PD-L1 staining

PD-L1 staining was done using a Dako clone 22C3 and Ventana clone SP263 as per manufacturers published assay SOP. Immunostaining for PDL1 was performed using an indirect immunoperoxidase method as described by the manufacturers. Staining with the 22C3 clone (Dako, UK) was performed on the Dako Autostainer Link 48 following pretreatment with EnVision FLEX Target Retrieval Solution Low pH using the Dako PT link. Staining with the SP263 clone (Ventana, USA) was performed on the Ventana BenchMark ULTRA following standard Cell Conditioning 1.

CD3 immunohistochemistry (IHC)

Staining was done using a CD3 polyclonal antibody (rabbit polyclonal, 1:150; Dako ref. A0452). IHC was performed on the Bond-RX automated staining platform (Leica Microsystems). Heat-based antigen retrieval was achieved with ER1 buffer, pH6.0 for 30 minutes. Reactions were visualised using the Bond Polymer Refine Detection Kit (#DS9800, Leica Microsystems). Slides were imaged using the Aperio ScanScope CS2 digital pathology slide scanner (Leica Biosystems) and image analysis performed using the HALO v2.218 software provided by Indica Labs. The density of cells of interest are presented as number of CD3 positive cells per mm^2 area of tumour.

T Cell Panel - Immunofluorescence (IF)

Multiplex sequential IF staining was performed on 3µm FFPE tissue sections using an automated staining platform (Bond-RX, Leica Microsystems). Briefly, antigen retrieval was achieved using Bond Epitope Retrieval Solution 2 (#AR9640, Leica Biosystems), pH9.0 for 20 minutes. Endogenous peroxidase was inactivated in 3% H_2O_2 for 10 minutes. Tissue sections were incubated for 1 hour at room temperature with antibodies against CD4 (#ab133616, clone EPR6855, 1:100, Abcam) and CD8 (#M7103, clone C8/144B, 1:200, Dako, Agilent Technologies). A second layer of antibodies using AlexaFluor 555-conjugated IgG (H+L) goat anti-rabbit (#A21429, Invitrogen) and AlexaFluor 488-conjugated IgG (H+L) goat anti-mouse (#A-11029, Invitrogen) were used to detect CD4 and CD8, respectively. Tissue sections were treated with an avidin/biotin blocking kit according to the manufacturer's protocol

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(#ab64212, Abcam). Next, tissue sections were incubated for 1 hour with antibodies against Foxp3 conjugated to biotin (#13-4777-82, clone 236A/E7, 1:100, eBioscience) and PanCK conjugated to AlexaFluor 647 (#4528S, clone C11, 1:100, Cell Signaling Technology). Tissue sections were incubated with streptavidin peroxidase (HRP) (#K5001, Dako, Agilent Technologies) for 15 minutes followed by TSA Coumarin detection system (#NEL703001KT, PerkinElmer) for 10 minutes. Nuclei were counterstained with DRAQ 7 (#DR71000, Biostatus) and tissue sections were mounted with ProLong Gold antifade reagent (#P36930, Molecular Probes).

After staining, slides were scanned using a multi-spectral camera provided by the Vectra® system (PerkinElmer). Whenever possible, more than one non-overlapping micrograph at 20x magnification was collected. Digital image analysis linear unmixing of multi-spectral images was done using inForm® Cell Analysis® software version 2.1.1. A tissue segmentation algorithm was developed using PanCK positivity as a tumor mask to separate neoplastic cells from adjacent stroma. A cell segmentation algorithm was developed using DRAQ7 as nuclear marker and phenotype determination was based on staining for CD4, FOXP3 and CD8. Cells in tumor areas selected by the algorithm were separated into bins as follows: CD4+/FOXP3- cells, CD4+/FOXP3+ cells, CD8+ cells and cells negative for these markers were classified as “other”. All tissue segmentation, cell segmentation, and phenotype determination maps were reviewed by a pathologist (BG). For each image, the tumor area (in mm^2) and the number of CD4+/FOXP3-, CD4+/FOXP3+, and CD8+ cells were determined to calculate the lymphocytic density of tumor infiltrating lymphocytes (LD-TIL) determined as: (Σ T lymphocytes from all images)/(Σ of areas from all images).

Whole Exome Sequencing

DNA was extracted from formalin-fixed and paraffin embedded (FFPE) blocks positive for tumor content using the FFPE Tissue DNA kit (Qiagen). DNA was quantified with the Quant-iT high-sensitivity PicoGreen double-stranded DNA Assay Kit (Invitrogen). Germline DNA was extracted from a buccal swab using Isohelix DNA Isolation kit DDK-50 protocol.

WES was performed using Kapa Hyper Plus Library Prep Kits and the Agilent SureSelectXT V6 target enrichment system. Paired-end sequencing was performed

using the NovaSeq S2 flowcell (2 × 100 cycles; Illumina). FASTQ files were generated from the sequencer's output using Illumina bcl2fastq2 software (v.2.17.1.14, Illumina)