Supplementary Methods

Pyrosequencing

The methylation status of *LINE-1* (long interspersed nuclear elements) and *IL22RA1* (Interleukin 22 Receptor Subunit Alpha 1) was determined following bisulfite modification of DNA using EZ DNA Methylation kit (Zymo Research) followed by PCR (polymerase chain reaction) amplification using an annealing temperature of 53°C and 58°C respectively with each primer pair (forward and reverse primers, latter with Biotin modification). Primers were designed using PyroMark Assay Design 2.0 Software (Qiagen). The biotinylated strand of the amplicons was captured and selected with streptavidin Sepharose beads (GE Healthcare) and purified using Vacuum Prep Tool (Qiagen) and subsequently annealed to corresponding sequencing primers. Pyrosequencing was performed using Pyromark Q96 MD instrument. In this study, two technical replicates were performed for each assay. The percentage methylation at individual CpG sites was analysed using Pyro Q-CpG software (Qiagen) and averaged across CpG sites and technical replicates.

Table 1s. Primer and CPG Sites for Pyrosequencing *LINE-1*

Abbreviations. LINE-1: long interspersed nuclear elements

Info *LINE-1*	**Sequence (5' to 3')**
Forward Primer	GGATTTTTTGAGTTAGGTGTGGG
Reverse Primer	BIOTIN-CAAAAAATCAAAAAATTCCCTTTCC
Sequencing Primer	AGGTGTGGGATATAGT
DNA Sequence to analyse (Bisulfite Converted)	TT**C**GTGGTG**C**GT**C**GTTTTTTAAGT**C**GGTTT
Number of CpG sites interrogated	4

Table 2s. Primer and CPG Sites for Pyrosequencing *IL22RA1*

Abbreviations. IL22RA1: Interleukin 22 Receptor Subunit Alpha 1

Info *IL22RA1*	**Sequence (5' to 3')**
Forward Primer	ATGGGTATTTATTAGTTAGGGATTTTATAG
Reverse Primer	BIOTIN- AACCCCAAAACTCCCAACCCT
Sequencing Primer	GGATTTTATAGTTAAGATGGTTAG
DNA Sequence to analyse (Bisulfite Converted)	TAGCGTTTTTATCGGGGTTGGTATAG

Number of CpG sites interrogated	2

EPIC array

Genome-wide DNA methylation at specific genomic loci of immunomodulatory genes of interest in tumour samples was analysed using Infinium Methylation EPIC BeadChip (Illumina) array which allows the interrogation of methylation patterns at a genome-wide level, covering over 850,000 CpG sites across the genome. 300 ng of genomic DNA was converted for EPIC array. Illumina Infinium HD FFPE QC Assay kit (WG-321-1001, Illumina), utilising real-time quantitive PCR (qPCR) to assess the quality of genomic DNA extracted from FFPE samples prior to bisulphite conversion. The average quantification cycle (Cq) value for the in-kit control DNA was subtracted from the average Cq for each sample to obtain a delta-Cq. Samples with delta-Cq<5 are considered good quality. The EPIC array also contains internal control probes to assess quality of different sample preparation steps including bisulphite conversion and hybridisation. Raw signal intensity data were processed from IDAT files through a standard pipeline using the Bioconductor package minfi in R platform (v.4.0.5). A number of pre-processing and quality assurance steps were performed to generate beta-density plots, median intensity and control strips. Data were then functional normalised for background adjustment and reducing technical variation. CpG positions were mapped against the human hg19 reference genome. DNA methylation at baseline and C2D8 was interrogated using probes for 426 immunomodulatory loci of interest. Beta-values and m-values were used to measure percentage methylation and $\log_2$ ratio of the intensity differences between methylated and unmethylated probes, respectively. Beta-values were grouped into bins, where 0 indicates all copies of the CpG site are unmethylated and 1 indicates methylated, and Gaussian distribution curves fitted for individual patients and all patients together to assess frequency distribution. The difference in beta-values, delta-beta, was calculated at each probe for individual patients. Differentially methylated positions (DMPs), with a biologically significant change in methylation, were defined using a cut-off of delta-beta |0.1| in at least three of six patients.

PD-L1 IHC

Formalin-fixed, paraffin-embedded (FFPE) samples were cut in 3-µm sections onto charged glass slides. PD-L1 IHC (programmed death ligand-1 immunohistochemistry) was performed using a rabbit anti-PD-L1 antibody (#13684; monoclonal [clone E1L3N]; Cell Signalling Technology). Heat-induced antigen retrieval was achieved by microwaving slides in antigen retrieval buffer (Tris-EDTA [ethylenediaminetetraacetic acid] buffer, pH 8.1) for 18 minutes at 800 W prior to incubation with anti-PD-L1 antibody (dilution 1:200) for 1-hour at room

temperature. Endogenous peroxidase was inactivated using 3% H_2O_2, and nonspecific staining was blocked using protein block serum-free solution (#X0909, Dako, Agilent Technologies). Reactions were visualized using the Dako REAL EnVision Detection System (#K5007, Dako, Agilent Technologies). Partial or complete membrane staining was considered a signal and cases were evaluated as a tumour proportion score, i.e., number of signal positive viable tumour cells/total number of viable tumour cells as previously described (Roach, Zhang et al. 2016). Comparison of baseline and on-treatment was done using Mann-Whitney test (GraphPad Prism v9).

CD3 IHC

FFPE samples were cut in 3-µm sections onto charged glass slides. CD3 IHC (cluster differentiation 3 immunohistochemistry) was performed using a rabbit anti-CD3 antibody (#A0452; rabbit polyclonal; Dako, Agilent Technologies) on the BOND RX automated staining platform (Leica Microsystems). Heat-induced antigen retrieval was achieved with BOND Epitope Retrieval Solution 1, pH6.0 (#AR9961, Leica Microsystems), for 30-minutes prior to incubation with anti-CD3 antibody (1:150 dilution) for 15-minutes at room temperature. Reactions were visualised using the BOND Polymer Refine Detection Kit (#DS9800, Leica Microsystems). CD3 IHC stained slides were scanned at high resolution (200x) using the VS200 digital slide scanner (Olympus, Tokyo, Japan). The digitized slides were then analysed with the HALO image analysis suite (HALO v2.218, Indica Labs, New Mexico, USA). The number of intratumoural and stromal CD3 positive cells were divided by the total area of tumour and stroma respectively, providing intratumoural and stromal CD3 density values (CD3+ cells per mm^2) for each sample.

Assessment of tumour infiltrating lymphocytes by Immunofluorescence (IF)

FFPE samples were cut in 3-µm sections onto charged glass slides. Multiplex sequential IF staining was performed on the BOND RX automated staining platform (Leica Microsystems). Briefly, heat-induced antigen retrieval was achieved with BOND Epitope Retrieval Solution 2, pH9.0 (#AR9640, Leica Biosystems), for 20-minutes. Endogenous peroxidase was inactivated in 3% H_2O_2 for 10-minutes. Tissue sections were then incubated for 1-hour at room temperature with antibodies against CD4 (#ab133616, rabbit monoclonal [clone EPR6855],

1:100, Abcam) and CD8 (#M7103, mouse monoclonal [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 then treated with an Avidin/Biotin blocking kit according to the manufacturer's protocol (#ab64212, Abcam). Next, tissue sections were incubated for 1-hour with a cocktail of biotinylated Foxp3 (#13-4777-82, mouse monoclonal, [clone 236A/E7], 1:100, eBioscience) and AlexaFluor 647 conjugated PanCK (#4528S, mouse monoclonal [clone C11], 1:100, Cell Signaling Technology) antibodies, followed by streptavidin peroxidase (HRP) (#K5001, Dako, Agilent Technologies) for 15 minutes and TSA Coumarin detection system (#NEL703001KT, Akoya Biosciences) 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 Vectra multi-spectral camera (Akoya Biosciences) under 20x magnification. The digitized images were then analysed with inForm® Cell Analysis® software (v2.2.1. Akoya Biosciences). Tissue segmentation was achieved using PanCK (pan-cytokeratin) positivity as a tumour mask to separate tumour cells from adjacent stroma. Cell segmentation was achieved using DRAQ7 as nuclear marker and immune cell phenotype determination was based on staining for CD4, FOXP3 (forkhead box protein P3) and CD8. All tissue segmentation, cell segmentation, and phenotype maps were reviewed by a pathologist (BG). For each image, the tumour area (in mm^2) and the number of $CD4^+FOXP3^-$, $CD4^+FOXP3^+$, and $CD8^+$ cells were determined to calculate the lymphocytic density of tumour infiltrating lymphocytes (Σ T lymphocytes from all images)/(Σ of areas from all images) as previously described (Rodrigues, Rescigno et al. 2018). Comparison of baseline and on-treatment was done using Wilcoxon matched-pairs signed rank test (GraphPad Prism v9).

Transcriptome Analysis

Tissues were lysed with QIAGEN TissueLyser II (QIAGEN) using 5 mm steel beads (cat# 69989, QIAGEN) 2 × 30 s at 18Hz settings, and processed for extraction using the AllPrep DNA/RNA kit (cat# 80224, QIAGEN). DNA and RNA quantity and quality was assessed using Agilent 4200 TapeStation (Agilent, USA) for RINe and DINe (RNA Integrity Number equivalent and DNA Integrity Number equivalent respectively). Tumour RNA-Seq libraries were prepared

according to the manufacturer's protocol using NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® NEB (#E7760) and ribo depletion using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #6310). All sequencing was performed on the Illumina NextSeq 500 platform (Illumina) with 2 × 75bp read length.

FASTQ files were generated using the BCL2FASTQ software. Transcriptomes reads were aligned to the human reference genome (GRCh37/hg19) using TopHat2 (version 2.0.7). Gene expression, fragments per kilobase of transcript per million mapped reads (FPKM), was calculated using Cufflinks. Expression fold change (Log2 transformed) was used for Gene Set Enrichment Analysis (GSEA) (pre-ranked HALLMARK gene list; http://software.broadinstitute.org/gsea/) with the default parameters.

Immunophenotyping

3.5mls. of peripheral blood were collected in EDTA transported at room temperature to the laboratory and assayed within 24-hours of collection; 200 ul of peripheral blood were incubated in an erythrocyte lysing buffered Sodium Chloride's (NaCl) solution for 10-minutes and washed once in PBS. The lysed cells were incubated with a pre-prepared lymphocyte subsets antibody cocktail for 15 min in the dark and washed twice. 30,000 lymphocytes were acquired on a FACSCanto II flow cytometer and analysed using FACSDiva software (BD Biosciences, San Jose, California, USA). Doublets were excluded and a CD45 gate was applied with a previous exclusion of doublets and a lymphocytes gate was applied to assess the T-lymphocytes subsets. Results were reported as percentage of lymphocytes for CD3 CD4 and CD8. NK cells (natural killer cells) were reported as percentage of CD45 positive cells. Comparison of cell percentages were compared using two-tailed paired t-test (GraphPad Prism v9).

Clinical Data

All analyses of clinical data was done using GraphPad Prism v9. Time to progression was calculated as time from cycle 1 day 1 until date of confirmed progressive disease. Kaplan-Meier curves were calculated for time to progression.

References

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