Supplementary Materials and Methods

Human PBMCs, T cells and tumor samples

All specimens were obtained following written informed consent approved by Hospital Pellegrin and Bergonié Institute. Patient consent forms for all samples were obtained at the time of tissue acquisition. Biopsies were de-identified. Matched whole blood and freshly resected colon tumor tissues and their non-tumoregenous counterparts were obtained from Bergonié Institute, Bordeaux, France. Fresh whole blood from healthy donors was obtained from Hospital Pellegrin, Bordeaux, France. Samples were processed for RNA and histology studies. Human peripheral blood mononuclear cells (hPBMCs) were isolated from healthy donors and colon cancer patients by density gradient centrifugation with Pancoll (PANBiotech; human, density 1,077g/ml). hPBMCs were cultured in RPMI 1640 (PAN-Biotech) supplemented with 10% FBS (Gibco), 2mM L-glutamine (Gibco) and penicillin/streptomycin solution (Dominique Dutscher). hPBMCs were directly used for RNA/protein extraction, cultured under indicated experimental conditions, cryopreserved or used for CD8+ T cell isolation. Tumor-infiltrating CD8+ T cells were isolated from colon tumor samples freshly harvested following manufacturer's instructions (Miltenyi Biotec). Briefly, tumor tissue was cut into small fragments and enzymatically digested. CD8+ T cells were then purified by negative selection with MACS magnetic beads (human CD8+T cell isolation kit from Miltenyi Biotec). CD8+ T cells were tested for purity by flow cytometry and used for RNA extraction or further experiments.

Cell lines

The human colon cancer cells HT29 (MSS) and HCT116 (MSI), BALB/c syngeneic colon carcinoma CT26 cell line, and human T cell lines Jurkat, J.RT3-T3.5 (JRT3), Myla, SeAx and HUT-78, were cultured in DMEM or RPMI 1640 complete media and grown at 37 °C in

a 95% air, 5% CO2 humidified incubator. The characteristics and the origin of the control (Ø) and stably α1-PDX-expresssing Jurkat cells (Jurkat-PDX) and CT26 cells (CT26-PDX) were described previously (1,2). For the generation of Jurkat-PDX and CT26-PDX cells, G418 resistant Jurkat-PDX and CT26-PDX cells were selected and screened for α1-PDX expression by western blotting. Further selection was performed by culturing Jurkat-PDX and CT26-PDX cells in the presence of 1 μg/ml Pseudomonas exotoxin A (3).This toxin mediates cell death only after its cleavage by PCs (4). Cells were grown in the presence of 200 μg/ml G418 to maintain selection. In some experiments, CT26-Ø and CT26-PDX cells were transiently transfected with pIRES2-EGFP-V5 empty vector or containing PDGF-A cDNA. PDGF-A is an established PC substrate used to assess the activity of the PCs in tumor cells (5). Jurkat cell transfections were performed by electroporation (2) and CT26 cell transfections were carried out using lipofectamine (Invitrogen) as recommended by the manufacturers (1).

Mouse model

All research animals were housed in our institution (Université de Bordeaux) in a temperature-controlled environment. All experimental procedures were approved by the Institutional Animal Care and Use Committee, Université de Bordeaux, and were conducted under the supervision of trained veterinarian. Female BALB/c mice (Charles Rivers) were maintained under pathogen-free conditions until used for experiments. All research animals were housed in our institution (University of Bordeaux) in a temperature-controlled environment. The group sizes used for *in vivo* studies were those estimated to be the smallest necessary to generate meaningful data. Mice were monitored regularly, and those requiring medical attention were provided with appropriate care and excluded from the studies. BALB/c mice were inoculated subcutaneously in the right flank with 1 x 106 syngeneic colon

carcinoma CT26- Ø cells or CT26-PDX cells. Tumors were measured three times per week using calipers, and tumor volume was calculated using the ellipsoid formula: Tumor volume = 1/2(length × width2)(6). Animals whose tumors grew larger than 2000mm3 were considered progressed and were euthanized. After the growth period, tumors were collected from previously euthanized animals, weighted and cut in several pieces depending on tumor size. These pieces were cryopreserved, used for RNA/protein extraction or immunohistochemistry, or freshly used for tumor-infiltrating CD8+ T studies. Tumor were dissociated to obtain single cell suspension with the tumor dissociation kit for mouse from Miltenyi Biotec.

NICD overexpression in Jurkat cells

Lentivirus containing human NICD-pHR-EGFP plasmid and its control Ø-pHR-EGFP (kind gifts of Dr. Raúl V. Durán) were produced at the Vectorology Core Facility, Université de Bordeaux. Jurkat-PDX cells were infected with the control (PDX/lenti Ø) or NICD (PDX/lentiNICD) lentiviruses for 24h and infection was confirmed by detection of NICD mRNA levels by RT-PCR. NICD primers were designed to amplified only the exogenous human NICD (exohNICD) and no the endogenous mRNA (**Supplementary Table S1**).

NFAT-TA-eGFP expression and quantification

Jurkat cells were transfected with the plasmid containing pNFAT-TA-eGFP using DharmaFECT™ kb transfection reagent (Dharmacon, Lafayette, CO, USA). Twenty four hours after transfection, Jurkat cells were treated with PMA/Io for 48h in RPMI medium. Cells expressing pNFAT-TA-eGFP were monitored with an epifluorescence inverted microscope (Nikon Eclipse Ti2, Amsterdam, The Netherlands). Cells were excited with light from a high-speed monochromator (Optoscan ELE 450, Cairn Research, Faversham, UK) at

488 nm. Fluorescence emission at 514 nm was detected by a cooled digital sCMOS camera (Zyla 4.2, Andor, Belfast, UK) and recorded with NIS-Elements AR software (Nikon, Amsterdam, The Netherlands). Samples were acquired with a nine parameters MACsQuant instrument (Miltenyi Biotech, BergischGladbach, Germany) and analyzed with FlowJo v X 10.0.7 software (TreeStar, Portland, OR, USA). Jurkat cells were gated by size and granularity (FSC vs SSC). Single cells were gated and doublets were excluded by forward scatter height versus forward scatter área. Percentage of GFP positive against total was assessed.

T cell activation

Activation of TCR signaling was performed either with phorbol myristate acetate (PMA) (100ng/ml) and Ionomycin (Io) (1ug/ml) or with 5µg/ml plate-bound anti-CD3 (clone OKT3, #317302, BioLegend). Activation time ranged between 10 min and 48 h, depending on the experiment. In hPBMCs experiments, PC activity was inhibited before TCR activation. hPBMCs were cultured with 100µM of the general PC inhibitor, Decanoyl-Arg-Val-Lys Argchloromethylketone (CMK) (Bachem), for 24-48 h. CMK-treated cells were activated with anti-CD3. hPBMCs and collected for flow cytometry analyses, RNA/protein extraction or cytotoxicity assay.

Apoptosis assay

Jurkat-Ø and PDX cells were incubated in the presence and absence of PMA and Ionomycin for 24h and 48h. Subsequently, cells were washed with PBS-5% BSA and stained with PEAnnexin V and 7AAD using the Annexin V Apoptosis Detection Kit (BioLegend), according to the manufacturer's instructions. Cells were analyzed by flow cytometry (BD Accuri C6). The populations Annexin−/7AAD−, Annexin+/7AAD−, Annexin−/7AAD+, and

Annexin+/7AAD+ that correspond to live cells, early apoptotic cells, necrotic cells and late apoptotic cells, respectively, were enumerated.

Proliferation assay

Jurkat-Ø and PDX cells were plated on 24 wells plate at 1 x105/well for 24h. For long-term activation, PMA and Ionomycin were added and cell number was counted at 0, 3, 24, 48 and 72h time points. For short-time activation, after 3h, PMA and Ionomycin treatment medium was replaced with fresh medium (no PMA or Ionomycin) for the same time points as for long-term activation. Cells were counted with a Countess II Automated Cell Counter (Invitrogen) and using trypan blue exclusion staining.

JRT3 functional assay

The Jurkat T cell line J.RT3-T3.5 (JRT3) stably expressing the human LES -γδ TCR (JRT3-LES) was incubated with the colon cancer cell line HT29 overexpressing the endothelial protein C receptor (HT29-EPCR) at 5:1 (effector:target) ratio for 4 h at 37°C. Specific recognition and binding of LES -γδ TCR to EPCR induces JRT3-LES TCR-mediated activation as previously reported (7). The activation of JRT3-LES cells was evaluated by the expression of CD69, as assessed by flow cytometry analysis using PE-conjugated anti-CD69 mAb (Beckman Coulter). Data were acquired using a LSR Fortessa and analyses were performed using Diva and FlowJo 9.3.2 softwares (flow cytometry facility of TBM Core).

Cytometric Bead Array (CBA)

CBA was used to measure the concentration of cytotoxins released by primary human CD8+ T cell populations. CD8+ T cells were isolated from hPBMCs from five different donors using a specific CD8+ T cell microbead-cocktail (Miltenyi Biotec). CD8+ T cells were

stimulated with plate-bound anti-CD3 for 6 h and supernatants collected for flow cytometry analysis using a LegendPlex human CD8/NK panel CBA (BioLegend) to detect six cytotoxicity-related molecules: Granzyme A, Granzyme B, Perforin-1, Granulysin, sFas, and sFasL. Flow cytometry data were acquired using a LSR Fortessa. Results were analyzed with the LEGENDplex™ software.

Cytotoxicity assay

Susceptibility of cancer target cells (HT29: microsatellite-instable (MSI) cells; and HCT116: microsatellite-stable (MSS) cells) to PBMC-mediated cytotoxicity was determined using a carboxyfluorescein diacetate succinimidyl ester (CFSE)-based assay. CFSE is a dye that irreversibly binds to intracellular proteins and is retained within living cells for a long period. Target cells were stained with 8 μM CFSE (BioLegend) for 20min at 37°C and plated at 3 x 105 cell/ml. In parallel, hPBMCs (effector cells) were cultured in the presence and absence of CMK for 24 h. hPBMCs were then stimulated with plate-bound anti-CD3 antibody (OKT3) 5 μg/ml for 6 h. CD3-stimulated hPBMCs were co-cultured at different target:effector (1:1, 1:5) ratios with CFSE-target cells for 24 h. CFSE-target cells were collected for flow cytometry analysis or protein extraction.

Real-Time qPCR

Total RNA (1μg) was isolated by the Nucleospin RNA kit (Macherey-Nagel) and used for reverse transcription in a 20μl reaction mixture containing 50mM Tris-HCl (pH 8.3), 30mM KCl, 8mM MgCl2, 1mM dNTPs, and 0.2U Superscript reverse transcriptase (Invitrogen). Reverse transcription cycle consisted of 25°C 10min, 2x 37°C 60min and 85°C 5s, in a Veriti Thermal Cycler (Applied Biosystem). Quantitative real-time PCR of cDNA samples was performed with SYBR or TaqMan primers (**Supplementary Table S1**) using respectively

SYBR MesaBlue Master Mix or TaqMan Master Mix (Eurogentec), in a StepOne Plus Real Time PCR system following manufacturer's instructions (Applied Biosystem). GAPDH was used as housekeeping gene for normalization.

Immunohistochemistry

Human colon cancer and its corresponding non-cancer colon tissues were cut into smaller pieces and snap frozen in liquid nitrogen until use. Frozen pieces were embedded in OCT (Sakura) prior to sectioning on a microtome-cryostat (Leica CM1900) at 10µM thickness. Sections were mounted in gelatine-coated slides and stored at -20°C. Syngeneic mouse tumors from CT26-Ø and CT26-PDX cells were collected, weighted and cut into smaller pieces depending on tumor size. For immunohistochemistry, tumor samples were fixed in 2% paraformaldehyde for 10min and cryopreserved in 30% sucrose solution. The samples were embedded in OCT to produce frozen blocks and stored at -80°C. Mouse section slides were washed 3 times for 5min in TBS-tween20 then treated with cold acetone for 5min, washed and incubated in a blocking solution containing 5% bovine serum albumin (BSA; Euromedex) for 1h at RT. Sections were then incubated in the primary antibodies diluted 1:100 in PBS-0.1% BSA, overnight at 4°C. For PD-1 and CD8 detection, anti-mouse PD-1 (#AF1021, RD Systems), anti-mouse CD8 (MCA1768T, BioRad), antihuman PD-1 (#ab52587, Abcam) and anti-human CD8 (#ab4055, Abcam) were used. Antibodies against PCs namely, anti-PC7 (V-20, #sc-22903), anti-PC5 (E-20, #sc-22901), anti-furin (H-220, #sc-20801; B6, #sc-133142) and anti-PACE4 (K-18, #sc-22898), were obtained from Santa Cruz Biotech. The sections were incubated with the appropriate fluorophore-conjugated secondary antibody at 1:500 (Fluoprobes and Interchim) for 1h at RT and sections were mounted with ProLong Gold Antifade mounting medium containing DAPI (Invitrogen).

Immunocytochemistry

Immunocytochemistry was performed in hPBMCs and Jurkat cells in suspension. Cells were washed in PBS with 2% FBS. Surface antigens were detected by incubating with the primary antibody 1:100 in TBS with 5% BSA for 1h at RT, followed by appropriate fluorophoreconjugated secondary antibodies (1:100) for 30min at RT. Cells were then fixed in cold methanol for 7min on ice. For detecting intracellular antigens, cells were fixed with methanol before antibody incubations. Finally cells were washed and mounted in Fluoroshield medium containing DAPI (Sigma). Confocal immunofluorescence images were taken using the inverted microscopes Nikon C2si Eclipse Ti-S with NIS-ElementsAR software (Nikon Instruments Europe B.V.) or Leica DM6 CFS TCS SP8 (Leica Microsystems, Bordeaux Imaging Center (BIC).

Flow cytometry analysis

Single cell suspension of hPBMCs and Jurkat cells were stained with fluorophore-conjugated antibodies: PE-anti-PD-1 mAb (MIH4, #560908 eBiosciences), FITC-anti-CD8a mAb (#130-110-677, Miltenyi Biotec), PE-anti-CD69 mAb (IM1943U, Beckman Coulter) or APC-anti-CD107a mAb-(#641581, BD Biosciences). For mouse-derived cells, the following mouse monoclonal antibodies from Miltenyi Biotec were used: PE-anti-PD-1 (#130-111-953), APCor FITC- anti-CD8a (#130-102-808). For PD-1, CD8 and CD69 staining of Jurkat cells and hPBMCs, 1-2 x 105 cells were collected by centrifugation, washed twice with PBS-5% BSA and incubated with primary antibody at 1:5 (anti-PD-1), 1:10 (anti-CD69) or 1:50 (anti-CD8) in PBS-5% BSA for 15min on ice in the dark. Cells were resuspended in PBS-5% BSA and a viability dye was added 5min before flow cytometry acquisition, either 7-amino-actinomycin (7AAD) 1:30 or DAPI 1:100. For CD107a staining, anti-CD107a antibody (1:50) and brefeldin A (1:100, BD Biosciences) were added to the cells right after

stimulation. Flow cytometry data was acquired either with BD Accuri C6 or LSR Fortessa (BD Biosciences). Flow cytometry analyses were performed using BD Accuri C6 software, or Diva (BD Biosciences) and FlowJo 9.3.2 (TreeStar) softwares (flow cytometry facility of TBM Core).

Measurement of PC activity

The effect of PC inhibitors (CMK, α1-PDX expression in cells) on PC activity in cells and tissues was assessed by the evaluation of the enzymes' ability to digest the universal PC substrate, the fluorogenic peptide pERTKR-MCA, as previously described (1). Briefly, cells were incubated with pERTKR-MCA (100μM) during the indicated time periods in 25mM Tris (pH 7.4), 25mM methyl-ethane-sulfonic acid, and 2.5mM CaCl2, at 37°C, and the fluorometric measurements were performed using a spectrofluorometer (Tecan Infinite® F200 PRO, Tecan Group Ltd. France).

Protein extraction and Western blot analysis

For total protein extraction, cells were washed with PBS prior to the addition of RIPA lyses buffer (50mM HEPES, 150mM NaCl, 1% Triton X-100, 2mM vanadate, 100mM NaF, and 0.40mg/ml phenylmethylsulfonyl fluoride). For nuclear fractionation, cells were lysed using a NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Scientific) following manufacturer's instructions. Protein extracts (20-30μg per lane) were separated by electrophoresis in reducing conditions (SDS-PAGE, BioRad Miniprotein) and transferred onto polyvinylidene difluoride membrane (PVDF, Amersham Pharmacia Biotech) for 1h at 100V. For immunoblotting, membranes were first incubated in blocking solution (10mM Tris-HCl, pH 7.6, and 150mM NaCl containing 5% non-fat milk powder and 0.1% Tween 20) for 1h followed by primary antibodies incubated overnight at 4°C against: IGF-IR (#sc-

713), NFAT2c (#sc-7296), NF-κB p65 (#sc-8008) from Santa Cruz Biotech; phospho-NF-κBp65 (#3033), ERK1/2 (#4695), phospho-ERK1/2 (#9106), cleaved-caspase-3 (#9664), Notch-1 (#3608), cleaved-Notch-1 (Val1744, #4147S), HDAC1 (#5356), BIM (#2933) from Cell Signaling; BCL11B (#650601) from BioLegend; and actin-β (#A5441) from Sigma. Membranes were then incubated with IgG-horseradish peroxidase conjugated secondary antibodies (Amersham Pharmacia Biotech) for 1h at RT. Protein-antibody binding was detected using chemiluminescence according to the manufacturer's instructions (Pierce ECL Plus, Thermo Scientific). Images were acquired with a Genegnome system and GeneSys software (Syngene). Membranes were reprobed with a blot restore solution following manufacturer's instructions (Millipore).

Cytosolic free-calcium measurement

Jurkat-Ø and PDX were loaded with fura-2 by incubation with 4μM fura-2/AM (Fura-2 acetoxymethyl ester, Molecular Probes) and 2.5mM probenecid for 30min at 37°C in the dark. Cells were washed and suspended in HEPES-buffered saline (HBS) containing (in mM): 125 NaCl, 5 KCl, 1 MgCl2, 5 glucose, 25 HEPES, and pH 7.3, supplemented with 0.1% (w/v) BSA. Fluorescence was recorded from 2 mL aliquots of magnetically stirred cellular suspension (2 × 106 cells/mL) at 37°C using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380nm and emission at 505nm, as described previously. Changes in [Ca++]c were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to Grynkiewicz et al. (8). Ca++ release and entry were estimated using the integral of the rise in [Ca++]c for 3min after the addition of PMA+Ionomycin or CaCl2, respectively.

Statistical analysis

Statistical details can be found in Results, Figure and Figure Legend sections. Data are shown as mean ± S.E.M. or mean ± SD. Analysis of statistical significance was performed using Student's t-test or one-way ANOVA followed by Bonferroni's comparison as a post hoc test. Statistical significance was estimated when $P<0.05$.

References

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