Supplementary material:

Chimeric PD-1:28 receptor upgrades low-avidity T cells and restores effector function of tumor-infiltrating lymphocytes for adoptive cell therapy

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Constructs, expression vectors, electroporation, retroviral transduction

For *in vitro*-transcribed (ivt)-RNA, sequences were cloned into the pGEM vector (provided by S. Milosevic, Medigene GmbH, Martinsried, Germany) using HindIII or HindII and EcoRI (New England Biolabs). Ivt-RNA was generated from pGEM plasmids using the mMESSAGEmMACHINE Kit (Ambion) according to the

manufacturer's protocol. Human T cells were electroporated with 20 µg ivt-RNA at 900 V for 2.3 ms using Gene Pulser Xcell (Bio-Rad).

For retroviral transduction, PD-1:28^{tm}, mPD-1:28^{tm} and Thy1.1 constructs were cloned into pMP71 vector using NotI and EcoRI. Human PBMCs were activated with 5 µg/ml of plate-bound OKT3 (provided by E. Kremmer, Helmholtz Center Munich, Germany) and 1 µg/ml of anti-CD28 (BD) for 2 days in T-cell medium with 100 U/ml IL-2. Virus production and transduction was performed as described (29). Activated T cells were cultivated on virus-coated plates for 4 days, harvested and cultivated for at least 13 days reducing the amount of IL-2 to 50 U/ml.

Murine splenocytes were activated with 1 µg/ml anti-CD3 and 0.1 µg/ml anti-CD28 antibodies (BD) in mouse medium with 10 U/ml IL-2 (Novartis) for 1 day. Activated cells were seeded in virus-coated plates in mouse medium supplemented with 10 U/ml IL-2 and CD3/CD28 beads (Invitrogen). Virus production was performed as described (29). One day after the first transduction, 1 ml of fresh supernatant was added to splenocytes and incubated for 6h. Splenocytes were harvested and cultivated in mouse medium supplemented with 50 ng/ml IL-15 (Miltenyi). Three days later, splenocytes were sorted for Thy1.1+ cells using magnetic beads (Miltenyi) according to the manufacturer's protocol.

Murine HCC model

LoxP-Tag mice are transgenic for the oncogenic large T antigen, which is controlled by a stop cassette (LoxP-TAg mice). The oncogenic process is initiated by infecting mice with adenoviruses coding for Cre recombinase. Due to the hepatic tropism of adenoviruses, TAg is predominantly activated in the liver and mice develop multinodular HCC within 8-24 weeks after virus injection. In the early phase of

oncogenesis, multiple malignant lesions develop in the liver, most of which are eradicated by T cells. Despite this initial antitumoral T-cell activation, tumors grow out leading to death over the next 8-35 weeks presumably because T cells become exhausted. Indeed, it was demonstrated that survival could be prolonged when mice received blocking antibodies to PD-L1 (28).

LoxP-Tag mice were injected with adenovirus coding for Cre recombinase (Ad.Cre) to activate the oncogenic process. 15 weeks after injection, splenocytes were isolated and transduced with Thy1.1 or Thy1.1 plus mPD-1:28^{tm} (Thy1.1/mPD-1:28^{tm}). 5 x 10^6 transduced splenocytes were injected r.o. into recipient mice who had received Ad.cre 11.5 weeks earlier. Mice were irradiated with 5 Gy one day prior to ATT. 11 mice received Thy1.1 splenocytes, 12 mice Thy1.1/mPD-1:28^{tm} splenocytes and 12 mice did not receive ATT. Mice were sacrificed when tumors reached a size of 1.5 cm. Single cell suspensions of spleens were prepared by pressing through a 40 µm filter followed by erythrocyte lysis using 0.15 M ammonium chloride (Merck). Tumors were dispersed through a 100 µm filter, digested with 800 U/ml collagenase IV (Sigma-Aldrich) and TILs separated by percoll-density-centrifugation.

Tumor cell spheroids and Imaging

All 3D cell culture steps were conducted with RPMI basic medium (see above) with 10 % FCS at 37°C/5% CO_2. Tumor spheroids were generated by seeding 800 SK-Mel23 cells each into hanging drops of 25 µl. T cells were stained with 0.5 µM Cell Tracker^{TM} CMFDA fluorescent dye (ThermoFisher) according to the manufacturer's protocol. Spheroids were harvested after 3 d and confronted with 1.5 × 10^4 T cells in a 1.5-ml reaction tube, containing a single spheroid, in a total volume of 50 µl cell

culture medium. The tube was shaken at 300 rpm for 30 min at 37°C, thus ensuring an even distribution and attachment of T cells to the spheroid surface. T cells that had not attached were washed away. T cell/spheroid samples were incubated in hanging drops of 25 µl culture medium with 5 µM CellEvent™ Caspase-3/7 Red Detection Reagent (ThermoFisher) for a period of 24 h. Spheroids were fixed using 4% formalin for 2 h at room temperature. Image stacks (18 planes, 4 µm step size) were acquired at 488 nm and 640 nm excitation wavelength with a Nikon TiE microscope equipped with perfect focus, Yokogawa CSU-W1 spinning disk unit (50 µm pinhole size), Andor ALC600 laser-beamcombiner: 405nm/488nm/561nm/640nm, Yokogawa CSU-W1 dichroic mirror 405/488/561/640 LD Quad, Andor Borealis illumination unit and Andor IXON 888 Ultra EMCCD camera using a Nikon CFI Apo Lambda S LWD 40x NA 1.15 water immersion objective. The microscope was controlled by software from Nikon (NIS elements, version 4.51.01). CMFDA-positive T cells and CellEvent™ Caspase-3/7 Red Detection Reagent positive apoptotic cells were counted within the 3-dimensional datasets with FIJI (Nature methods 9(7) 676-682).

Human renal cell carcinoma tissue

Tumors were post-surgery material provided by Urologic Clinic Dr. Castringius, Planegg, Germany. They were classified as clear cell RCC according to the TNM guidelines (UICC 2010): RCC102: pT3aN0M0G2; RCC105: pT3aN0M0G3; RCC106: pT1aN0M0G2; RCC110: pT1bN0M0G2; RCC112: pT1aN0M0G2.