**Supplementary methods**

**Mice.**

Female C57BL/6 and BALB/c mice were obtained from Harlan Laboratories. All animal procedures were conducted under institutional guidelines that comply with national laws and policies approved (study number 117/14). BATF3-/- mice were bred at Center for Applied Medical Research (CIMA), University of Navarra, in specific pathogen-free conditions. Batf3−/− on C57BL/6 background (kindly provided by Dr. Kenneth M. Murphy, Washington University, St. Louis, MO) were further back-crossed with C57BL/6 mice at the CNIC (Dr. David Sancho) to establish WT and Batf3−/− cousin colonies from the heterozygotes. Animal studies (protocol approval 150/12) were approved by the local ethics committee. All animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes (Real Decreto 1201/2005). IFNAR−/− mice backcrossed to C57Bl/6 background for 12 generations were obtained through Matthew Albert (Institute Pasteur, Paris, France).

**Tumor radiotherapy procedures.**

All mice were lightly anesthetized by i.p. injection of Ketamine (Imalgene Merial Laboratorios S.L. at dose of 100mg/kg, positioned on a dedicated methacrylate platform over the linac couch. Field delimiting light was to correctly orient the beam and a CT-SCANS were performed with a Siemens Emotion Somatron using the narrowest thickness allowed (0.6 mm) and sequential (non-helical) capture, to increase spatial accuracy on axis direction for dose planning. Radiotherapy was delivered to a field including the tumor with 0,5 mm margins using a Linear Accelerator (Oncor Impression Plus Siemens, Palo Alto, Ca.) fitted with a 10 mm RadioSurgery conical collimator (BrainLAB AG, Feldkirchen, Germany), which is designed to deliver very sharp and limited radiation dosed fields. Superflab bolus (5mm in thickness tissue equivalent material) was placed over the tumor as a skin surrogate, and a source to skin distance (SSD) of 85 cm was set (to reduce processing time). The use of collimator-based radiosurgery allows a more homogenous and more concentrated estimation of tumor dosimetry (supple figure 1). 98% of treatment volume was covered by the prescription dose (8 Gy) and the average distance between the isodoses of 8 Gy and 2 Gy, inside the mouse, was approximately 2 mm. Radiation was delivered at 180 cGy/min with 6 MV x-rays. In our Oncor Impression Plus, the quality index of that energy is TPR20.10=0.67 Mice received 3 fractions of 8 Gy on alternate days (days 12,14 and 16), or a single dose of 20 Gy.

**Flow cytometry.**

To obtain unicellular cell suspensions, tissues were incubated in collagenase and DNase (Roche) for 15 min at 37°C, mechanically disrupted and passed through a 70-μm cell strainer (BD Falcon, BD Bioscience) by pressing with a plunger. Cells dissociated from tissues were centrifuged with Percoll© (GE Healthcare) at 35% (500 g, 10 min, 20°C) and a gradient established in order to eliminate parenchymal cells. Single-cell suspensions were treated with FcR-Block in a PBS-based buffer containing 10% fetal calf serum to avoid unspecific staining. Fluorochrome-conjugated mAbs to the following mouse antigens were used: CD45.2 PerCP/Cy5.5 (clone 104 from Biolegend), CD45.2 PB (clone 104 from Biolegend), CD4 FITC (clone RM4-4 fromBiolegend), CD4 BV421 (clone RM4-5 from Biolegend), CD8 BV510 (clone 53-6.7 from Biolegend), CD25 APC (clone PC61 from Biolegend), CD11b FITC (clone M1/70 from BDPharmingen), CD11b APC (clone M1/70 from Biolengend), CD11c FITC (clone N418 from Biolegend), CD137 biotin (clone 17B5 from Biolegend), CD137 PE (clone 17B5 from Biolegend), PD-1 FITC (clone 29F.1A12 from Biolenged), PD-L1 APC (clone 10F.9G2 from Biolegend), FoxP3 PE (clone PFJK-16Ca from eBioscience), FoxP3 AF647 (clone 150D from Biolegend), Iab PE (clone AF6-120.1 from Biolegend), F4/80 APC (clone BM8 from Biolegend), F4/80 BV421 (clone BM8 from Biolegend), Ly6C PerCP/Cy5.5 (clone HK1.4 from Biolegend), Ly6G BV510 (clone 1A8 from Biolegend), GR1 PE (clone RB6-8C5 from Biolegend), NK1.1 PE (clone PK-136 from Biolegend), Streptavidin APC (from Biolegend).

Cytofix/Cytoperm (BD Biosciences) was used for FoxP3 and IFN γ intracellular staining. Blood cells were centrifuged and the supernatant was discarded. Erythrocytes were lysed with RBC Lysis buffer 1X (10 minutes, 37°C) from eBioscience. Thereafter, cells were stained as above. For IFN γ intracellular staining, lymphocytes were cultured at 37°C for 4 hour in the presence of PMA plus ionomycin and golgi plug.

FACS-Canto II (BD-Biosciences) was used for cell acquisition and data analysis was carried out using FlowJo software (Tree Star Inc).

**Tissue specimens and Immunohistochemistry**

Tumor tissue was freshly taken from patients and processed for IHC. Samples were obtained following protocols approved by our institutional ethics board and patients signed informed consent. For ex-vivo irradiation minced fragments of the tumor approximately 5 x 5 mm were placed in culture medium and irradiated with a single dose of 20 Gy (15MV x-rays). Fragments were placed in culture for 48 hours in complete (10%FCS) medium and used for IHC.

IHC was performed on formaldehyde-fixed and paraffin-embedded tissue sections 3 to 4 μm thick, using monoclonal antibody antagonists against PD1 (clone NAT105), B7-H1(clone SP142), CD137(clone BBK-2), CD4(clone SP35, Cell Marque, 104R-14) and CD8(clone SP6, Neomarkers, RM9116). After deparaffinization and rehydration, the sections were washed in Tris-buffered saline (TBS) 0.55M. Antigen retrieval for CD137 was performed with sections submerged in citrate buffer, pH 7, for 12 minutes at 98ºC (PTLink Dako), for CD4 and CD8, sections were submerged in Tris/EDTA (pH 9) for 30 minutes at 95°C in a Pascal pressure chamber (Dako). After blocking, the sections were incubated with the primary antibody; CD137 overnight at room temperature; CD4 and CD8, overnight at 4°C. After washing with TBS 0.55M, the sections were incubated with EnVisionFLEX/HRP (Dako) for 30 minutes at room temperature. The sections were stained using the Liquid DAB + Substrate Chromogen System kit (Dako) and were contrasted with Harris Hematoxylin. The immunoreactivity of the tumour cells was assessed qualitatively taking as negative the absence of immunostaining, and positive when membrane staining was observed. Multiplexed immunofluorescence was performed as described in supplementary methods section.

**Multiplexed Immunofluorescence**

Multiplexed quantitative immunofluorescence (QIF) was performed as recently reported ([1](#_ENREF_1)) using mAb against hPD-L1 (clone E1L3N, Cell Signaling Technologies). Briefly, fresh full-face, whole tissue sections from the tumor samples were deparaffinized and subjected to antigen retrieval in citrate buffer pH=8.0 and boiling for 20 ­min at 102­°C in a pressure-boiling container (PT module, Lab Vision). Slides were then blocked with 0.3% BSA in TBS for 30 min at room temperature followed by overnight incubation at 4oC with a solution containing primary rabbit anti hPD-L1 (1:1000) mAb and a mouse monoclonal anti-human pancytokeratin antibody (clone M3515; DAKO). Sections were incubated for 1 h at room temperature with Alexa 546-conjugated anti-rabbit (A11010; Molecular Probes) secondary antibody diluted 1:100 in rabbit EnVision amplification reagent, (K4003, Dako). Cyanine 5 (Cy5) directly conjugated to tyramide (FP1117; Perkin-Elmer) at a 1:50 dilution was used for target antibody detection. Prolong mounting medium (ProLong Gold, P36931; Molecular Probes) with 40,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei in the preparations. Objective signal quantification was performed using multispectral fluorescence imaginig and the AQUA method of QIF (Genoptix Inc.), as previously described.

**In vivo Depletion of CD4, CD8 and Natural Killer (NKT- Cells)**

Depletion of CD4, CD8 T cells or NK 1.1+ cells was achieved by intraperitoneal injection of anti-mouse CD4 (clone GK1.5), CD8 (clone 2.43) and NK 1.1+ (clone PK136) monoclonal antibodies, produced and purified by affinity chromatography on protein-G from the corresponding hybridomas. Anti-mouse CD4 and anti-mouse CD8β monoclonal antibodies were administrated at days 10, 14, 19 and every 7 days at 100ug per dose and anti-NK1.1 monoclonal antibody at days 10, 12, 14, 16, 19 and every 7 days until end of the experiment. During the experiment, blood samples were taken and analysed by flow cytometry, showing that mice were completely depleted of the corresponding lymphocyte subset.

Reference:

1. Velcheti V, Schalper KA, Carvajal DE, Anagnostou VK, Syrigos KN, Sznol M, et al. Programmed death ligand-1 expression in non-small cell lung cancer. Lab Invest. 2014;94:107-16.

 **Supplementary Figures Legends**

**Supplementary figure 1: Mouse external beam irradiation setting and combinations of radiotherapy and anti-CD137 and/or anti-PD1 immunostimulatory monoclonal antibodies are safe and effective to treat MC38-derived bilateral tumors of which only one of the tumor sites is irradiated.**  (A) 3D-reconstruction of the subcutaneous tumor-bearing mouse under general anesthesia with the irradiation beam represented in green. Lateral view of a reconstruction mouse on a CT-SCAN (sagital section) with the ipsilateral (to be irradiated tumor in blue) and the contralateral tumor out of the field in purple. Tumors are located in opposite sides of the mice to maximize distance and ensure that one of the lesions does not receive irradiation. Isodose curves on a representative tumor-bearing mouse. The clinical linear accelerator equipment used for mouse irradiation is shown in the right picture.(B) Scheme of MC38 tumor cell engraftment in syngenic mice and sequential treatment interventions represented by arrows. External beam radiotherapy was only provided to the right (primary) tumor when indicated (3 fractions of 8 Gy). (C) Follow-up of tumor sizes of the irradiated (primary) and non-irradiated tumor (contralateral) that received the indicated schemes of treatment. Statistical comparisons are in supplementary table I. (D) Overall survival of the mice. (E) To study immune memory, 90 days after the first tumor engraftment tumor-free mice of the indicated groups were bilaterally s.c rechallenged with MC38 cells and B16-OVA cells in the contralateral side. Tumor growth curves (mean ± SD) and fraction of mice developing tumors are shown.

**Supplementary figure 2: 4T1-derived spontaneous lung metastases** with representative sections of the CT-SCANS summarized for figure 2C and representative photographs of metastatic lungs taken following necropsy.

**Suplementary figure 3: Radiotherapy reduced the density and absolute numbers of CD4+, CD8+ and CD25+FOXP3+ T cells and Myeloid-derived suppressor cells both in the irradiated and non-irradiated lesions**. (A) Scheme of repeated unilateral radiotherapy treatments. Mice were sacrificed on day +17 and tumor-derived cell suspensions were analyzed by multicolor immunofluorescence and flow cytometry. (B and C) Absolute numbers and density (cells per mg) of the indicated T-cell subsets are presented in the primary irradiated tumor (B) and contralateral (non-irradiated) tumor (C). (D and E)total, M-MDCSC and Gr-MDSC were measured in tumor-derived cell suspensions, showing absolute numbers and density. A tendency to a decrease in Gr-MDSC in the irradiated tumor (D) and of M-MDSC in the contralateral tumor (E) were observed, but did not reach statistical significance in any of the two performed experiments.

**Supplementary figure 4: A course of radiotherapy plus immunostimulatory monoclonal antibodies anti-CD137 and anti-PD-1 mAb increases CD4 and CD8 T cells in the tumor infiltrate as well as the number of tumor antigen-specific CD8 T cells.** (A) Scheme of treatment of MC38-derived tumors as in supplementary figure 2 in which mice of all the experimental groups were sacrificed on day 17 to generate cell suspensions for the irradiated and contralateral tumors as indicated. (B) Tumor size follow-up prior to tumor excision. (C) Absolute numbers of CD4 and CD8 T cells and number of T lymphocytes (per gram of tumor) as recovered from the tumor cell suspensions. (D) Numbers of gp70 specific CD8 T lymphocytes stained with H-Kb KSPWFTTL tetramers. (E) Numbers of gp70 tetramer-positive cells expressing PD-1 and CD137 as potential targets for the immunostimulatory mAb. To estimate absolute numbers perfect count microspheres were used as an internal standard.

**Supplementary figure 5: single dose radiotherapy and hypofractionated doses increase the level of CD137 and PD-1 expression on the surface of tumor-infiltrating T cells.** (A) Scheme of unilateral single dose irradiation (20 Gy) in mice bearing bilateral MC38-derived tumors that were excised 48 hour later to obtain single cell suspensions. (B) Levels of surface expression (MFI) of CD137, PD-1 and PD-L1 on the gated CD8+ or CD4+ T cells among tumor infiltrating T lymphocytes. Left bar graphs represents primary irradiated lesions and right graphs represent the corresponding contralateral non-irradiated tumors. Experiments shown in C and D were performed a following three fractionated doses of 8Gy (C) in the irradiated tumor instead of a single radiotherapy dose, Graphs in (D) show the surface expression levels of CD137 and PD-1 as indicated.

**Supplementary figure 6. Ex-vivo irradiation of human carcinoma surgical expecimens gives rise to more intense expression of CD137 and PD-1 on infiltrating lymphocytes.** Two colon adenocarcinomas (different from those in figure 7) and two gastric carcinomas were excised and freshly minced to (5x5 mm pieces), that were put in tissue culture to be either irradiated with 20 Gy or left mock irradiated. 48 h later tissues were fixed and paraffin embedded and IHC stained for CD4, CD8, PD-1 and CD137. (A) percentage of positively stained cells per high power microscopic field in the four analysed tumors and (B) representative microphotographs of a colon carcinoma case.