SUPPLEMENTARY METHODS

Tissue digestion and cell isolation

Tumour tissues, tumour-associated lymph nodes, and colorectal healthy mucosa were collected in IMDM+Glutamax medium (Gibco) complemented with 20% fetal calf serum (FCS) (Greiner Bio-One) after surgical resection. Samples were cut into small fragments in a petri dish, and enzymatically digested with 1 mg/mL collagenase D (Roche Diagnostics) and 50 µg/mL DNase I (Roche Diagnostics) in 5 mL of IMDM+Glutamax medium for 30 min at 37°C in gentleMACS C tubes (Miltenyi Biotec). During and after incubation, cell suspensions were mechanically dissociated on the gentleMACS Dissociator (Miltenyi Biotec). Cell suspensions were filtered through a 70-µm cell strainer (Corning) and washed in IMDM+Glutamax medium. Cell number and viability was determined with the Muse Count & Viability Kit (Merck) on the Muse Cell Analyser (Merck). Cells were cryopreserved based on the number of viable cells in liquid nitrogen until time of analysis in 50% FCS and 10% dimethyl sulfoxide (DMSO) (Merck). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (provided by apothecary LUMC) density-gradient centrifugation and cryopreserved in liquid nitrogen until time of analysis in 20% FCS and 10% DMSO.

Mass cytometry antibodies

Heavy metal isotope-tagged monoclonal antibodies are listed in Table S2. Purified antibodies were conjugated with heavy metal reporters in-house using the MaxPar X8 Antibody Labeling Kit (Fluidigm) according to the manufacturer's instructions. All antibodies were titrated to determine the optimal labelling concentration.

Mass cytometry antibody staining and data acquisition

Percoll (GE Healthcare) density-gradient centrifugation was performed to isolate immune cells from tumour tissues and colorectal healthy mucosa. Cells were washed in Maxpar Cell Staining Buffer (CSB, Fluidigm), and incubated with 1 mL CSB containing 1 µM Cell-ID intercalator-^{103}Rh (Fluidigm) for 15 min at room temperature (rT). Cells were washed, incubated with human Fc receptor block (BioLegend) for 10 min at rT, and stained with cell surface antibodies for 45 min at rT in a final volume of 100 uL. After washing, cells were incubated with 1 mL Maxpar Fix and Perm buffer (Fluidigm Sciences) containing 0.125 µM Cell-ID intercalator-Ir (Fluidigm) overnight at 4°C. Cells were acquired on a Helios-upgraded CyTOF2 and Helios mass cytometer (Fluidigm) at an event rate of <500 events/sec in de-ionized water containing 10x diluted EQ Four Element Calibration Beads (Fluidigm). Data were normalized with the normalization passport EQ-P13H2302_ver2 for each experiment.

Mass cytometry data analysis

Correlation analysis of the presence of immune cell clusters across samples (Figure 7) was performed using Spearman's rank correlation in the Corrplot R package. PD-1 subsets in CRCs (Figure S9) were gated based on a healthy control PBMC sample into PD-1 negative cells (no PD-1 expression), PD-1 intermediate cells (PD-1 expression levels to a similar extent as healthy control PBMCs), and PD-1 high cells (PD-1 expression levels surpassing that of healthy control PBMCs) as described in a previous study on functional diversity between PD-1 subsets.[1] Tumours with less than 100 cells in one of the PD-1 subsets were excluded from this analysis. Sample and immune cell cluster t-distributed Stochastic Neighbour Embedding (t-SNE) maps (Figure S8) were computed as described previously.[2]

Flow cytometry antibody staining

Cells were first incubated with human Fc receptor block and cell surface antibodies for 45 min at 4°C. After washing, cells were stained for intracellular proteases and cytokines using Fixation Buffer and Intracellular Staining Perm Wash Buffer (BioLegend) or for FOXP3 expression using FOXP3 Transcription Factor Staining Buffer Set (eBioscience). Compensation was carried out with CompBeads (BD Biosciences) and ArC reactive beads (Life Technologies). Cells were acquired on a LSR II and LSR Fortessa flow cytometer (BD Biosciences) running FACSDiva software version 8.0 (BD Biosciences). Data were analysed with FlowJo software version 10.2 (Tree Star Inc). Antibody details are listed in Table S3.

Immunohistochemical staining

FFPE blocks from colorectal cancers (CRCs) were obtained from the department of Pathology at the Leiden University Medical Centre (Leiden, The Netherlands). Tumour mismatch repair (MMR) status (MMR-proficient or MMR-deficient) was determined by immunohistochemical detection of PMS2 (anti-PMS2 antibodies; clone EP51, DAKO) and MSH6 (anti-MSH6 antibodies; clone EPR3945, Abcam) proteins.[3] MMR-deficiency was determined by lack of expression of at least one of the MMR-proteins in the presence of an internal positive control. Immunohistochemical detection of human leukocyte antigen (HLA) class I expression was performed with HCA2 and HC10 monoclonal antibodies (Nordic-MUbio), and classified as HLA class I positive, weak, or loss as described previously[4].

Multispectral immunofluorescence detection

Five-μm frozen tissue sections were cut on adhesive immunohistochemistry slides. Following fixation in ice-cold 100% methanol for 5 min and wash in PBS, the tissues were incubated with Superblock buffer (Thermo Fisher Scientific) for 30 min at rT. For the detection of CD127, TSA signal amplification was performed with Opal520 from the Opal 7-colour manual IHC kit (Perkin Elmer), according to manufacturer's instructions. Thereafter, the tissues were incubated with primary TCR V beta F1 and CD45RO antibodies to be detected indirectly overnight at rT, followed by incubation with corresponding fluorescent secondary antibodies CF680 and CF633, respectively, for 1h at rT. The tissues were then incubated with directly conjugated primary CD3-AF594 and CD7-AF647 antibodies for 5h at rT. CD3 was conjugated to AF594 using the Alexa Fluor 594 antibody labelling kit (Thermo Fisher Scientific). Lastly, the tissues were incubated with 1 μM DAPI for nuclear counterstaining, and mounted with Prolong® Gold Antifade Reagent (Cell Signaling Technology). Antibody details are listed in Table S4.

References

1. Thommen DS, Koelzer VH, Herzig P, et al. A transcriptionally and functionally distinct PD-1(+) CD8(+) T cell pool with predictive potential in non-small-cell lung cancer treated with PD-1 blockade. *Nat Med* 2018 doi: 10.1038/s41591-018-0057-z
2. van Unen V, Li N, Molendijk I, et al. Mass Cytometry of the Human Mucosal Immune System Identifies Tissue- and Disease-Associated Immune Subsets. *Immunity* 2016;44(5):1227-39. doi: 10.1016/j.immuni.2016.04.014
3. Hall G, Clarkson A, Shi A, et al. Immunohistochemistry for PMS2 and MSH6 alone can replace a four antibody panel for mismatch repair deficiency screening in colorectal adenocarcinoma. *Pathology* 2010;42(5):409-13. doi: 10.3109/00313025.2010.493871 [published Online First: 2010/07/17]
4. Ijsselsteijn ME, Petitprez F, Lacroix L, et al. Revisiting immune escape in colorectal cancer in the era of immunotherapy. *Br J Cancer* 2019;120(8):815-18. doi: 10.1038/s41416-019-0421-x [published Online First: 2019/03/14]