SUPPLEMENTAL MATERIAL

Materials

FAP-targeted murine 4-1BB Ligand fusion protein (muFAP-4-1BBL, P1AE8721) and the non-tumor targeted DP47 murine 4-1BB Ligand fusion protein (muDP47-4-1BBL, P1AG3059), were generated by Roche Innovation Centers Zurich. The molecules display a mouse IgG1 like format. The Fc-fragment is based on a IgG1 κ including D265A P329G (DAPG) mutation to abrogate mouse Fcγ-receptor binding while remaining binding to mouse Fc-natal receptor (FcRn) to extend antibody like half-life [28]. Further the Fc region contains a charged aminoacid pairs (DD-KK) to improve molecule assembly [29]. One arm of the antibody-like fusion proteins is an anti-FAP specific or non-targeting DP47 (germline) Fab domain whereas the other arm consists of the dimeric mouse 4-1BBL ectodomain amino acids 140-309 [30].

CAF isolation

MMTV-Neu tumor was sliced with scalpel blades and into small fragments, together with PBS (10% FBS and 1% penicillin-streptomycin (Life Technologies)). Fragments were placed on a previously settled gel containing matrigel (2mg/mL, BD Bioscience), collagen (4mg/mL, Gibco), 5x collagen buffer, 10% FBS and DMEM+GlutaMAX medium (Gibco). 5x Collagen buffer contained 5x DMEM powder, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (0.1M, Gibco), 2% $NaHCO_3$ (Merck) and distilled water. After fragments were covered in gel, CAF medium was added, containing DMEM+GlutaMAX (Gibco), supplemented with 10% FBS, 1% antibiotic-antimycotic (Gibco), 50 µM 2-mercaptoethanol (Gibco), 5 µg/ml hydrocortisone (Sigma-Aldrich), 10 ng/ml epidermal growth factor (EGF) (Tebubio or

PeproTech), and 1% Insulin-Transferrin-Selenium (ITS) (Gibco). Within 4 days, spindle-shaped fibroblast cells could be observed. After 8 days, gel under the fragment was taken and digested with TM-TH liberase (Roche) in DPBS (Gibco) at 37ºC for 45 minutes. The solution was centrifuged at 283 x g for 4 minutes and the cell pellet was resuspended in CAF medium. In order to enrich sample in CAFs and get rid of tumor cells, sequential pipetting was performed by pipetting sample from well to well and letting settle for short periods, so that only CAFs get attached to the wells.

mCherry, GFP, and SV40 Large T-Antigen Transfectant Cell Lines

4T1-mCherry cell line was provided by Dr. Montserrat Arrasate (CIMA, Pamplona). CAFs were transfected with the SV40 large T-antigen expression plasmid and/or GFP. GFP-positive cells were single-cell sorted using a MoFlo Astrios EQ cell sorter (Beckmann Coulter), and single-cell clones were expanded in culture using the respective medium.

RNA extraction and quantitative RT-PCR

RNA from tumor samples was extracted using RNA Maxwell RSC simply RNA extraction kit (Promega) according to the manufacturer's instructions and followed by retrotranscription into cDNA with M-MLV enzyme kit (Invitrogen). For lung samples Trizol (Invitrogen) was used following manufacturer's instructions.

Real-time PCR reaction was performed with Bio-rad CFX qPCR system with the following primers for FAP (FW 5'- GGAAATGCTTGCCACAAAAT -3' and RV 5'-AAACACGAACAACCGGATTC -3') whose expression was normalized with the mouse Tbp levels (FW: 5'- CCTTGTACCCTTCACCAATGAC -3', RV: 5'-

ACAGCCAAGATTCACGGTAGA -3') and gp70 (FW 5'-TGACCTTGTCCGAAGTGACC -3' and RV 5'- TAGGACCCATCGCTTGTCTT -3') whose expression was normalized with the mouse H3 levels (FW: 5'-AAAGCCGCTCGCAAGAGTGCG -3', RV: 5'-ACTTGCCTCCTGCAAAGCAC -3').

Luminex multiplex assay

After tumor harvest, minced tumors were incubated with PBS at 37ºC in agitation and supernatants were collected. Cytokine and chemokine concentrations in tumor were measured with mouse ProcartaPlex Mix&Match 10-plex (Invitrogen) following manufacturer's instructions. For analysis, Luminex xMAP Technology (MAGPIX) data analysis software was used.

ELISPOT assay

gp70 specific CD8 T cell response was assessed ex vivo by a mouse IFNγ Enzyme-linked Immunosorbent Spot (ELISpot) Assay kit (BD Biosciences #551083). 96–well Multiscreen IP Plates (BD Biosciences) were coated with 100 µL of assay diluent containing anti–IFNγ monoclonal antibody (BD Biosciences) and incubated overnight at 4 ºC. The plates were washed and then blocked with RPMI1640 medium containing 10% FBS for 2 hours at room temperature. Splenocytes depleted of erythrocytes with ACK lysing buffer (Gibco) were added to the wells (800.000 cells per well) where they were stimulated with synthetic $gp70_{421-431}$ (SPSYVYHQF) peptide (10 µg/mL) for 24 hours. IFNγ-producing cells were assessed by counting the spots referred to input cells according to the manufacturer's instructions.

Immunohistochemistry (IHC) staining and analysis of human tumor samples

Fibroblast activation protein alpha (FAP) and alpha-smooth muscle actin (αSMA) expression was evaluated with a monoclonal anti-human FAP rabbit IgG (clone SP325, 1:100 dilution, Abcam) and monoclonal anti-human αSMAmouse IgG2a (clone 1A4, ready-to-use, Cell-marque, product number 202M-90) detection antibodies. In brief, IHC was performed on 4-microns-thick formalin-fixed paraffin-embedded (FFPE) tissue sections on a BOND RX autostainer (Leica Biosystems). Sections were deparaffinized (Bond DeWax, Leica Biosystems) and rehydrated per standard protocols. For both antibodies, antigen retrieval was performed (ER1, Leica Biosystems, product number AR9961) at pH 6 for 10 minutes at 98 °C. Bound detection antibodies were detected using the Bond polymer refine detection kit (Leica Biosystems, product number DS9800) according to the manufacturer's recommendations. The slides were mounted with glass coverslips and scanned using an Aperio CS2 scanner (Leica Biosystems). Image analysis was performed using the open-source digital pathology software QuPath version 0.4.4. The built-in cell segmentation algorithm of QuPath was used to identify each cell. After intensive quality control, intensity thresholds based on FAP- and αSMA-positive staining were set for cellular DAB detection. The percentage of FAP- and αSMA-positive cells in total cells was evaluated from the FAP-positive post-CRT resections.