR)-deficient (MMRd) versus MMR-proficient (MMRp) metastatic colorectal cancer (mCRC), the overall response rate was 52% for MMRd mCRC with a 2-year progression-free survival of 53% (3). However, advanced colorectal cancer with DNA MMR deficiency represents less than 5% of the total number of patients with mCRC. Although MMRp mCRC did not exhibit either high mutational density or objective responses to anti-PD-1 therapy, the disease control rate was

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Translational Relevance

Less than 5% of patients with metastatic colorectal cancer (mCRC) are mismatch repair deficient and thus candidates for anti-PD-1 as standard of care. The finding that approximately 10% of immunoreactive MMRp mCRC could also benefit from anti-PD-1 as monotherapy or as combinations could more than double the impact of immunotherapy on colorectal cancer treatment. Extensive efforts are underway to identify biomarkers that may define tumor immune microenvironments in patients with colorectal cancer deriving clinical benefit from anti-PD-1 treatment, and therefore may delineate patients with colorectal cancer eligible for immunotherapy. The association of IL17 detection with resistance to immune checkpoint blockade could provide one mechanistic explanation for the large proportion of patients who still remain resistant to anti-PD-1 therapy despite exhibiting preexisting antitumor immune response. This could also be the foundation for a new avenue of immunotherapy in colorectal cancer aimed at combining T-cell checkpoint blockade with inhibitors of the IL23/Stat3/IL17 signaling axis.

12% with 3 of 25 patients stabilizing their disease (4). In addition, although the phase III clinical trial IMblaze370 testing the efficacy of atezolizumab (anti-PD-L1)/cobimetinib (MEK inhibitor) combination to treat MMRp mCRC did not meet its primary endpoints (5), Bendell and colleagues initially reported partial responses for 7 of 84 patients in the phase 1b NCT01988896 clinical trial (6). Overall, these clinical results together with the findings by our group and others that a subset of MMRp colorectal tumors is characterized by a MMRd-like tumor immune environment (*Ti*ME; refs. 7, 8), support the concept that MMRp colorectal cancer with low tumor mutational burden can trigger antitumor immune responses and can benefit from immune checkpoint blockade–based immunotherapy. Extensive efforts are needed to identify biomarkers that delineate these patients with immunoreactive colorectal cancer and to explore new combinatorial immunotherapy strategies that can impact MMRp colorectal cancer (2).

Herein, we performed a comparative analysis of immune-related gene expression in the *Ti*ME of primary colon tumor specimens obtained from patients with mCRC who responded versus those who did not respond to anti-PD-1 therapy (NCT01876511; clinicaltrial.gov) in an attempt to define an immune signature associated with clinical response to immunotherapy and that could ultimately help delineate immunoreactive MMRp colorectal cancer from nonimmunoreactive conventional colorectal cancer. We found that a subset of patients with MMRp colorectal cancer characterized by immune correlates of intratumor immunosuppression [IFNγ^{+}PD-1^{hi}CD8^{+} T-cell infiltration, programmed death-ligand 1 (PD-L1)/indoleamine-pyrrole 2, 3-dioxygenase (IDO)-1 counter-expression on tumor cells] may be limited in their capacity to derive clinical benefit from immune checkpoint blockade because of the concomitant presence of intratumoral Th17 immunity. Our results brought evidence that a high mutational burden and/or detection of preexisting antitumor CTLs in mCRC may not be sufficient to predict clinical response to immune checkpoint blockade and other features of the *Ti*ME, including IL17 production, may negatively alter the clinical outcome of patients with colorectal cancer.

Materials and Methods

Clinical trial, patient selection, and tumor samples

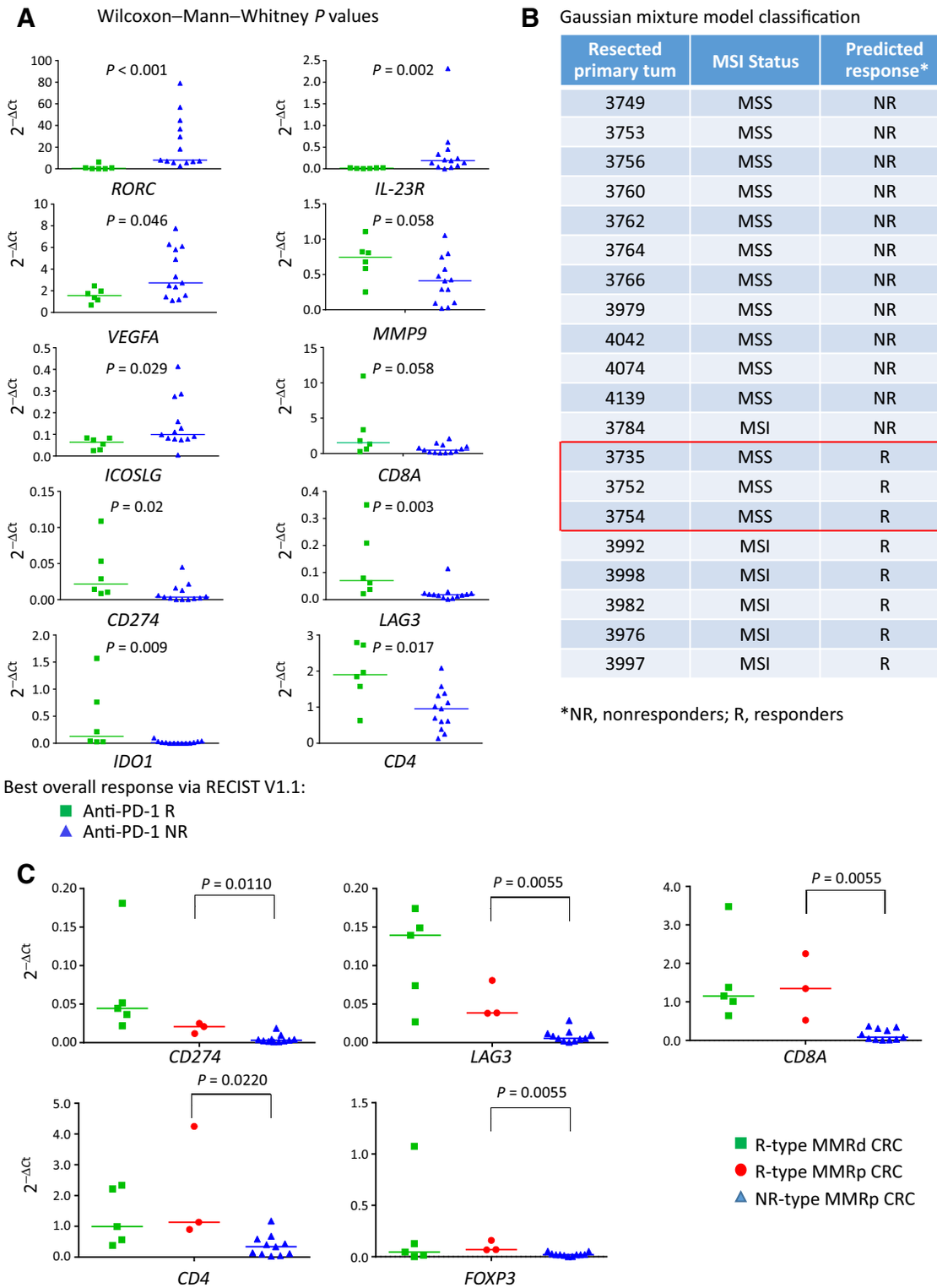
Patients with treatment refractory progressive mCRC were recruited from three centers for this phase II study using pembrolizumab as published previously (4). Patients are described in Supplementary Table S1. Fourteen microsatellite stable (MSS, also called MMRp) and six microsatellite instable (MSI, also called MMRd) colorectal cancer specimens were randomly selected for *in situ* gene expression analysis. Fresh primary sporadic colorectal cancer tissue specimens along with patient-matched normal distal colon tissue were collected at Johns Hopkins Hospital (Baltimore, MD; ref. 7). Specimens are described in Supplementary Table S2. The MSI status was determined as previously described and according to the revised Bethesda guidelines (7). This study was approved by the Institutional Review Board of Johns Hopkins University and was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice guidelines. The patients described in this study provided written informed consent and all samples were obtained in accordance with the Health Insurance and Accountability Act.

Histopathology, IHC, and image analysis

Formalin-fixed, paraformaldehyde-embedded (FFPE) specimens were stained with hematoxylin and eosin combination, CD3 (clone PS1, Leica Biosystems), CD8 (clone C8144B, Cell Marque), and IDO1 (clone SP260, Abcam/Spring Bio) according to the standard protocols. IHC for PD-L1 (clone 5H1) stain and scoring technic were described previously (7). For retinoic acid receptor–related orphan nuclear receptor gamma T (RORγT) staining, slides were baked and dewaxed followed by high pH EDTA buffer antigen retrieval for 40 minutes at 100°C. Endogenous peroxidase was blocked, subsequently anti-RORγT (clone 6F3.1, Biocare Medical, LLC) was applied for 60 minutes at a concentration of 0.0835 μg/mL at room temperature. Detection was performed using the Bond Polymer Refine Kit (Leica Biosystems). Slides were counterstained, dehydrated through a graded alcohol, and coverslipped using EcoMount (Biocare Medical). 20× whole-slides scanning used ScanScope XT and were annotated by the pathologist (R.A. Anders) for invasive (IF) and tumor-infiltrating lymphocytes (TIL) areas. Digital whole-image analysis was performed via the Indica Labs HALO platform and immune cells densities were quantified accordingly.

RNA extraction from FFPE tissue and TaqMan qRT-PCR

FFPE tissue was obtained by laser capture microdissection using the Leica LMD 7000 System (Leica) or Pinpoint Slide RNA Isolation Procedure (Zymo Research). RNA isolation, cDNA synthesis, and preamplification for TaqMan RT-PCR–based gene expression analysis was described previously (7). The selected genes were previously tested and validated to distinguish the TiME of MMRp from MMRd colorectal cancer (7). Data are expressed as $2^{-\Delta C_t}$ where $\Delta C_t = C_{t_{gene}} - C_{t_{ctrl}}$. For our calculation $C_{t_{ctrl}}$ is the average of C_t for two ubiquitous genes (*GAPDH*, *GUSB*). When undetectable, a value of 40 was arbitrarily assigned as C_t (higher number of amplification cycles). Details of the



Resected primary tum	MSI Status	Predicted response*
3749	MSS	NR
3753	MSS	NR
3756	MSS	NR
3760	MSS	NR
3762	MSS	NR
3764	MSS	NR
3766	MSS	NR
3979	MSS	NR
4042	MSS	NR
4074	MSS	NR
4139	MSS	NR
3784	MSI	NR
3735	MSS	R
3752	MSS	R
3754	MSS	R
3992	MSI	R
3998	MSI	R
3982	MSI	R
3976	MSI	R
3997	MSI	R

*NR, nonresponders; R, responders

Figure 1.

A 10 immune-related gene expression signature associated with clinical response to pembrolizumab treatment delineates a group of immunoreactive MMRP colorectal cancer among resected patients with primary colorectal cancer. **A,** Immune-related gene expression comparison between responders (R; $n = 6$, green squares) and nonresponders (NR; $n = 13$, blue triangles) mCRC to pembrolizumab treatment. Ten of 44 top genes (*Continued on the following page.*)

TaqMan Assays (Thermo Fisher Scientific) are shown in Supplementary Table S3A and S3B.

Tumor processing and flow cytometry

TILs were isolated from freshly dissociated tissues using an enzymatic digestion cocktail (DNase I, 2,500 U/mL and Liberase 400 U/mL, MilliporeSigma) and a Percoll Density Gradient (Thermo Fisher Scientific; ref. 7). Multiparameter flow cytometry and cytokine intracellular staining for IFNγ were performed following a 3-hour *in vitro* stimulation in the presence of stimulation cocktail (PMA and ionomycin; EBioscience/Thermo Fisher Scientific) and GolgiStop (Monensin; BD Biosciences) according to the manufacturer's instructions (7). Data were analyzed using DIVA 6.1 Software (BD Biosciences).

Statistical analysis

The gene expressions were used to build a model to predict clinical responses to anti-PD-1 coded as R (complete response/partial response; R) and NR (no response). Gene expression levels were on the log scale and were centered across patients for each gene. Summary statistics were calculated for all genes, and compared between response status using Wilcoxon–Mann–Whitney tests. Heatmaps of gene expression levels were depicted for those genes with the 20 lowest *P* values based on the Wilcoxon–Mann–Whitney test comparing R versus NR. An unsupervised Gaussian mixture model of response was performed with a limited top 10 smallest *P* values (*RORC*, *IL23R*, *LAG3*, *IDO1*, *CD4*, *CD274*, *ICOSLG*, *VEGFA*, *CD8A*, and *MMP9*). The mixture model assumed a Multivariate Gaussian distribution for each of two groups and assumes equal volume and shape across the two groups. Assessment of the model performance in predicting response includes the sensitivity/specificity. The mixture model was first developed from trial patients, and then was used to predict response status for those patients in the untreated primary colorectal cancer cohort. Colorectal cancer patients' gene expression levels were depicted in heatmaps and separated by predicted response status (Supplementary Fig. S1). Individual gene expressions were further compared between the predicted response status (R vs. NR) of the MMRp colorectal cancer cases using the Wilcoxon–Mann–Whitney test. Statistical analyses were performed using the R statistical package (version 3.4.0). *P* values were not adjusted for multiplicity.

Results

Identification of immunoreactive MMRp colorectal cancer

An immune-related gene expression signature in the T*i*ME of primary colon tumors of patients with mCRC who responded to anti-PD-1 was used to define patients with immunoreactive MMRp colorectal cancer who benefited from immune checkpoint blockade–based immunotherapy (7, 8). For this purpose, we tested the expression of 44 genes in the primary colon tumors (prior to standard-of-care therapy) of patients with mCRC treated with pembrolizumab (Supplementary Table S3A). Total RNA was extracted and analyzed in 19 primary colon tumor specimens obtained from patients with mCRC treated with pembrolizumab under our study (NCT01876511; clinicaltrial.gov). Six patients have an objective response including complete response/partial response. Nine patients had progressive disease (PD) and 4 had stable disease (SD; ref. 4). Samples were tested with this 44 gene panel and then narrowed down to the top 10 differentially expressed genes (the lowest *P* values; median, Mann–Whitney nonparametric test) between responder (R) and nonresponder (NR; PD and SD) patients with mCRC. Significantly upregulated genes were *CD4* and *CD8A* ($P = 0.017$ and 0.058, respectively), *RORC* (coding RORγt) and *IL23R* ($P < 0.001$ and $= 0.002$, respectively), *CD274* (coding PD-L1), *LAG3*, and *IDO1* ($P = 0.02$, 0.003, and 0.009, respectively), *VEGF* and *MMP9* ($P = 0.046$ and 0.058, respectively), and *ICOSLG* ($P = 0.029$; Fig. 1A; Supplementary Table S4 for detailed analysis). We observed that responder mCRC patients exhibited a stronger T-cell infiltration (indicative of inflamed MMRp colorectal cancer) and checkpoint gene expression (indicative of adaptive immunosuppression) signatures, as well as a lower Th17-associated gene expression compared with nonresponder mCRC (Fig. 1A).

A Gaussian mixture model was built to define how this 10 gene signature would separate responding from nonresponding patients. The model achieved a sensitivity of 0.67 [95% confidence interval (CI), 0.22–0.96] and a specificity of 0.92 (95% CI, 0.64–1.00), respectively (Supplementary Fig. S1). We further validated this model in a separate cohort of untreated patients' primary colon tumors ($n = 20$). We wanted to test whether we would be able to identify inflamed colorectal cancer including MMRd and immunoreactive MMRp colon tumors (4). Therefore, based on our immune gene signature, untreated primary colon tumors were predicted to exhibit a R or NR phenotype based on the resemblance of their T*i*ME with that of primary colon tumor in patients with mCRC responsive (R-type T*i*ME) or not responsive to anti-PD-1(NR-type T*i*ME) therapy. The resected colon specimens served as a validation set to test the association of the predicted status (R and NR) with the immune-related gene expression, the phenotype and the function of TILs freshly isolated from the same tumors. Three of 14 resected MMRp tumor specimens (∼21%; #3735, #3752, and #3754) exhibited an R-type T*i*ME (Fig. 1B). Five of the six MMRd-resected tumor specimens (∼83%) were associated with R-type T*i*ME and therefore suggested the accuracy of the model used because it would be predicted that approximately 80% of MMRd colorectal cancer would show clinical response to anti-PD-1 therapy (4, 9).

(*Continued.*) with the lowest *P* values when comparing R- and NR-derived primary colon tumor tissues of mCRC are shown. The full analysis including the 44 genes is shown in Supplementary Table S4. A Gaussian mixture model was used to model the relationship between the 10 genes and the R/NR status. The model achieved a sensitivity and specificity of 0.83 (0.36–1.00) and 0.54 (0.25–0.81), respectively (Supplementary Fig. S1). **B,** The Gaussian mixture model using the 10 genes based on *P* values was used to predict response status (R vs. NR) in resected primary colorectal cancer specimens (untreated patients). The red open box indicates the MSS colorectal cancer (i.e., MMRp) with an R-type immune signature. **C,** Differential gene expression between R-type MMRp (red circles) and NR-type MMRp (blue triangles) primary colorectal cancer. Gene expression analysis performed on FFPE tumor tissue showed a higher expression of T-cell checkpoints (*CD274, LAG3*, and *CTLA4*), CTL (*CD8A*), and Treg (*CD4* and *FOXP3*) associated genes in R-type MMRp (red circles) versus NR-type MMRp (blue triangles). MMRd colorectal cancer (green squares) are shown for representing highly immunogenic specimens. Graphs show $2^{-\Delta C_t}$ where ΔC_t are the genes of interest C_t normalized by GAPDH and GUSB C_t.

R-type MMRp colon tumors from the validation set are MMRp tumors infiltrated with exhausted IFNγ-producing PD-1^{hi} CD8^{+} T cells

The R-type MMRp colorectal cancer from the validation cohort expressed a higher level of genes associated with immune infiltration (*CD4*, $P = 0.0220$ and *CD8A*, $P = 0.0055$) T-cell checkpoints (*CD274*, $P = 0.0110$ and *LAG3*, $P = 0.0055$), regulatory T cells (Treg; *FOXP3*, $P = 0.0055$), compared with other MMRp colorectal cancer cases (Fig. 1C and detailed in Supplementary Table S5). TIL freshly isolated from resected specimens were evaluated by intracellular cytokine staining, multiparameter flow cytometry, and cell sorting of the populations of interest. Two of the three R-type MMRp colon tumors were highly infiltrated with IFNγ^{+}PD-1^{hi}–activated CD8^{+} T cells and considered to be immunoreactive (unavailable fresh TILs for #3754; Fig. 2). Notably, flow cytometry analysis of these immunoreactive MMRp colorectal cancer specimens showed a dramatic difference in IFNγ production by PD-1^{hi} versus PD-1^{low} CD8^{+} cells, with PD-1^{low} CD8^{+} T cells having systematically a higher IFNγ mean of fluorescence compared with PD-1^{hi} CD8^{+} cells (Supplementary Fig. S2A). This suggests an exhausted function of PD-1^{hi}CD8^{+} T cells compared with PD-1^{lo}CD8^{+} T cells (10). IFNγ^{+}PD-1^{hi} CD8^{+} T cells are, at the most, sparse in the corresponding patient-matched distal normal tissue (Supplementary Fig. S2B). We then sorted the PD-1^{hi}, PD-1^{lo}, and PD-1^{neg} CD8^{+} TIL ($n = 6$) and compared gene expression between PD-1^{hi} and PD-1^{lo} to PD-1^{neg} CD8^{+} TIL

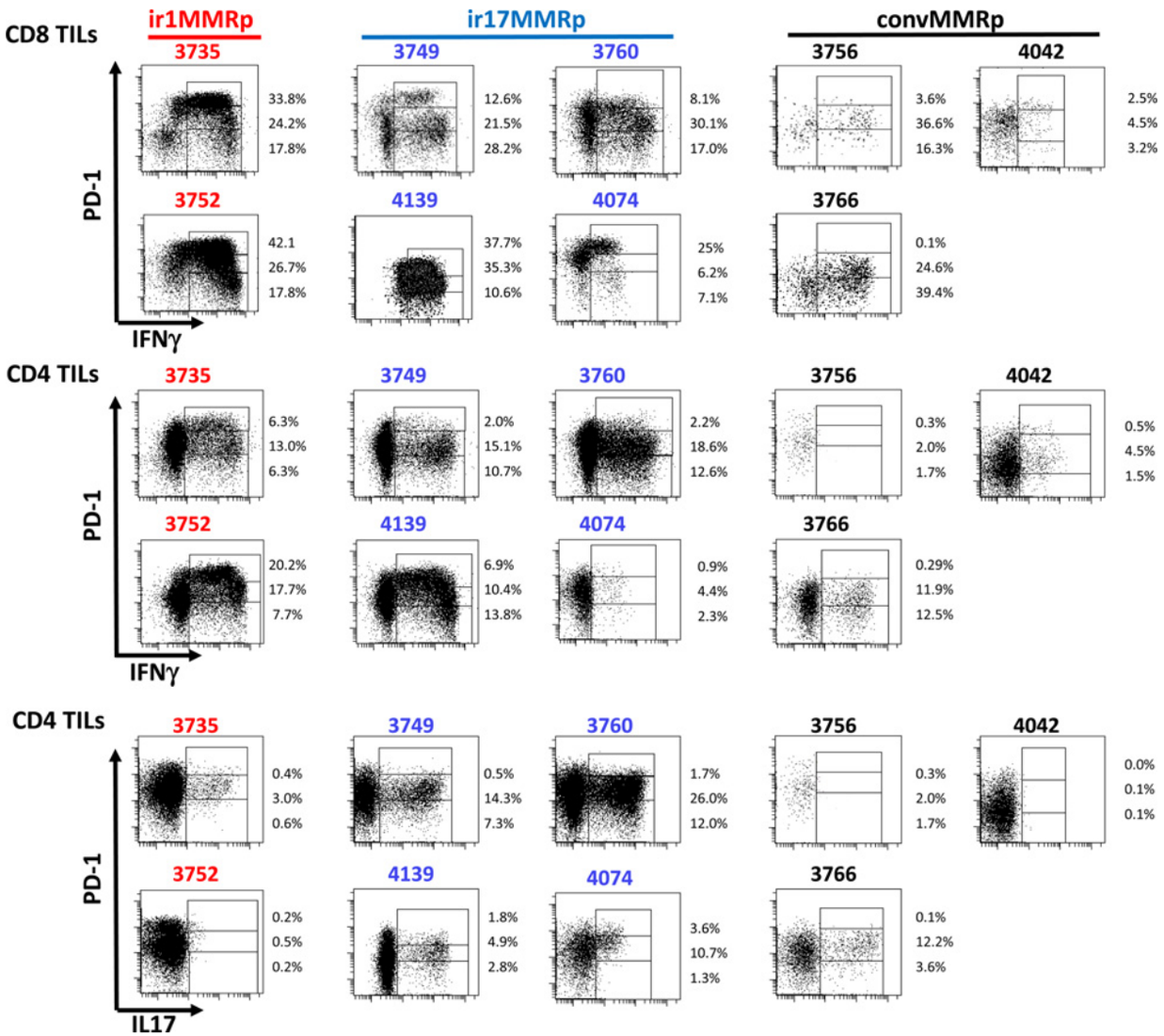


Figure 2.

Flow cytometry analysis of IFNγ and IL17 expression by freshly isolated immunoreactive and conventional MMRp colon TILs. The figure shows PD-1 expression versus IFNγ production by CD8 (top two rows) and CD4 (two middle rows), and PD-1 expression versus IL17A by CD4 cells (bottom two rows) in type 1 (#3735 and #3752), type 17 (#3749, #3760, #4139, and #4074) immunoreactive (ir1MMRp and ir17MMRp, respectively) and conventional (convMMRp; # 3756, #3766, and #4042) MMRp colon tumors. CD4 and CD8 cells are distinguished by their level of PD-1 expression, high, low, and negative (Hi, Lo, and Neg gates in each plot), and percent in each gate is indicated. The PD-1 gates are defined relatively to the level of PD-1 in the patient-matched normal colon.

using TaqMan qRT-PCR on a defined immune-related gene array (Supplementary Tables S3B and S6). PD-1$^+$ TILs (PD-1^{hi} and PD-1^{Lo}) exhibited an exhausted/effector memory gene expression profile because these cells lacked of effector cytokines (*IL2*, *IL15*, and *TNFA*) and had a lower *CD28*, *CCR7*, *IL7R*, and *CD62L* gene expressions than PD-1^{neg} CD8 cells (Supplementary Fig. S3). We observed that *CXCL13* expression was increased in PD-1$^+$CD8$^+$ TILs versus PD-1^{neg}CD8$^+$ TILs. CXCL13 is a chemoattractant protein involved in the recruitment of immune cells and the formation of tertiary lymphoid structures (11, 12). PD-1$^+$CD8$^+$ TILs also demonstrated higher expression of the T-cell checkpoints *CTLA4*, *LAG3*, and *HAVR2* (coding Tim3), costimulatory molecules, TNFRSF4 (coding OX40), *TNFRSF9* (coding 4-1BB), and *TNFRSF18* (coding *GITR)*, as well as higher expression of *EOMES* and *GZMB* mRNA (Supplementary Fig. S3). Importantly, genes involved in senescence (*KLRG1*) or anergy (*EGR3*) were not upregulated in the PD-1^{hi} fraction (Supplementary Fig. S3). Our analysis therefore suggested that, higher PD-1 expression (negative < low < high PD-1 expression) is associated with a deeper functional exhaustion of CD8 TILs. MMRd colorectal cancer #3784, which was associated with a NR-type T*i*ME (Fig. 1B), had no detectable IFNγ$^+$PD-1^{hi} CD8$^+$TILs by flow cytometry (Supplementary Fig. S4). Therefore, TILs signature in immunoreactive MMRp colon tumors resembles the one recently described in MMRd tumors and characterized by a vigorous CD8$^+$ T-cell infiltration with a Th1/Tc1 polarization, as well as T-cell checkpoints expression (7). However, Fig. 2 showed that despite being associated with a NR-type T*i*ME, four MMRp colorectal cancer cases (#3749, #3760, #4074, and #4139) were unexpectedly characterized by PD-1^{hi}IFNγ$^+$ CD4$^+$ and/or CD8$^+$ TILs (Fig. 2) suggesting that a subset of NR-type MMRp colorectal cancer are nevertheless immunoreactive. In conclusion, the sole detection of activated CD8$^+$ T cells in MMRp colon tumors was not sufficient to define R-type T*i*ME guaranteeing the efficacy of immune checkpoint blockade treatment.

NR-type immunoreactive MMRp colon tumors are infiltrated with IL17$^+$ T cells

Because MMRp colorectal cancer cases #3735, #3752, and #3754 were characterized by a significantly higher expression of the *CD274* gene (coding PD-L1) compared with other MMRp colorectal cancer specimens (Fig. 1C) and by the presence of PD-1^{hi}IFNγ$^+$ TILs (Fig. 2), we sought first to confirm the expression of PD-L1 protein by IHC on FFPE tumor tissue sections. Figure 3A shows that two of the R-type MMRp colorectal cancer (#3735 and #3752) exhibited a remarkable high expression of PD-L1 (80% and 100%, respectively; $P = 0.0182$ compared with NR-type MMRp tumor). The third specimen (#3754) did not have an identifiable invasive front region in the examined specimen and therefore could not be scored for PD-L1 expression. However, four MMRp colorectal cancer cases (#3749, #3760, #4074, and #4139) with a NR-type T*i*ME (Fig. 1), and reported earlier to be infiltrated with IFNγ$^+$ PD-1^{hi}CD8$^+$T cells (Fig. 2) also exhibited a strong PD-L1 staining (>20%; Fig. 3A). This finding reinforced the concept that CTL infiltration and activation of the PD-1/PD-L1 axis in colorectal cancer may not be sufficient to grant efficacy to immune checkpoint blockade (or R-type immune signature in primary colon tumor) and that other components of the T*i*ME may interfere with the clinical response. When testing the TILs freshly isolated from the colorectal cancer specimens, we found that the four PD-L1^{hi} MMRp specimens (3760, 4139, 4074, and 3749) which were modeled as nonresponder in Fig. 1, produced IFNγ (55%, 84%, 38%, and 63% of CD8$^+$ T cells; 33%, 31%, 8%, and 28% of CD4$^+$T cells, respectively) and IL17 (40%, 10%, 16%, and 22% of CD4$^+$ T cells, respectively) upon PMA/Ionomycin stimulation (Fig. 2). With the exception of patient #3749, detection of IL17-producing TILs was associated with the *in situ* expression of IL17-associated genes (*IL17A*, *RORC*, and/or *IL23R*) for 3 of these patients (Table 1). Therefore, although these MMRp specimens were immunoreactive and characterized by IFNγ$^+$PD-1^{hi} CD8$^+$ TIL (Fig. 2) along with a high level of PD-L1 expression (Fig. 3A), the detection of IL17-producing cells (4/4) and/or an IL17-associated transcriptomes (3/4, Table 1) prevailed over their classification as possible R-type immunoreactive MMRp cases (Fig. 1B). None or low IL17 protein expression was found for the MMRp colorectal cancer cases 3752 and 3735 (1% and 4% of CD4$^+$ T cells, respectively; Fig. 2). In sum, our findings predict a deleterious impact of IL17 in immunoreactive MMRp colon tumors interfering with their clinical response to immune checkpoint blockade. Altogether, we propose to distinguish conventional MMRp colorectal cancer (PD-L1$^-$, IFNγ$^-$IL17$^-$) and type 17 (PD-L1$^+$, IFNγ$^+$IL17^{hi}) from the type 1 (PD-L1$^+$, IFNγ$^+$IL17^{lo}) immunoreactive MMRp colorectal cancer group, the latter being expected to benefit from immune checkpoint blockade. Conventional MMRp and type 17 immunoreactive MMRp colorectal cancer would be expected to progress upon checkpoint blockade monotherapy. Both type 1 and 17 immunoreactive MMRp tumor specimens had significantly higher expression of PD-L1 at the IF compared with conventional MMRp colorectal cancer ($P =$ 0.0476 and 0.0159; Fig. 3A). Both also expressed higher levels of genes associated with a type 1 immune signature, including *IFNG* ($P = 0.0354$ and 0.0079, respectively), *CD8A* ($P = 0.0357$ and 0.0159, respectively), and genes coding T-cell checkpoints, including *CD274* ($P = 0.035$ for ir1MMRp but not significant for ir17MMRp) and LAG3 ($P = 0.0357$ and 0.0159, respectively; Supplementary Fig. S5).

Immunopathologic definition of type 1 and 17 immunoreactive MMRp colorectal cancer

Type 1 and 17 MMRp colon tumors were further distinguished by CD8/IDO1/PD-L1 and RORγt IHC studies. Representative cases of both groups of immunoreactive and conventional MMRp colon tumors are shown in Fig. 3B and Supplementary Figs S6 and S7. *IDO1* and *CD274* (coding PD-L1) are both IFNγ target genes, and the colocalization of encoded proteins IDO1 and PD-L1 with CD8 cells (in #3752, #3735, #3760, and #3749) is thought to reflect the intratumor adaptive immunosuppression phenomenon (13). Importantly, we found a remarkable correlation between the proportions of Th1 but not of Th17 cells in freshly isolated TIL and the PD-L1 scoring obtained via IHC (Supplementary Fig. S8) validating the use of PD-L1 IHC as a correlate of intratumoral type-1 immunity. RORγt is a transcription factor critical for IL17 expression and we used its detection in tumor tissue as a correlate of intratumoral type 17-immunity. Figure 3B and Supplementary Figs S6 and S7 show that the detection of PD-L1 and RORγt allows the distinction between type 1 (PD-L1 high, RORγT low) and type 17 (PD-L1 high, RORγT high) MMRp colorectal cancer. Conventional MMRp colorectal cancer exhibited no or low PD-L1 and RORγT staining (Supplementary Fig. S7). Although the increased gene expression of *IDO1* in types 1 and 17 immunoreactive MMRp tumors

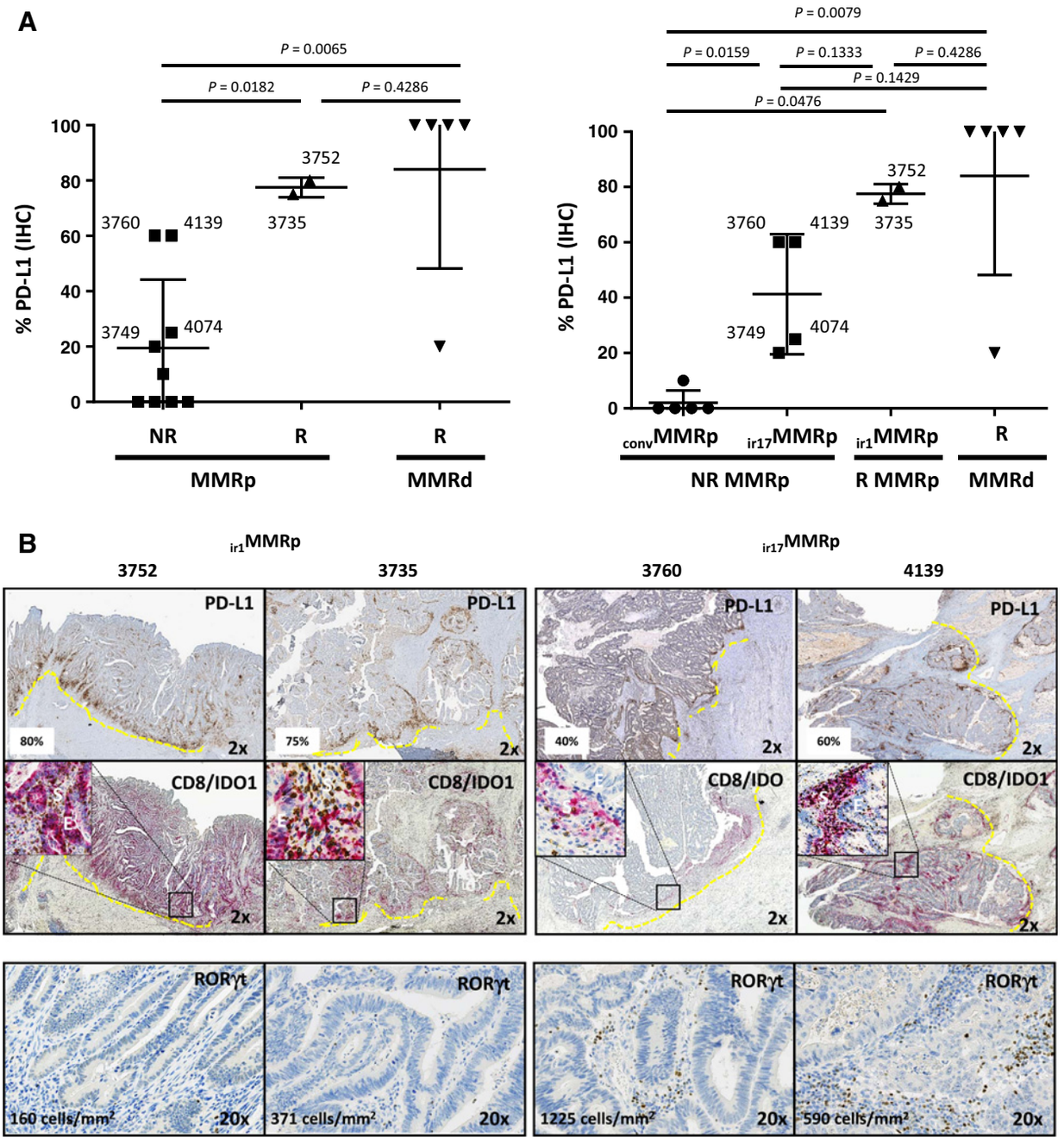


Figure 3.

PD-L1, IDO-1, and RORγt protein expressions in immunoreactive MMRp and conventional MMRp colorectal cancer. **A,** The left graph shows PD-L1 IHC performed on FFPE tumor tissue sections of R-type MMRd (R MMRd; inversed triangles), R-type MMRp (R MMRp; triangles), and NR-type MMRp (NR MMRp; squares) primary colorectal cancer specimens. The graph on the right shows the PD-L1 expression when distinguishing NR MMRp as conventional MMRp (convMMRp; circles) and type 17 immunoreactive MMRp (ir17MMRp; squares) colon tumors. R-type MMRp are type 1 immunoreactive MMRp (ir1MMRp; triangles) colon tumors. R MMRds are represented as inversed triangles. PD-L1–expressing ir17MMRp specimens (#3760, #3749, #4139, and #4074) were found to be infiltrated with $CD8^+PD\text{-}1^{hi}IFN\gamma^+$ TIL in Fig. 2. **B,** IHC staining for PD-L1, CD8/IDO1 (dual staining), and RORγT in representative R-type ir1MMRp colorectal cancer (#3735 and #3752) and NR-type ir17MMRp (#3760 and #4139) colon tumors. RORγT cell densities expressed as number of cells/mm^2 in annotated tumor invasive front. Insets in CD8/IDO-1 pictures represent 20× magnification of epithelial (E) versus stromal (S) patterns of expression of IDO1.

compared with conventional MMRp tumors was not significant, we found that the IDO1 protein IHC staining was not detected in conventional MMRp colon tumors pointing out a discrepancy between mRNA and protein expression (Supplementary Fig. S7). Furthermore, the pattern of expression of IDO1 in tumor tissue distinguished types 1 and 17

Table 1. Characteristics of the untreated primary MMRp colorectal cancer resections

			Genomics		IHC			MFC		Gene expression signature[c]			
Id	TiME Signature	MMRp Groups	MSI	# Mutations	CD8	IDO1[a]	PD-L1[b]	Th1	Th17	Th1		Th17	
3735	R	ir1MMRp	Neg	72	290	+	75%	26%	4%	+++	*IFNG/TBX21/IDO1*	+++	*IL17A/RORC/IL23R*
3752	R	ir1MMRp	Neg	76	1563	+	80%	46%	<1%	+++	*IFNG/TBX21/IDO1*	–	NA
3754	R	ir1MMRp	Neg	nd	25	Nd	10%	Nd	nd	+	*IDO1*	++	*RORC/IL23R*
3749	NR	ir17MMRp	Neg	120	325	–	20%	26%	22%	–	NA	–	NA
3760	NR	ir17MMRp	Neg	95	170	–	50%	33%	40%	++	*IFNG/TBX21*	+++	*IL17A/RORC/IL23R*
4074	NR	ir17MMRp	Neg	122	336	–	10%	8%	15%	+++	*IFNγ/TBX21/IDO1*	++	*RORC/IL23R*
4139	NR	ir17MMRp	Neg	188	159	–	50%	31%	9%	++	*IFNG/TBX21*	+++	*IL17A/RORC/IL23R*
3756	NR	convMMRp	Neg	nd	18	–	1%	4%	4%	+	*TBX21*	+	*IL23R*
3762	NR	convMMRp	Neg	nd	138	Nd	5%	Nd	nd	–	NA	++	*IL17A/IL23R*
3764	NR	convMMRp	Neg	nd	182	Nd	0%	Nd	nd	++	*TBX21/IDO1*	++	*IL17A/IL23R*
3766	NR	convMMRp	Neg	nd	130	–	1%	25%	16%	–	NA	++	*IL17A/IL23R*
4042	NR	convMMRp	Neg	127	48	Nd	10%	6%	0%	+++	*IFNγ/TBX21/IDO1*	++	*RORC/IL23R*

Abbreviations: ir1MMRp, type 1 immunoreactive MMRp; ir17MMRp, type 17 immunoreactive MMRp; convMMRp, conventional MMRp.

[a]Detection of IDO1 on epithelial cells.

[b]Scoring of PD-L1 at the invasive front.

[c]+, one gene expressed; ++, two genes expressed; +++, three genes expressed; –, no genes expressed.

immunoreactive MMRp colon tumors, IDO1 was strongly expressed in epithelial cells of type 1 immunoreactive MMRp tumors and conversely, mainly expressed in stromal cells of type 17 immunoreactive MMRp tumors (Fig. 3B). Whereas number of mutations and CD8 density in the invasive front and tumor area are significantly higher in MMRd colon tumors, in our case, these biomarkers did not clearly distinguish conventional MMRp from type 1 and type 17 immunoreactive MMRp tumors (Supplementary Fig. S9). Neither immunoreactive MMRp (resected primary tumors from untreated patients) nor patients with MMRp mCRC with durable SD upon anti-PD-1 therapy, showed POLD or POLE mutations (not shown) or displayed high numbers of mutations (Supplementary Tables S1 and S2).

Discussion

It is predicted that about 101,420 new cases of colorectal cancer will be diagnosed in 2019. Approximately 20% will be metastatic and 51,020 deaths will occur due to mCRC ("Key statistic for colorectal cancer," American Cancer Society, www.Cancer.org). Only 2%–3% of mCRC are MMRd and currently eligible for pembrolizumab treatment as the standard of care (3). We established that MMRp colorectal cancer which express high levels of PD-L1, infiltrated by $CD8^+PD\text{-}1^{hi}IFN\gamma^+$ and no IL17 producing TIL have a T*i*ME which resembles that of advanced MMRd mCRC responsive to anti-PD-1 therapy in the setting of our pembrolizumab trial (4). $PD\text{-}1^{hi}CD8^+$ TILs detected in patients with MMRp colorectal cancer were characterized by an exhausted/memory transcriptome suggesting the presence of an antitumor T-cell repertoire, as previously reported in melanoma and digestive system cancers (10). Endogenous antitumor T-cell immunity is largely restricted to $PD\text{-}1^{hi}$ TILs and infiltration of non–small cell lung carcinoma with such $PD\text{-}1^{hi}CD8^+$ T cells has recently been associated with clinical response to anti-PD-1 (10, 11). Our findings, therefore, point out that low mutational density cancers may still exhibit one or several immunogenic mutations that can be recognized by the patient's immune system (14). On that note, we recently reported that 1 of the patient with MMRp mCRC who developed a durable SD upon anti-PD-1 treatment in the clinical trial NCT01876511 was indeed characterized by an immune response targeting the hotspot mutation *AKT1* E17K (15). Unfortunately, patients with immunoreactive MMRp mCRC are not captured by the currently FDA-approved microsatellite-high/DNA MMRd biomarker and are therefore unable to receive immune checkpoint blockade as part of their care despite being highly immunogenic and having benefited from pembrolizumab treatment in our phase II clinical trial (4).

Recent advances in the understanding of the role of the cancer-associated immune contexture into clinical prognosis have fueled efforts at stratifying patients with primary and mCRC into different subsets with the final aim of defining eligibility of patients with colorectal cancer for immunotherapeutic interventions (16). Extensive and sophisticated gene expression profiling and Immunoscore analysis of colorectal cancer recently helped to predict clinical outcomes of patients with colorectal cancer (8, 17). However, none of these complex characterization approaches of colorectal cancer have thus far been tested as predictive biomarkers of response to immune checkpoint blockade. Herein, taking advantage of a landmark clinical trial comparing the clinical response of patients with MMRp and MMRd mCRC to pembrolizumab (4), we found ourselves in a unique position to interrogate the T*i*ME of the primary colon tumors of patients with colorectal cancer who responded versus those who did not. By associating the clinical response to anti-PD-1 with the nature of the T*i*ME, we further identified a significant proportion (3/14 or ∼21%) of patients with MMRp colorectal cancer (type 1 immunoreactive MMRp colorectal cancer) who may be targeted via immune checkpoint blockade–based immunotherapy. Most importantly, our findings also highlight that the detection of IL17 and/or an IL17 gene expression signature should be investigated in cohorts of patients with colorectal cancer treated with PD-1 blockade as a critical component of the colon T*i*ME, which might be able to distinguish R from NR patients. Th17 cells were recently found to be enriched in MMRp compared with MMRd colon tumors and their detection was associated with a worse clinical outcome, but they have not yet been evaluated for their impact on the clinical response to checkpoint inhibitors (18, 19). Our findings in untreated primary MMRp colorectal cancer confirmed that despite the fact that several MMRp cases were characterized by a strong PD-L1 expression and an IFNγ signature, their corresponding IL17 signature distinguished them from MMRp predicted to respond to immune checkpoint blockade treatment. In sum, we have defined on resected colorectal cancer specimens the analytical tools that, when validated on larger cohort of treatment refractory mCRC receiving immune checkpoint inhibitors, could

help identify a broader population of mCRC more likely to benefit from T-cell checkpoint blockade. Mining MMRp colorectal cancer RNA sequencing in The Cancer Genome Atlas datasets, we found that about 10% of patients with primary MMRp colorectal cancer are characterized by a type 1 immune signature without IL17-related gene expression (Supplementary Fig. S10). Furthermore, Tosolini and colleagues found that 4% of patients with colorectal cancer had a high Th1/cytotoxic gene signature with low expression of gene from the Th17 cluster (*IL17A/RORC*) with the best disease-free survival at 5 years (19). The perspective that up to 10% of immunoreactive MMRp mCRC could also benefit from anti-PD-1 as monotherapy or as combinations could more than double the impact of immunotherapy on colorectal cancer treatment. About 40% of MMRp colorectal cancers have a mixed Th1/Th17 signature (Supplementary Fig. S10). The mechanisms associated with the generation of an intratumoral IL17 response in certain subsets of colon tumors remain unclear. The microbiota (including biofilm formation) and the tumor metabolism (including IDO/Kynurenine/AHR pathway), two critical components of the tumor microenvironment are thought to be key in shaping the intratumoral immunity and responses to immunotherapy in colorectal cancer by switching on/off intratumoral IL17 production (20, 21). Although the contribution of IL17 into the mechanisms of resistance to immunotherapy is largely unknown, IL17 and Th17 cells are critical inflammatory components for the defense against invading pathogens (recruitment of phagocytic and granulocytic immune cells or induction of IL22-dependent bactericidal peptides), as well as for maintaining the integrity of the intestinal barrier by promoting epithelial regeneration upon tissue damage (IL22 and Stat3-dependent epithelial cells proliferation and survival), features that all could contribute to tumor growth when unchecked (22).

In conclusion, our analysis of the T*i*ME of patients with MMRp colorectal cancer highlights two critical findings for the selection of patients with cancer for immune checkpoint blockade. First, a substantial number of MMRp colorectal cancer exhibit a T*i*ME similar to MMRd colorectal cancer despite their low tumor mutation number. Second, the detection of an IL17 immune signature is a predominant element in the T*i*ME of patients with colorectal cancer that may impact their response to immune checkpoint blockade and which should be taken into account for guiding immunotherapy decisions as suggested previously (23, 24). If validated in a larger cohort of patients as part of a biomarker-driven clinical trial, the association of IL17 detection with resistance to immune checkpoint blockade could provide one mechanistic explanation for the large proportion of patients who still remain resistant to anti-PD-1 therapy despite exhibiting a high level of PD-L1 and a strong CD8 infiltration in their tumors, both canonical hallmarks of a preexisting antitumor immune response (13). This could also be the foundation for a new avenue of immunotherapy in colorectal cancer aiming at combining T-cell checkpoint blockade with inhibitors of the IL23/Stat3/IL17 signaling axis known to be detrimental in colorectal cancer clinical outcomes (19). Previous experimental data showed that tumor-bearing mice treated with 5-fluouracil had increased intratumoral IL17 and IL17A-neutralizing antibodies improved tumor reduction (25). Drugs targeting the IL23/IL17 have already proven clinical efficacy or are currently tested in clinical trials in the treatment of autoimmune disorders, such as psoriasis, rheumatoid arthritis, or spondyloarthritis (26, 27) and could therefore be tested in combination with immune checkpoint blockade to improve the clinical responses of colorectal cancer to immunotherapy.

Disclosure of Potential Conflicts of Interest

N.J. Llosa reports receiving other commercial research support from and is a consultant/advisory board member for Bristol-Myers Squibb. K.N. Smith reports receiving speakers bureau honoraria from Neon Therapeutics. J.M. Taube reports receiving commercial research grants from Bristol-Myers Squibb, and is a consultant/advisory board member for Bristol-Myers Squibb, Merck, Amgen, and AstraZeneca. C.L. Sears reports receiving commercial research grants from Bristol-Myers Squibb, and is a consultant/advisory board member for Merck. D.T. Le reports receiving commercial research grants from Bristol-Myers Squibb and Merck, speakers bureau honoraria from Merck, and is a consultant/advisory board member for Bristol-Myers Squibb. L.A. Diaz is an employee of Jounce Therapeutics and Personal Genome Diagnostics (PGDx), reports receiving commercial research grants from Merck, holds ownership interest (including patents) in Personal Genome Diagnostics (PGDx), PapGene, Jounce Therapeutics, and NeoPhore, and is a consultant/advisory board member for Merck, Personal Genome Diagnostics (PGDx), and Neophore. D.M. Pardoll reports receiving commercial research grants from AstraZeneca, Bristol-Myers Squibb, Compugen, Ervaxx, and Potenza, holds ownership interest (including patents) in Aduro Biotech, DNAtrix, Potenza, Tizona, Trieza, WindMil, and Dracen, and is a consultant/advisory board member for Rock Springs, Merck, Janssen, Immunomic, FLX Bio, Five Prime, Dynavax, Camden Partners, Bayer, and Amgen. R.A. Anders reports receiving speakers bureau honoraria from Bristol-Myers Squibb, Merck, and AstraZeneca. F. Housseau reports receiving commercial research grants from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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