MATERIALS AND METHODS

I. Human study

1. Patient characteristics

French cohort of patients with prostate cancer

Stool metagenomic analysis: 32 Patients with CRPC (castration refractory prostate cancer) and 10 Patients with HSPC (hormone sensitive prostate cancer) were enrolled at Gustave Roussy Cancer Campus, France in "Oncobiotics" clinical study, B2M ethics protocol number PP: 15-013 (see flow diagram of patient inclusion Figure S3). Written informed consent in accordance with the Declaration of Helsinki was obtained from all patients. Samples were collected at baseline, before treatment initiation regardless of the treatment line.

Blood samples: 27 paired blood samples were also collected from previous patients, 17 CRPC and 10 HSPC (5 pre-ADT and 5 post-ADT) in Oncobiotics clinical study.

Lymphocyte count analysis: We retrospectively collected lymphocyte count for 21 mCRPC from Oncobiotics study (ethics protocol number PP: 15-013) and 31 hormone sensitive patients from PEACE ONE (NCT01957436), LATTITUDE (NCT01715285) studies at the Gustave Roussy site and standard of care patients.

The lymphocyte count was collected at baseline before treatment with androgen deprivation therapy (GNRH agonists or antagonists) for the 32 patients and paired 17 patients at 4-6 months after ADT initiation.

2. Sequence preprocessing and taxonomic profiling:

DNA have been extracted with Qiagen DNeasy PowerSoil Pro Kit following the manufacture's instruction.

Sequencing libraries were prepared using the Illumina Nextera DNA Flex Library Prep Kit according to manufacturer's protocols. Libraries were multiplexed using dual

indexing and sequenced for 300 bp paired-end reads using the Illumina NovaSeq6000 platform according to manufacturer's protocols. All sequenced metagenomes were QCed using the pre-processing pipeline implemented in https://github.com/SegataLab/preprocessing. This pre-processing pipeline consists of three main stages: **(1)** Initial quality control by removing low-quality reads (quality score <Q20), fragmented short reads (<75 bp), and reads with >2 ambiguous nucleotides; **(2)** contaminant DNA removal by using Bowtie2 and the --sensitive-local parameter, removing both the phiX174 Illumina spike-in and human-associated reads (hg19); **(3)** Sorting and splitting for the creation of standard forward, reverse, and unpaired reads output files for each metagenome. We obtained an average of 76,823,598 reads/sample after quality control and pre-processing. Taxonomic profiling and quantification of organisms' relative abundances of all metagenomic samples was performed using MetaPhlAn 3.0 (F. Beghini et al) with default parameters.

3. Stastistics:

Exploratory analysis of β-diversity was calculated using the Bray-Curtis measure of dissimilarity on species-level relative abundances and represented in Principal Coordinate Analyses (PCoA). P-values were determined by using 10,000 permutations in the function adonis from the vegan R package (v2.5-7). Non-parametric Wilcoxon tests were used for univariate differential abundance analysis using SIAMCAT (J.Wirbel et al.) after abundance filtering. Spearman’s correlations between immune cell profiles and species’ abundances were computed using the pcor.test function from the ppcor R package controlling for time-point, age and patient. P values were

corrected using false discovery rate (FDR) through the Benjamini-Hochberg procedure.

4. Quantification of T-cell Receptor (TCR) Excision Circles (TRECs):

TREC quantification was performed as in Clave et al. (E. Clave et al.). Briefly, real time quantification was made using ViiA7 (Applied Biosystems by Life Technologies, Austin, TX, USA) in 384-well plates loaded with 20 µl containing 500 ng of genomic DNA,1x Takyon Low Rox Probe MM (Eurogentec) and 200 nM sjTREC LNA probe (FAM-ACACCTCTGGTTTTTGTAAAGGTG-EclipseDQ with N= LNA base), 100 nM Alb probe VIC-CCTGTCATGCCCACACAAATCTCTCC-TAMRA, 400 nM sjTREC-F CACATCCCTTTCAACCATGCT, 400 nM sjTREC-R, GCCAGCTGCAGGGTTTAGG, 20 nM ALB-F, GCTGTCATCTCTTGTGGGCTGT and 20 nM ALB-R, ACTCATGGGAGCTGCTGGTTC. All primers and probes are from Eurogentec except the Alb probe from Applied Biosystems. sjTRECs were normalized to 150 000 cells (around 1 µg of DNA) using the Albumin gene quantification. Male aged matched controls are from a 150 healthy adults aged between 19 and 95 years old (Pitié Salpétrière Hospital, Paris) (Nicoli et al, submitted)

5. Flow cytometry analysis:

Preparation.

PBMCs were stained for flow cytometry analysis at +4°C for 20 minutes. Antibodies used for flow cytometry analysis are included in the table below. All stainings were performed using a FACS buffer made with 2% FBS 10 uM EDTA in PBS.

Target	Fluorochrome	Company	Clone	Staining
CD8	Brillant UltraViolet395	BD	RPTA8	Extracellular
CD4	Brillant UltraViolet496	BD	RPTA4	Extracellular
CD45	Brillant UltraViolet563	BD	HI30	Extracellular
CD3	Briallant UltraViolet805	BD	UCHT1	Extracellular
CD279/PD-1	Brillant Violet421	BD	EH12.1	Extracellular
CD45RO	Pacific Blue	BioLegend	UCHL1	Extracellular
CD62L	Brillant Violet480	BD	DREG	Extracellular
TCRδ	Brillant Violet510	BD	11F2	Extracellular
CD25	Brillant Violet605	BD	2A3	Extracellular
CD197/CCR7	Brillant Violet650	BioLegend	G043H7	Extracellular
CD31	Brillant Violet711	BD	L133.1	Extracellular
CD28	Brillant Violet785	BioLegend	CD28.2	Extracellular
Vβ11	FITC	Beckman Coulter	C21	Extracellular
CD44	PerCP/Cyanine5.5	BioLegend	BJ18	Extracellular
CD95	BB700	BD	DX2	Extracellular
TOX1	PE	Miltenyi	REA473	Intracellular
CD127	PE/Cyanine5.5	ThermoFisher	eBioRDR5	Extracellular
Vα24	PE/Dazzle 594	BioLegend	6b11	Extracellular
CD27	PE/Cyanine 7	BioLegend	O323	Extracellular
Foxp3	APC	Thermo Fisher	PCH101	Intracellular
TCF-1	Alexa Fluor 647	BioLegend	7F11A10	Intracellular
CD45RA	Alexa Fluo700	BD	HI100	Extracellular
TCRαβ	APC/Cyanine 7	BD	IP26	Extracellular

Acquisition.

Stained samples were acquired on a CyTEK Aurora flow cytometer (Cytek Biosciences).

Analysis.

Fcs files were exported and analysed using FlowJo software (Tree Star, Ashland, OR, USA) and Prism 9.

II. Pre-clinical study details

1. Mice

All animal experiments were carried out in compliance with French and European laws and regulations. The local institutional animal ethics board and French Ministère de la Recherche approved all mouse experiments (permission numbers: 2018_026_14002). Experiments were performed in accordance with Government and institutional guidelines and regulations.

Immunocompetent mice: for main experiment, FVB/N males were purchased from Charles River (France). Mice were used between 8 and 10 weeks of age. All mouse experiments were performed at the animal facility in Gustave Roussy. Cancer Campus where animals were housed in specific pathogen-free conditions.

Nude-athymic mice were purchased from Gustave Roussy (France).

2. Tumor cell line

Myc-CaP cells, derived from spontaneous prostate cancer in c-Myc transgenic mice, were a generous gift from Dr. Charles. L. Sawyers and were maintained as previously described (Watson, P. A. et al)

3. Animal model

Immunocompetent mice:

FVB/NJ mice were subcutaneously inoculated with Myc-CaP cells (1×10^6 cells/mouse) in the right flank as previously described (Shen, Y.-C. et al). Tumor diameters were measured every 3 days with an electronic caliper. Tumor surface were calculated using the formula: longest diameter × shortest diameter. When tumor surface reached approximately 60 mm^2, mice were randomly allocated to treatment groups as indicated. Euthanasia was performed for tumor ulceration, tumor surface > 300 mm^2, or any other sign of animal suffering. Time to progression is defined as the time between the first treatment and the regrowth of tumor to a size greater than 20 mm2

that was confirmed on 2 successive measurements. Overall survival (OS) was defined as the time period between castration and death.

Immunocompromised mice:

Athymic nude mice lacking T cells were used (nu/nu mice): the same process as described above.

4. Androgen deprivation therapy (ADT)

Androgen deprivation therapy is a subcutaneous (sc) injection of degarelix acetate (a GnRH receptor antagonist; Ferring Pharmaceuticals Inc., Parsippany, NJ) at a dosage of 0.625 mg in 100 µl physiologic serum every 28 days.

5. Immune checkpoint Inhibitors (ICIs)

Five or ten days after ADT, Mice were treated intraperitonaly (ip), with 4 injections of anti-PD-1 mAb (250µg/mouse, clone RMP1-14), anti-CTLA-4 (100µg of anti-CTLA-4 mAb (clone 9D9) or isotype control (clone 2A3 and clone MPC11 respectively) every 3-days.

6. T- Cell depletion

For in vivo depletion of CD4 and CD8 T lymphocytes, we performed i.p. injection of 200 µg/ mice of monoclonal Antibodies (mAbs) or isotype controls. Mice were treated 3 days prior to ADT and then weekly.

We used anti-mouse Abs (clone GK1.5) for CD4 depletion, anti-mouse Abs (clone 53–6.72) for CD8 depletion and isotype controls (LTF-2 and 2A3). All antibodies were from Bioxcell).

The depletion was confirmed after 3 injections by flow cytometric (FC) analysis of blood samples.

7. Antibiotic treatments

Mice were treated with an antibiotic solution (ATB) containing ampicillin (1 mg/ml), streptomycin (5 mg/ml), and colistin (1 mg/ml) (Sigma-Aldrich), added in the drinking

water of mice. Antibiotic activity was confirmed by cultivating fecal pellets suspended in BHI+15% glycerol at 0.1 g/ml on COS (Columbia Agar with 5% Sheep Blood) plates for 48 h at 37°C in aerobic and anaerobic conditions.

In the context of antibiotic induced dysbiosis: mice received 3 days of ATB before ADT and for 14 days, then 1 week on/ 1 week off.

In the context of fecal microbial transplantation experiments: mice received 3 days of ATB before undergoing fecal microbial transplantation the next day by oral gavage using animal feeding needles.

8. Cohousing experiment

We used the cohousing method, a gold standard for the analysis of intestinal microbiota phenotype transfer (Rakoff-Nahoum et al.). Tumour-bearing mice were cohoused with healthy tumour-free mice prior to treatment with ADT.

Animals were randomly assigned to experimental groups (i.e., cohoused or non-cohoused). During cohousing experiments, the investigators were not blinded to allocation during experiments.

9. Fecal microbiota transfer experiments (FMT)

Fecal microbiota transfer (FMT) was performed by thawing fecal material. Two hundred µL of the suspension was then transferred by oral gavage into ATB pre-treated recipient. In addition, another 100µL was applied on the fur of each animal. Two weeks after FMT, tumor cells were injected subcutaneously and mice were treated with ADT or isotype controls with or without oral gavage of fecal samples from responding patients or of commensal species, as mentioned above.

10. Gut colonization with Akkermansia

Akkermansia CSUR P2261 (Akkp2261) was provided by the Institut hospital-universitaire Méditerranée Infection, Marseille, France.

Akkp2261 was grown on COS plates in an anaerobic atmosphere created using 3 anaerobic generators (Biomerieux) at 37°C for at least 72h. Akkp2261 was verified using a Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometer (Microflex LT analyser, Bruker Daltonics, Germany).

Akkp2261 was inactived by pasteurization (30 min at 70°C) and then immediately frozen and stored at -80°C.

Colonization of mice was performed by oral gavage with 100uL of suspension containing 1x10^8 bacteria. For bacterial gavage: suspension of 10^9 CFU/mL were obtained using a fluorescence spectrophotometer (Eppendorf) at an optical density of 600 mn in NaCl. Two bacterial gavages were performed for each mouse, the first 24h before the androgen deprivation therapy (ADT) and subsequently 6 hours after this injection.

11. Analysis of immunohistochemistry biomarkers.

A slide scanner Olympus VS120 at 20x objectives was used to obtain a whole slide image (WSI). WSIs were imported into QuPath software (version 0.2.0-m8). Total tissue area was calculated using 'Simple Tissue Detection' in QuPath and modified manually. A region of interest (ROI) for cortex area was identified manually or by an algorithm using ImageJ software (version 1.52r). Briefly, green channel of WSIs was extracted and the region of interest (ROI) was calculated by binary threshold defined for each image. The ROI was modified by hand in QuPath software and defined as cortex area. The area of medulla was calculated by deduction of the total area by cortex area.

12. Sequence preprocessing and taxonomic profiling:

DNA extraction and 16S rRNA sequencing of mouse stools. Preparation and sequencing of mouse fecal samples were performed at IHU Méditerranée Infection,

Marseille, France. Briefly, DNA was extracted using two protocols. The first protocol consisted of physical and chemical lysis, using glass powder and proteinase K respectively, then processing using the Macherey-Nagel DNA Tissue Extraction kit (Duren, Germany). The second protocol was identical to the first protocol, with the addition of glycoprotein lysis and deglycosylation steps. The resulting DNA was sequenced, targeting the V3–V4 regions of the 16S rRNA gene as previously described. 16S rRNA gene sequence processing and analysis was performed using R v3.6.1 and GraphPad Prism v8.3.1. DADA2 R package v1.14.0 (B.J Callahan et al.) was used to generate exact amplicon sequence variants (ASV) of each sample from raw amplicon sequences. Sequences were corrected for Illumina amplicon sequence errors, de-replicated, chimera removed, and merged of paired-end reads with 240-bases for forward reads and 220-bases for reverse reads. The taxonomy assignment was performed against the SILVA reference database (v132) (C. Quast et al.). Archea and Eukaryota residual sequences were removed. Alpha-diversity, defined as the number of distinguishable taxa, was analyzed at the genus-level and computed with phyloseq R package26 v1.30.0 (McMurdie PJ, et al.). The alpha-diversity, a mathematical value that summarizes an ecological (e.g. microbial) community according to the count of unique species and how evenly their frequencies are distributed, was estimated with different metrics at the genus-level: observed ASV, Shannon index, Inverse Simpson index, as well as weighted and unweighted Faith's Phylogenetic Diversity. Bray–Curtis distance and weighted UniFrac distance were used as beta diversity metrics (which shows the difference in taxonomic abundance profiles from different samples) and visualized through NMDS method (Chengsong Zhu et al.). The Mann–Whitney U test and the Wilcoxon signed-rank test were used to determine significant differences among the different groups according to alpha-

diversity respectively for paired samples. The DESeq2 (Love MI et al.) R package was used to performed differential abundances analysis at the genus-level.

13. Flow cytometry analyses:

Tissues processing

Myc-CaP Tumors. Sub-cutaneous tumors were harvested at D20-D28. Excised tumors were cut into small pieces and digested in RPMI medium containing LiberaseTM at 25 µg/mL (Roche) and DNase1 at 150 UI/mL (Roche) for 30 minutes at 37°C and then crushed and filtered twice using 70 and 40 µm cell strainers (Becton & Dickinson).

Thymus: Thymus were mechanically disaggregated and passed into a sterile filter (40 µm) then rinsed with RPMI and centrifuged. The pellets were resuspended in 2 mL of complete media.

For both organs, the total count was established with Vi-Cell (Beckmann Coulter).

Flow cytometry

Preparation

Depending on the timepoint of the experiment (tumor size and treatment), from one to ten million of cells from tumor samples were used. Five million of thymocytes were stained in each experiment. Cells were pre-incubated with purified anti-mouse CD16/CD32 (1 µg per test, clone 93; eBioscience) and viability marker (1:100 per test, Zombie Aqua™ Fixable Viabikity Kit, BioLegend) for 10 minutes at +4°C in PBS 1X, before membrane staining. Cells were then stained with a panel of extracellular antibodies (see below) for 20 minutes at +4°C. Samples were washed, fixed and permeabilized (Foxp3/Transcription Factor Staining Buffer Set eBiosciences) for 40 min at +4°C before being stained with intracellular antibodies (see below) for 30 min at +4°C. Intracellular and extracellular labelling were performed in FACS buffer made with 2% FBS 10 uM EDTA in PBS. Cells were pre-incubated with a viability marker

(LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit, ThermoFisher) for 20 minutes at +4°C in PBS 1X, before membrane staining.

Target	Fluorochrome	Company	Clone	Staining	Tissue
CD25	Pacific Blue	BioLegend	PC61	extracellular	Tumor and Thymus
CD3	Brillant Violet 650	BD	145-2C11	extracellular	Tumor and Thymus
CD44	PerCP/Cyanine 5.5	BioLegend	IM7	extracellular	Tumor and Thymus
CD45	APC-R700	BD	30-F11	extracellular	Tumor
CD8a	PE	eBiosciences	53-6.7	extracellular	Tumor and Thymus
CD4	PE/Dazzle 594	BioLegend	GK1.5	extracellular	Tumor and Thymus
CD4	PerCP/Cyanine 5.5	BioLegend	GK1.5	extracellular	Tumor
CD137/4-1BB	PE	BioLegend	17B5	extracellular	Tumor
CD366/TIM 3	PE/Dazzle 594	BioLegend	B8.2C12	extracellular	Tumor
CD8	PE-Cy7	BioLegend	53-6.7	extracellular	Tumor
CD183/CX CR3	APC	eBiosciences	173	extracellular	Tumor
CD45	Alexa Fluor 700	BioLegend	104	extracellular	Tumor
CD3	APC/Cyanine7	BD	145-2C11	extracellular	Tumor
CD279/PD-1	Brillant Violet421	BioLegend	RMP1-30	extracellular	Tumor
CD223/LA G3	Brillant Violet650	BioLegend	C9B7W	extracellular	Tumor
CD152/CT LA4	Brillant Violet605	BioLegend	UC10-4B9	intracellular	Tumor
TCRβ	APC/Cyanine7	BioLegend	H57-597	extracellular	Thymus
TCRδ	APC	eBiosciences	eBioGL3	extracellular	Thymus

Acquisition:

Stained samples were acquired on a Cytoflex (Beckman Coulter) cytometer.

Analysis

Fcs files were exported and analysed using FlowJo software (Tree Star, Ashland, OR, USA), R Studio and Prism.

Blood Flow cytometry :

Red blood cell was lysed with ACK, a cocktail of 8,29g of chlorure of amonium, 0,037g of ethyl diamine tetraacetic acid, 1g of bicarbonate of potassium and water. Cells was resuspended in FACS Buffer and stained with a mix of anti-mouse antibodies (same as thymus panel above).