

JITC-2020-002319.R2 Supplemental Methods

Mice

C57BL/6-Ly5.1 (CD45.1+, *Ptprca*) mice were bred in animal facilities of the German Cancer Research Center. OT-I mice (C57BL/6-Ly5.2/CD45.2+; *Tg(TcraTcrb)1100Mjb*) were purchased from Charles River. The mice were aged between 8 and 12 weeks and grouped after tumor injection prior to treatment in order to equalize tumor size within groups. Mice were held in individually ventilated cages in groups of up to five animals. Food and water were provided ad libitum. Mice were maintained on a 12 hour light/dark cycle and environmental enrichment was provided; temperature was maintained between 20 and 24 °C. Mice were visually checked daily and euthanized if adverse effects were anticipated or if mice were nearing an endpoints.

PDA30364 cell line, generation and use

The murine PDA cell line was generated from primary pancreatic tumors of PDA genetically engineered mouse model *Elas-tTA/TetO-Cre Kras^{+/LSL-G12D} Tp53^{+/LSL-R172H}* after cessation of doxycycline, which induces DNA recombination, and chronic pancreatitis induced by repetitive injections (three times per week, hourly injection for 6 hours) of 1 µg cholecystokinin analog cearulein (Sigma). This tumor induction protocol is based on the published work by Guerra et al.⁵⁹. The presence of the KRAS and P53 driver mutations in the cell line was verified by whole-exome sequencing. PDA30364 were cultured in DMEM, 10% FBS, 100 units (U) ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin (Thermo Fisher). PDA30364-OVA were cultured in DMEM, 10% FBS, 100 units (U) ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin.

Handling of small molecule drugs

Inhibitor stocks (10 and 100 mM) for in vitro use were generated by dissolving lyophilized inhibitor in dimethyl sulfoxide (DMSO). Inhibitors were stored at -20 °C until use and thawed only prior to use. For in vivo experiments, GDC-0623 was suspended in methylcellulose (0.5% w/v)/tween (MCT) using a laboratory dispenser.

Production of anti-CD40 antibodies

Chimeric agonist anti-mouse CD40 antibody (mIgG1, clone 3–23) sequences were obtained from Martin Glennie (University of Southampton, UK). Codon optimization for the production in HEK (Homo sapiens) and CHO (*Cricetulus griseus*), as well as sub-cloning into pCEP4_A164 expression vector, was performed by GeneArt (Thermo Fisher Scientific). Plasmids stocks were maintained in form of regular MaxiPreps using the Endofree Plasmid Maxi kit (Qiagen) according to the manufacturer's protocol. HEK293-F suspension cells were transfected with the indicated constructs using the 293-Free transfection reagent kit (Novagen) with equimolar amounts of plasmid DNA for heavy and light chain. HEK293-F cells were seeded on day prior to transfection at 0.3×10^6 viable cells ml⁻¹ prewarmed FreeStyle293 expression medium. At the day of transfection, the cell number was adjusted to 1×10^6 cells ml⁻¹ in FreeStyle293 expression medium. Plasmid mix with equimolar amounts of heavy and light chain were diluted in mixed with 293-transfection reagent (Novagen). After 15 minutes incubation, the transfection mix was added to the cells in a dropwise fashion. Viability of cells was monitored for one week using a ViCell cell counter (Beckman Coulter). Transfected cells were cultured on a shaking platform at 37 °C, 8% CO₂ for ~1 week. Antibodies were purified with an ÄKTA pure chromatography system in conjunction with a HiTrap protein G column (1 or 5 ml) according to the manufacturer's (GE Healthcare) instructions. Bound antibodies were eluted from the columns with a glycine-based elution buffer (pH 2,7–3). Buffer exchange from Tris-HCl to sterile PBS was performed using a Slide-a-Lyzer dialysis cassettes (Thermo Fisher). Antibody

concentration was determined using a NanoDrop 8000 device. Low levels of endotoxin ($< 1 \text{ EU ml}^{-1}$) were confirmed by Limulus amoebocyte lysate-based test with the Endosafe-PTS testing system (Charles River).

Tumor experiments and treatment protocols

Murine tumor cell lines were passaged at least four times after thawing and grown as a monolayer with a maximal confluency of 70–80%. Cells were detached using 0,25% trypsin-ethylenediaminetetraacetic acid (EDTA) solutions, centrifuged, and washed once with cell culture medium. $0,5 \times 10^6$ PDA30364 tumor cells were injected in PBS/Matrigel (1:1, Corning) subcutaneously into the flank. Tumor growth was measured twice a week with a caliper. The tumor volume was calculated by multiplying length by width by height. Tumor growth curves were generated using GraphPad Prism 7 software and formatting was finalized using Adobe Illustrator imaging software. Quantification of immune cell populations was visualized in bar graphs using GraphPad Prism 7 software. Treatment schedules and administration of drugs are indicated in the respective figure legends. In general, treatment was initiated one week after tumor implant when tumors were palpable and started to grow. Mice were treated daily by oral gavage with MEK inhibitor GDC-0623 (200 μl per mouse). Control groups received 0.5% methylcellulose tween (MCT). For combination therapy experiments, 200 μg of anti-CD40 mIgG1 (clone 3-23, in-house production) was administered intraperitoneally. As isotype controls mouse IgG1 isotype control (BioXCell, clone MOPC-21, BP0083) was used. Treatment with anti-CSF1R antibody (BioXCell, BP0213) comprises one bolus injection of 1 mg, followed by 0,5 mg every other day as indicated in the figure legend. Mice were killed if signs of distress were noticed, when termination criteria were reached, or analyses were performed at specific time points.

In vitro assays with mouse macrophages and tumor cells

Macrophage precursors were isolated from bone marrow of C57BL/6-Ly5.1 mice and differentiated ex vivo with cytokines into M1- or M2-like macrophages, respectively. For this, bones from femurs and tibias were isolated and crushed with a mortar in RPMI-1640 medium. The medium containing bone marrow cells were filtered through a 70 μm cell strainer. For red blood cell lysis, the cell suspension were resuspended in ACK lysis buffer (Lonza) for 2 minutes at RT. Cells (200.000 monocytes per well of a 96-well plate) were resuspended in macrophage medium (RPMI-1640 supplemented with 10% FCS, 100 U ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin and 20 ng ml^{-1} M-CSF). Medium change was performed on day 4 by replacing 50% of the medium with 2-fold concentrated cytokine solutions. In some experiments, full medium changes on day 4 were performed. On day 7, macrophages were polarized into M1- or M2-like macrophages by replacing the basal macrophage medium by respective polarization media. Concentrations of stimulants ranged between 1-20 ng/ml as indicated in the figure legends.

For in vitro cytotoxicity assays, 1.000 PDA30364 tumor or 200.000 bone marrow-derived monocytes were seeded in 200 μl growth medium per well into a 96-well flat bottom plate using a VIAFLO96 handheld 96-channel electronic pipette device (Integra). Monocytes were differentiated into macrophages as described above. On the day of macrophage polarization (day 7), medium was replaced by 80 μl fresh M1 or M2 polarization medium as indicated in the figure legends. Inhibitor stock solutions were diluted to 500 μM in growth medium followed by a serial 1:10 dilution using the VIAFLO96 device. 20 μl of diluted inhibitor were added to the tumor cells (one day after seeding) or differentiated macrophages (on day 7). Medium and DMSO controls equivalent to the highest inhibitor concentrations were included. Cells were incubated for 72 h at 37 $^{\circ}\text{C}$, 5% CO_2 . On day 10, cells and media were equilibrated to room temperature (RT) for 30 minutes and 100 μl of CellTiter-Glo reagent (Promega) were added to each well and agitated on a shaking platform for 2 minutes. After 10 minutes of incubation, 100 μl were transferred to a 96-well white OptiPlate and

luminescence was measured at 490 nm using the EnVision 2014 (Perkin Elmer) multilabel reader. Cell viability was calculated as percent of medium control plotted using GraphPad Prism 7. The concentrations of half-maximal inhibition (IC_{50}) were computed using a non-linear fit with bottom being constraint to greater than 0. The dose–response curve plots were exported and formatting was finalized using Adobe Illustrator imaging software.

In vitro assays with human macrophages

Monocytes were isolated from human PBMCs by using the pan monocyte isolation kit (Miltenyi Biotec) and differentiated ex vivo with cytokines into M1- or M2-like macrophages, respectively. For this, the PBMC single cell suspension was incubated with an Fc receptor blocking reagent followed by a biotin-antibody cocktail and subsequently magnetically labeled with anti-biotin microbeads. These magnetically labeled cells underwent magnetic cell separation on a LS column. Untouched, unlabeled monocytes were collected, resuspended in macrophage medium (RPMI-1640 GlutaMax supplemented with 10% FCS, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 50 µM beta-mercaptoethanol, and 50 ng ml⁻¹ M-CSF), and seeded in 96-well plates (200.00 monocytes per well). The entire medium was replaced by fresh macrophage medium on day 4. On day 8, macrophages were polarized into M1- or M2-like macrophages by replacing the basal macrophage medium by respective polarization media. Concentrations of stimulants ranged between 1-50 ng/ml as indicated in the figure legends.

For in vitro cytotoxicity assays, 200.000 PBMC-derived monocytes were seeded in 200 µl macrophage medium per well of a 96-well flat bottom plate. Monocytes were isolated and differentiated to macrophages as described above. On the day of macrophage polarization (day 8), medium was replaced by 80 µl fresh M1 or M2 polarization medium as indicated in the figure legends. Inhibitor stock solutions were diluted to 500 µM in respective M1 or M2 polarization medium followed by a serial 1:10 dilution using the VIAFLO96 device. 20 µl of diluted inhibitor were added to differentiated macrophages (day 8). Medium and DMSO controls equivalent to the highest inhibitor concentrations were included. Cells were incubated for 72 h at 37 °C, 5% CO₂. On day 10, cells and media were equilibrated to room temperature (RT) for 30 minutes. 100 µl of CellTiter-Glo reagent (Promega) were added to each well and agitated on a shaking platform for 2 minutes. After 10 minutes of incubation, 100 µl were transferred to a 96-well white OptiPlate and luminescence was measured at 490 nm using the EnVision 2014 (Perkin Elmer) multilabel reader. Cell viability was calculated as percent of medium control plotted using GraphPad Prism 7. The concentrations of half-maximal inhibition (IC_{50}) were computed using a non-linear fit with bottom being constraint to greater than 0. The dose–response curve plots were exported and formatting was finalized using Adobe Illustrator imaging software.

Flow cytometric analysis of macrophage phenotype after MEKi treatment

For analysis of tumor-derived macrophages, tumor tissue (50–200 mg) was digested using a human tumor dissociation kit (Miltenyi) according to manufacturer's instructions in conjunction with the gentleMACS Octo tissue dissociator (Miltenyi) with the program '37C_h_TDK_3'. After enzymatic digestion and homogenization, tumor cell suspensions were poured through a 100 µm pre-coated with 3% BSA/PBS. Flow cytometry staining and data analysis were performed as described in ⁵.

Ex vivo generated murine and human macrophages were treated with MEKi GDC-0623 as described above. MEKi was added during macrophage polarization to M1- or M2-like subtypes with respective polarization media as indicated in the figure legends. The next day, cell surface expression of costimulatory molecules as well as M1 and M2 markers were analyzed by flow cytometry. Staining and data analysis were performed as described in ⁵, with the following adjustment in case of the human macrophages: HumanTrueStain FcX (BD) was used to block Fc receptors (1:50, 10 min, 4°C). The following antibodies against surface and intracellular epitopes were applied for human samples:

CD14-BV711 (Biolegend, 1:50, cat. # 301838), CD45-BV786 (BD, 1:50, cat. # 563716), CD11b-FITC (Biolegend, 1:50, cat. # 301330), CD163-BV605 (Biozol, 1:50, 333615), CD40-PE (BD, 1:20, cat. # 560963), HLA-DR-APC/Cy7 (Biolegend, 1:50, cat. # 307618), CD80-PerCP/Cy5.5 (Biolegend, 1:50, cat. # 305232), CD86-PE/Cy7 (Biolegend, 1:50, cat. # 374210), CD68-AF647 (Biolegend, 1:50, cat. # 333820), CD206-BV421 (Biolegend, 1:50, cat. # 321126).

The following antibodies against surface and intracellular epitopes were applied for additional murine samples, in particular the MEK inhibitor treated murine bone marrow-derived macrophages: CD11b-PerCP/Cy5.5 (Biolegend, 1:1000, cat. # 101228), CD45-BV785 (Biolegend, 1:1000, cat. # 103149), F4/80-BV605 (Biolegend, 1:200, cat. # 123133), Gr1-PE/Dazzle594 (Biolegend, 1:1000, cat. # 108451), CD40-PE (Biolegend, 1:200, cat. # 124610), I-A/I-E-AF700 (Biolegend, 1:1000, cat. # 107621), CD86-FITC (Biolegend, 1:1000, cat. # 105005), CD11c-BV711 (Biolegend, 1:1000, cat. # 117349), iNOS-APC (eBiosciences, 1:200, cat. # 17-5920-82), CD206-BV421 (Biolegend, 1:200, cat. # 141717).

The flow cytometry data were analyzed with the FlowJo software (version, FlowJo LLC). Resulting quantification of immune cell populations was visualized in bar graphs using GraphPad Prism 7 software. Histograms and pseudocolor plots were exported and formatting was finalized using Adobe Illustrator imaging software.