T-cell mediated targeted delivery of anti-PD-L1 nanobody overcomes poor antibody penetration and improves PD-L1 blocking at the tumor site

Petit Pierre-Florent[a,b], Raphaële Bombart[a,b], Pierre-Hubert Desimpel[a,b], Stefan Naulaerts[a,b], Laurie Thouvenel[b], Jean-François Collet[b,c], Benoit J. Van den Eynde[a,b,c,d], Jingjing Zhu[a,b,c,1]

[a]Ludwig Institute for Cancer Research, Brussels B-1200, Belgium.

[b]de Duve Institute, UCLouvain, Brussels B-1200, Belgium.

[c]Walloon Excellence in Life Sciences and Biotechnology, Brussels, Belgium.

[d]Ludwig Institute for Cancer Research, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom.

[1]To whom correspondence should be addressed:
Email: Jingjing.Zhu@bru.licr.org

Running title: Nanobody delivery improves intratumoral PD-L1 blocking

Synopsis: Local delivery of anti-PD-L1 nanobody by tumor-specific T cells increases therapeutic efficacy and reduces systemic exposure as compared to systemic injection of anti-PD-L1 antibody. Poor intratumoral penetration of anti-PD-L1 antibody is a factor limiting efficacy in vivo.

Keywords: Nanobody, PD-L1, antibody, penetration, immunotherapy

Additional information

Financial support:
P-F. Petit was supported by FNRS-aspirant (Grant number 1.A.818.18F).
R. Bombart was supported by FRIA (Grant number 1.E.002.21F).
P-H. Desimpel was supported by Televie (Grant number 7.4598.21).
S. Naulaerts was supported by FNRS-EOS DECODE (Research program O000518F).
J. Zhu and B.J. Van den Eynde were supported by Ludwig Institute for Cancer Research.
J. Zhu was supported by Fondation Contre le Cancer (Grant number 2019-094).

Corresponding author info:
Jingjing Zhu
Ludwig Institute for Cancer Research, de Duve Institute, UCLouvain,
Avenue Hippocrate, 74 B1.74.03, Brussels B-1200, Belgium.
Phone: 32-2-764-7558; Fax: 32-2-764-7590
E-mail: Jingjing.Zhu@bru.licr.org

Disclosure statement: The authors declare no potential conflicts of interest.

Other notes: Word count (4159). Fig number (5).

Abstract

Monoclonal antibodies blocking immune checkpoints, such as PD-L1 have brought strong clinical benefits in many cancer types. Still, the current limitations are the lack of clinical response in a majority of patients and the development of immune-related adverse events in some of them. As an alternative to anti-PD-L1 antibody injection, we developed an approach based on the engineering of tumor-targeting T cells to deliver intratumorally an anti-PD-L1 nanobody. In the MC38-OVA model, our strategy enhanced tumor control as compared to injection of anti-PD-L1 antibody combined with adoptive transfer of tumor-targeting T cells. Furthermore, we demonstrate the detrimental distribution pattern of anti-PD-L1 antibody which massively occupies PD-L1 in the periphery but fails to penetrate at the tumor site. In sharp contrast, locally delivered anti-PD-L1 nanobody improved PD-L1 blocking at the tumor site while avoiding systemic exposure. Our approach appears promising to overcome the limitations of immunotherapy based on anti-PD-L1 antibody treatment.

Introduction

During the last decade, monoclonal antibodies (mAb) targeting receptors and ligands regulating the adaptive immune system have emerged as a powerful therapy against cancer. Among the so-called immune checkpoint inhibitors (ICI), antibodies targeting the receptor Programmed Death 1 (PD-1) and its ligand (PD-L1) are currently the milestones of cancer immunotherapy. PD-L1 is expressed by some tumor cells and different immune cells. Its interaction with PD-1 expressed on T cells negatively regulates T cell activity(1). Anti-PD-L1 antibodies have therefore been used to restore CD8-T cell activation resulting in unprecedented survival improvements in patients suffering from non-small cell lung cancer or urothelial carcinoma(2, 3). Their clinical activity has also been proven in breast cancer, renal cell carcinoma and melanoma(4-6). Still, a fraction of patients receiving immune checkpoint blockade do not respond to the treatment or undergo disease progression after an initial response phase(7). Different resistance mechanisms have been identified, including the lack of tumor-specific T cell generation(8), the dysfunction of T cells(9), or the presence of immunosuppressive cells in the tumor microenvironment (TME)(10). Discovery of novel strategies to overcome resistance and increase the number of responding patients remains the current main challenge in cancer immunotherapy.

A major drawback of ICI treatment is the development of immune-related adverse events (irAEs) related to the activation of T cells recognizing healthy tissue(11). These events can lead to death in a fraction of patients(12). Different strategies were therefore developed to reduce the systemic toxicity of immune checkpoint blockade. Among these, intra or peritumoral injection of ICI enabled toxicity reduction while preserving or even increasing therapeutic efficacy compared to systemically injected antibody(13, 14). Although these approaches represent an interesting proof of concept, their clinical value is limited since patients with advanced disease typically present high number of metastases mostly located in organs such as the lung or liver.

Recently, it has been proposed that tumor-targeting T cells could be used as delivery vehicles to bring immune checkpoint inhibitors at the tumor site. While promising results were reported in the context of adoptive transfer of chimeric antigen receptor T cells (CAR-T), only few studies are available so far(15-17). Further research and development are needed to demonstrate the therapeutic potential of local delivery strategies and bring them closer to clinical use.

Nanobodies are single variable domain (VHH) fragments derived from camelid heavy chain antibodies. They represent the smallest antigen-binding antibody fragments, with a molecular size of around 15 kDa, and are known for their excellent stability and solubility(18, 19). Their good tumor penetration ability is counterbalanced by a very short half-life in the blood stream, due to renal filtration, and these characteristics have rather supported their development as imaging tools(20). In this study, we propose to use tumor-specific CD8 T cells to bring PD-L1 blocking nanobodies to the tumor site, so that we capitalize on the properties of nanobodies to ensure both a good tumor penetration and a minimal systemic exposure.

Using a mouse colon tumor model, we compared the therapeutic efficacy as well as the intratumoral delivery of PD-L1 blocking therapy based on local secretion of nanobodies or systemic injection of antibody. We demonstrated that our local delivery approach improved tumor control compared to classical antibody injection. We also observed a limited tumor penetration of systemically injected anti-PD-L1 antibody, preventing it from reaching PD-L1-expressing cells in the TME. This barrier was overcome by local secretion of PD-L1 blocking nanobodies.

Material and methods

Generation of viral expression vectors

MSCV-Thy1.1-DEST retroviral vector was obtained from Addgene (plasmid #17442). IRES-Thy1.1 cassette was removed by double digestion with SalI-MluI restriction enzymes. Plasmid was then ligated with sticky end paired oligos to obtain the "MSCV empty vector". The four nanobody encoding DNA fragments were ordered as gBlocks from IDT (https://www.idtdna.com) and cloned into MSCV or pET-21b+ vector. Sequence for truncated ovalbumin missing 49 AAs in the N-terminal part to avoid secretion(21) was ordered as a gblock from IDT and cloned into pTM898 vector provided by Professor Thomas Michiels (De Duve Institute, Brussels, Belgium). DNA fragments encoding *Cd274* or *Pdcd1lg2* genes were ordered as gblocks from IDT and cloned into pTM899 lentiviral vector, provided by Professor Thomas Michiels (De Duve Institute, Brussels, Belgium).

Expression and purification of the anti PD-L1 nanobody

E. coli ClearColi BL21 (DE3) cells harboring the pET-21b+ plasmid expressing the C-terminally His-tagged PD-L1 nanobody were grown in Lysogeny Broth (LB Miller) medium (Sigma) supplemented with 50 µg/ml of kanamycin, at 37°C. When cells reached an OD600 of 0.6, expression of the nanobody was induced with 1 mM IPTG for 3h at 37°C. After harvest at 4,000 g, 15 min, 4°C, the bacterial pellets were frozen and stored at -20°C. Cells were resuspended in buffer A (100 mM Tris pH8, 300 mM NaCl) supplemented with a protease inhibitor cocktail (Complete EDTA-free, Roche) and lysed by two passages through a French press at 1,500 psi. After clearing the lysate at 40,000 g for 30 min, 4°C, the soluble protein extract was filtered through 0.22 µm filters (Merck Millipore) and applied on a 1 ml His-Trap HP column (Cytiva), using the AKTÄ pure system (Cytiva). After washing the column with buffer A, the protein was eluted with a linear gradient up to 100% buffer B (100 mM Tris pH8, 300 mM NaCl, 300 mM imidazole) over 25 column volumes. To complete the purification, the protein was then applied on a HiLoad 16/60 Superdex 75 size-exclusion chromatography column (GE Healthcare), using phosphate-buffered saline (PBS) (MP Biomedicals) as running buffer.

Cell lines and cell culture

B16F10-OVA cell line was a gift from Professor Muriel Moser (Université Libre de Bruxelles, Belgium). B16F10-OVA cells were transfected with pTM899-PD-L1 lentiviral vector using TransIT-LT1 (Mirus, #MIR 2306) and kept under Puromycin (Invivogen, #ant-pr-1) selection. MC38 cell line was a gift from Professor Mark Hull (University of Leeds, UK). MC38 cells were transduced with pTM898-Ova lentiviral plasmid, and were then selected with Geneticin (G-418, Roche, #04727878001) to obtain in house MC38-OVA cell line, no clonal selection was performed. MC38-OVA PD-L1^{+} cell line was obtained by transducing MC38-OVA cells with pTM899-mPD-L1 lentiviral plasmid and MC38-OVA PD-L2^{+} cell line was obtained by transducing MC38-OVA cells with pTM899-mPD-L2 lentiviral plasmid. T429.18 clone was derived from an induced Amela TiRP tumor referred to as T429(22). Murine tumor cells and HEK 293T cells were cultured in IMDM (Gibco) supplemented with L-arginine (0.55 mM, Merck, #181003), L-asparagine (0.24 mM, Merck, #101566), glutamine (1.5 mM, Merck, #100289), beta-mercaptoethanol (50 µM, Sigma, #M3148), 10% Foetal Bovine Serum (Sigma-Aldrich, #F7524), 100 IU/ml penicillin and 100 µg/ml streptomycin (Pen Strep, Gibco, #15140148). OT-I and TCRP1A CD8 T cells were cultured in the same medium supplemented with 25 U/ml IL-2 (Proleukin, Novartis). HEK 293T and tumor adherent cells were detached with PBS buffer supplemented with 2 mM EDTA. Cells were routinely tested for Mycoplasma using MycoAlert detection kit (Lonza, #LT07-118).

Mice

OT-I transgenic mice (C57BL/6-Tg(TcraTcrb)1100Mjb/Crl, Charles River Laboratory) and C57BL/6J Ola Hsd mice (Envigo) were purchased and bred at the animal facility of the Ludwig Institute for Cancer Research (Brussels, Belgium). B10.D2;Ink4a/Arf$^{flox/flox}$ (TiRP-10B$^{-/-}$) mice were used as recipients for T429.18 tumor transplantation experiments. TCRP1A mice heterozygous for the H-2L^{d}/P1A$_{35-43}$-specific TCR transgene were kept on the B10.D2;Rag1$^{-/-}$ background(22, 23). All animal procedures were performed in accordance with national and institutional guidelines for animal care, and with the approval of the Comité d'Ethique pour l'Expérimentation Animale from the Secteur des Sciences de la Santé, UCLouvain [2019/UCL/MD/24.

PD-L1 gene knock-out

PD-L1 knock-out in MC38-OVA cells was performed by electroporation of Cas9 ribonucleoprotein (RNP) complexes. Alt-R crRNA for murine PD-L1 (Mm.Cas9.CD274.1.AQ) was obtained from IDT. Alt-R crRNA and Alt-tracrRNA (IDT) were annealed in a thermocycler and then mixed with Alt-R S.p. Cas9 Nuclease V3 (IDT, #1081058). Tumor cells were nucleofected with Cas9-RNP complex in supplemented SF Cell Line Nucleofector Solution (Lonza), using Lonza 4D-Nucleofector (HEK-293 program). After twelve days, cells were incubated with 100 ng/ml recombinant mouse interferon gamma (Gibco, #PMC4031) during 48h. Cells were then collected and surface stained for PD-L1. PD-L1-negative cells were sorted with FACS Aria III.

HEK 293T transfection and viral production

HEK 293T cells were transiently transfected with retroviral vectors for nanobody expression using TransIT-LT1 (Mirus, #MIR 2306) according to manufacturer protocol. For viral productions, HEK 293T cells were transfected with TransIT-LT1 mixed with packaging and expression plasmids. For retrovirus, we used a mixture of PCL-Eco plasmid (Addgene, #12371)

and MSCV empty vector or MSCV-5DXW-T61V nanobody-expression vector; for lentivirus, we used a mixture of MD2.G-pSPAX2 plasmids (Addgene, #12259 and 12260) and pTM898-Ova, pTM899-mPD-L1 or pTM899-mPD-L2 lentiviral vectors.

T cell isolation and transduction

Spleen and lymph nodes from OT-I and TCRP1A transgenic female mice aged between 8 and 12 weeks were smashed using the plunger of a syringe. After red blood cells lysis (RBC lysis buffer, eBioscience, #00-4300-54), CD8 T cells were isolated using CD8a (Ly-2) mouse Microbeads (Miltenyi, #130-117-044). OT-I T cells were stimulated with CD3/CD28 Dynabeads Mouse T activator (Gibco, #11453D). TCRP1A CD8 T cells were stimulated with irradiated L1210.P1A.B7-1 cells[22]. Two or three days after activation, T cells were transduced with produced virus in presence of 50 µg/ml Protamine Sulfate (Sigma, #P3369-10G) and centrifuged for 4h at 32°C at 1200 G. T cells were collected 6 days after activation for adoptive cell transfer to mice. Before injection, for OT-I T cells, stimulation beads were removed with a magnet. For TCRP1A CD8 T cells, dead cells were removed by performing ficoll gradient centrifugation using the Lymphoprep medium (Stemcell, #07851) before injection. For *in vitro* assays and flow cytometry analysis, OT-I T cells were used 7 days after activation.

***In vitro* binding and blocking assays**

For nanobody binding assay, B16F10-OVA PD-L1$^+$ cells were incubated with supernatant from nanobody-expressing HEK 293T cells and then stained with anti-HA Tag antibody. Amount of supernatant was normalized according to nanobody quantity detected in the supernatant by western blot analysis (quantification was performed by Image J). For recombinant PD-1 blocking assays, B16F10-OVA PD-L1$^+$ or MC38-OVA PD-L1$^+$ were used as indicated. Cells were first incubated either with supernatant from transfected HEK 293T cells, anti-PD-L1 monoclonal antibody (BioLegend, clone 10F.9G2, #124318), or sera from treated mice as indicated. Cells were then washed and stained with recombinant PD-1-PE (rPD-1 PE) obtained by coupling recombinant mouse PD-1 Fc chimera (R&D, #1021-PD) to R-phycoerythrin, using Lightning-Link® R-PE Labeling Kit (Abcam, #ab102918). Percentage of rPD-1 blocking activity in sera from treated mice was calculated as [1-(rPD-1 Median FI with serum from treated mouse/mean rPD-1 Median FI with sera from PBS-treated mice)]x100.

To validate that the binding of anti-PD-L1 antibody clone MIH7 to PD-L1 is not impaired by the presence of the nanobody or the anti-PD-L1 injected mAb, MC38-OVA PD-L1$^+$ were first incubated with increasing amounts of supernatant from 5DXW-T61V-secreting OT-I T cells or increasing concentrations of injected mAb (BioLegend, clone 10F.9G2, #124318). The cells were then washed and stained with anti-PD-L1 antibody clone MIH7. PBS buffer supplemented with 1 mM EDTA and 1% FBS was used for incubations and washings through all experiments. Incubations for binding, blocking, and staining were performed during 20 minutes at 4°C.

Western blot analysis

Culture medium from transfected HEK 293T cells was collected, centrifuged to pellet cell debris, and supernatant was used for western blot analysis. Transduced OT-I T cells at day 7 from activation were centrifuged, supernatant was collected while cell pellet was lysed in Pierce RIPA buffer (ThermoFisher, #89901) supplemented with Halt Protease-phosphatase inhibitor cocktail (ThermoFisher, #78429). Protein concentration of OT-I T cells lysates was evaluated by Pierce BCA Protein assay (ThermoFisher, #23225). Lysates, supernatants, or

mice sera were added with Pierce Lane Marker Reducing Sample Buffer (ThermoFisher, #39000), heated (95°C, 10min) and loaded on polyacrylamide gel (Bolt 4-12%, Invitrogen, #NW04122). After migration, proteins were transferred to iBlot NC stacks (Invitrogen, #IB23002). Membrane was blocked with 5% milk and stained with anti-HA Tag antibody (Invitrogen, clone 2-2.2.14, #26183, 1:5000), or anti-Vinculin antibody (Cell Signaling, clone E1E9V, #13901S, 1:5000). Secondary antibodies used were anti-mouse IgG HRP (Cell signaling, #7076, 1:2500) or anti-rabbit IgG HRP (Cell signaling, #7074, 1:2500), respectively. Protein detection was performed with the chemiluminescent SuperSignal WestPico substrate (ThermoScientific, #34578). Pictures were captured with Fusion FX camera (Vilbert Lourmat).

***In vitro* cytotoxicity assay**

Transduced OT-I T cells were collected 7 days after activation. T cells were plated with different amounts of MC38-OVA PD-L1$^+$ or MC38 tumor cells. After 24h, cells were detached with PBS supplemented with 2 mM EDTA, first stained with Viability dye eFluor 780 (eBioScience, #65-0865-14) and blocked with Trustain FcX (BioLegend, #101320), then stained with anti-CD8 BV421 (BioLegend) and rPD1-PE. Tumor cells were gated as eFluor780-negative, CD8-negative cells and percentage of killing was calculated as [(Tumor cells alone-Tumor cells with OT-I)/Tumor cells alone] x 100.

***In vivo* experiments and samples collection**

C57BL/6 female mice aged between 8 and 12 weeks were inoculated subcutaneously with 1.5 x 10^6 MC38 or MC38-OVA tumor cells as indicated. After ten days, mice were randomized according to tumor size. Mice received either one single injection of 10 x 10^6 transduced OT-I cells by retro-orbital IV injection, and/or anti-PD-L1 mAb (BioXCell, clone 10F.9G2, #BE0101) or 100 µL of PBS by intraperitoneal (*i.p.*) injection, every 3-4 days for a total of 4 injections. Mice from different treatment groups were co-housed in same cages. Tumor dimensions were measured every 2 to 3 days with a caliper until mice reached humane endpoint. Tumor volume was calculated with the formula: (Length x width x width/2). Last recorded tumor volume of dead or sacrificed mice was considered for the calculation of mean tumor volumes at subsequent time points. For the T429.18 tumor model, B10.D2 mice were inoculated subcutaneously with 1.5 x 10^6 T429.18 tumor cells. After fourteen days, mice received one single injection of 10 x 10^6 transduced TCRP1A CD8 T cells. To evaluate nanobody or antibody distribution during treatment, mice were sacrificed 7 days after starting the treatment for sample collection as indicated. Parts of tumors and spleens were embedded in TissueTek O.C.T. compound (Sakura, #94-4583) in a mold and frozen on carbonic ice. Blood was collected by heart puncture, one part was collected in IMDM with 40 U/ml heparine (Heparine Leo®) and used for flow cytometry, the rest remained at RT for 1h. Blood clot was then centrifuged (15 minutes, 1500 G) and serum supernatant was collected. For flow cytometry analysis, tumors, spleens and distant lymph nodes (contralateral from tumor bed) were smashed with a syringe plunger to obtain a cell suspension. Cell suspension of splenocytes and blood cells in heparin underwent red blood cell lysis (eBioscience, #00-4300-54) during 5 minutes at room temperature. For the systemic injection of anti-PD-L1 nanobody, MC38-OVA tumor-bearing mice received 10 µg or 50 µg of purified anti-PD-L1 nanobody intraperitoneally, 1 hour, 14 hours, or 24 hours before sacrifice. Tumors, non-tumor draining lymph nodes, spleens, and serum were collected and processed as described above.

FACS analysis

PBS supplemented with 1 mM EDTA and 1% FBS was used through all experiments. Samples were first incubated with Fixable Viability Dye eFluor 780 (eBioscience, #65-0865-14) and Trustain FcX (BioLegend, #101320). Samples were then surface stained with different antibodies obtained from Biolgend: Anti-HA.11 Epitope Tag (16B12, PE), Anti-CD45(30F11, Alexa700), Anti-CD11b (M1/70, BV711), Anti-CD11c (N418, BV421), Anti-F4/80 (BM8, BV510), Anti-Gr1 (RB6-8C5, APC), Anti-CD3e (145-2C11, FITC, BV510, AF700), Anti-CD8a (56-6.7, PE-Cy7, BV421), Anti-PD-L1 (10F.9G2, PE), Anti-PD-L1 (MIH7, PE), Anti-PD-L2 (TY25, APC), Rat IgG2bk Isotype (RTK 4530, PE), Rat IgG2ak Isotype (RTK 2758, APC), Mouse IgG1 isotype (MOPC-21, PE). For intracellular staining, cells were fixed using Fixation Buffer (Biolegend, #420801,) and permeabilized with Perm/wash Permeabilization buffer (BioLegend, #421002). Staining was performed with anti-HA Tag antibody or isotype control diluted in Permeabilization buffer. Samples were acquired with LSR Fortessa Cytometer (BD). Data were analyzed with FlowJo software.

Frozen cell pellets for IHC

MC38-OVA PD-L1^{+} or PD-L1^{KO} tumor cells were incubated with anti-PD-L1 antibody (BioLegend, clone 10F.9G2, #124318), the supernatant of 5DXW-T61V-secreting OT-I T cells, or PBS for 25 minutes at 4°C. Cells were washed twice and cell pellets were resuspended in TissueTek O.C.T. compound (Sakura, #94-4583) in a mold, then frozen on carbonic ice.

Immunohistochemistry and immunofluorescence

Frozen cell pellets and frozen samples from treated mice were cut in 6 µm slices with a cryostat (CryoStar NX70, Thermo Fisher Scientific). Sections were thawed and directly fixed for 5 min in 4% formaldehyde. All the following steps were performed at room temperature. Endogenous peroxidases were blocked with IHC/ISG Peroxidase Block (Enzo, #ACC107-0100) for 15 minutes. Protein blocking was performed for 1h with a solution of TBS-Tween containing 2% milk, 5% biotin-free BSA and 1% human immunoglobulins (Privigen®, CSL Behring). For sections to be stained with anti-rat IgG-HRP secondary, 1.25% goat serum was added to the mixture. Primary antibodies were diluted in TBS-Tween containing 1% BSA and incubated for 1h at room temperature. Rabbit anti-HA Tag antibody (Cell signaling, clone C29F4, #3724, 1:500) was used to detect the nanobody. Rabbit anti-PD-L1 antibody (R&D, MAB90781, 1:250) or rat anti-PD-L1 antibody (eBioscience, clone MIH5, #14-5982-82, 1:250) were used for detecting PD-L1. Rat anti-CD8 antibody (Biolegend, clone 53-6.7, #100701, 1:50) was used to detect CD8. These primary antibodies were followed by Dako Envision+ Polymer HRP anti-rabbit (Dako, #K4003) or ImmPRESS HRP Goat anti-rat IgG, mouse adsorbed (VectorLab, #MP-7444) secondary antibody. For the detection of anti-PD-L1 antibody clone 10F.9G2 (injected mAb), staining with ImmPRESS HRP Goat anti-rat IgG was performed directly after blocking. For cell pellets, staining was revealed with DAB (Abcam, #64238), nuclei were counterstained with hematoxylin and slides were mounted with HIGHDEF IHC mount (Enzo). Images were acquired with PANNORAMIC confocal (3DHISTECH). For mouse samples, staining was revealed with the Tyramide Signal Amplification system (TSA). Tyramide hydrochloride (Sigma, #T2879-5G) was conjugated with FITC-NHS (Sigma, #21878) or Sulfo-Cyanine3 NHS ester (Lumiprobe, #21320). Fluorochrome-coupled tyramide was diluted in a buffer containing 0.1 M boric acid, 3 M NaCl, 0.1% Tween 20 (pH 7.8), 0.003% H_2O_2 and applied directly for 10 minutes. For multiplex staining on mouse samples, the whole

procedure was performed for each staining. Finally, anti-CD146 staining was added as a one-step incubation with Alexa Fluor 647 anti-mouse CD146 antibody 10 µg/ml (Biolegend, #134718). Nuclei were couterstained with Hoescht 33342 (Invitrogen, #H3570) 10 µg/ml in TBS-Tween containing 10% BSA. Slides were mounted with HIGHDEF IHC Fluoromount (Enzo). Images were acquired with Oyster PANNORAMIC 250 Flash III (3DHISTECH).

HALO analysis of IF results

For the calculation of colocalized areas, Indica labs module Area Quantification FL v1.2 was used. Staining threshold was defined based on negative controls: samples from mice treated with PBS (for injected mAb analysis) or samples from mice treated with adoptive cell transfer (ACT) of control OT-I (for nanobody analysis). Samples stained with secondary alone (anti-rabbit or anti-rat IgG HRP) were used to set threshold for anti-PD-L1 staining using rabbit or rat anti-PD-L1 monoclonal antibody, respectively. Single stained surface areas, colocalized surface, and total surface areas were recorded for each sample and were used for the calculation of percentage of PD-L1 area covered by treatment. To calculate the correlation of anti-PD-L1 injected mAb/Nanobody versus PD-L1, Indica labs module CytoNuclear FL v2.0 was used to recognize each cell in the sample based on DAPI signal and the raw signal intensities for each staining were recorded.

Free PD-L1 calculation

MC38-OVA PD-L1^{+} tumor cells incubated or not with anti-PD-L1 antibody clone 10F.9G2 were stained with anti-PD-L1-PE (clone 10F.9G2). Average PE mean fluorescence intensity (MFI) on tumor cells not incubated with anti-PD-L1 antibody was considered as the control condition with 100% free PD-L1. Free PD-L1 on cells incubated with the antibody was then calculated as: mean fluorescence intensity (MFI) of anti-PD-L1(10F.9G2)-PE staining of cells pre-incubated with anti-PD-L1 antibody clone 10F.9G2 ('injected mAb') as a percentage of MFI from control tumor cells. For cell populations from anti-PD-L1 mAb-treated or PBS-treated mice, the calculation was based on mean fluorescence intensity (MFI) of anti-PD-L1(10F.9G2)-PE staining of cells from mAb-injected mice as a percentage of MFI from PBS-injected mice.

Statistics

Statistical analyses were performed using Prism 6 (GraphPad Software) or Python 3.7. Comparison between two groups was performed using the paired or unpaired Student t-test as indicated. Two-way analysis of variance (ANOVA) was used to compare tumor growth curves. Density plots were visualized using Seaborn 0.11.1. Pearson correlations were calculated with scipy 1.6.1. Pairwise comparisons indicating the significance of differences in boxplots were calculated using Welch's two-sided t-test. ANCOVA was used to compared linear regression slopes. P-value significance in figures is marked as: ns $p \geqslant 0.05$, * $0.01<p<0.05$, ** $0.001<p<0.01$, *** $0.0001<p<0.001$, **** $p<0.0001$, unless explicitly stated otherwise.

Data availability

The data generated in this study are available within the article and its supplementary data files. Code used for IF staining intensities analysis is available on Code Ocean.

Results

Generation of PD-L1-blocking nanobody-secreting CD8 T cells

For the targeted delivery of PD-L1 blockade at the tumor site, we sought to engineer tumor-targeting CD8 T cells for the secretion of an anti-PD-L1 nanobody. We identified two nanobodies targeting murine PD-L1 from the literature: C7 nanobody, described as an imaging tool by Broos et al.(24), and the nanobody 5DXW from the RCSB open access protein database(25). Amino acid sequences were reverse translated and DNA sequences were flanked by IL-2 signaling peptide (to ensure nanobody secretion) and HA Tag (to allow detection) coding sequences. The constructs were then inserted into a retroviral vector (Suppl. Fig S1A). The secretion of nanobodies upon vector expression was first confirmed in HEK 293T cells, as shown in Suppl. Fig. S1B. However, molecular weight of expressed nanobodies was higher than expected, related to N-linked glycosylation. Selective point mutations were introduced in both 5DXW and C7 nanobody sequences to modify the *NXT* amino acid motif and upon expression, these mutant nanobodies reached the expected 15 kDa size (Suppl. Fig. S1A). By flow cytometry, we confirmed that all secreted nanobodies bound PD-L1 (Suppl. Fig. S1C). Importantly, only 5DXW nanobody and its mutant version were able to block PD-1/PD-L1 interaction (Suppl. Fig. S1D). The non-glycosylated 5DXW-T61V nanobody was chosen for further use, as its smaller size might maximize its penetration ability. Finally, we validated that 5DXW-T61V nanobody was specific for PD-L1 and did not bind murine PD-L2 (Suppl. Fig. S1E).

We then engineered tumor-specific CD8 T cells for the secretion of the selected nanobody. In that aim, anti-ovalbumin transgenic TCR CD8 T cells (OT-I T cells) were stimulated and transduced with MSCV-5DXW-T61V plasmid, or the empty MSCV plasmid as a control (Fig. 1A). As shown in Figure 1B, MSCV-5DXW-T61V was successfully introduced into OT-I T cells with a transduction rate of around eighty percent, as detected by intracellular anti-HA Tag staining. The production and secretion of 5DXW-T61V nanobody were confirmed by western blot analysis (Fig. 1C). When cultivating transduced OT-I T cells with MC38-OVA PD-L1^{+} tumor cells, secreted 5DXW-T61V impaired the binding of recombinant PD-1 labeled with PE fluorochrome (rPD-1 PE) to tumor cells, confirming its PD-L1 blocking capacity (Fig. 1D). Secretion of 5DXW-T61V did not improve the excellent killing capacity of OT-I T cells (Suppl. Fig. S1F).

We then evaluated the ability of transduced OT-I T cells to deliver anti-PD-L1 nanobody *in vivo*. 5DXW-T61V-secreting OT-I or control OT-I T cells were adoptively transferred into MC38-OVA tumor-bearing mice and tumor samples were collected seven days later. As tumor macrophages are known to represent the major PD-L1^{+} immune cell population in the TME(26), we assessed the abundance of secreted 5DXW-T61V nanobody on their surface by flow cytometry. The whole population of tumor macrophages were stained by anti-HA Tag antibody in mice treated with 5DXW-T61V-secreting OT-I T cells (Fig. 1E). 5DXW-T61V nanobody was also found on tumor cells (Fig. 1E).

These results confirmed that adoptively transferred engineered tumor-specific T cells efficiently secreted anti-PD-L1 nanobody in the TME.

Intratumoral delivery of anti-PD-L1 nanobody outperforms anti-PD-L1 antibody in controlling tumor growth

We next evaluated the therapeutic efficacy of 5DXW-T61V-secreting OT-I T cells in the MC38-OVA model and compared it to the standard intraperitoneal (*i.p.*) injection of anti-PD-L1 antibody. Treatment was initiated ten days after tumor implantation, when tumor was well established (Fig. 2A). As shown in Fig. 2B-C, adoptive cell transfer (ACT) of 5DXW-T61V-secreting OT-I T cells strongly improved tumor control as compared to ACT of control OT-I T cells ($p<0.0001$). In contrast, the addition of anti-PD-L1 antibody *i.p.* administration to ACT of control OT-I T cells only moderately improved tumor control as compared to ACT alone ($p=0.0080$). ACT of 5DXW-T61V-secreting OT-I T cells showed better tumor inhibition as compared to the combination of ACT of control OT-I T cells plus anti-PD-L1 antibody ($p=0.023$). These results therefore demonstrate that PD-L1 blocking therapy achieved by targeted delivery of an anti-PD-L1 nanobody is superior to the standard *i.p.* injection of anti-PD-L1 antibody.

Secreted nanobody exhibits enhanced tumor penetration compared to injected antibody

We next evaluated the intratumoral delivery of anti-PD-L1 treatment by performing immunofluorescence (IF) analysis on tumor samples from MC38-OVA tumor-bearing mice treated with *i.p.* injection of anti-PD-L1 antibody or treated with ACT of 5DXW-T61V-secreting OT-I T cells. Antibody validations for IF are depicted in Suppl. Fig. S2. Anti-PD-L1 mAb ('Injected mAb') or nanobody were co-stained with PD-L1 and IF images were processed further with HALO software for quantification. As shown in Fig. 3A, secreted nanobody was found to colocalize with PD-L1 in wide areas (in yellow, right upper panel), representing on-target distribution of secreted anti-PD-L1 nanobody. In sharp contrast, tumor samples of mice treated with anti-PD-L1 antibody showed large areas (in red, left upper panel) where PD-L1 was expressed but no injected mAb was detected. In these samples, only few colocalizing areas representing on-target antibody distribution were found. This was repeatedly observed in multiple tumor samples (Fig. 3B) and was not related to differential expression of PD-L1 across samples (Fig. 3C). Increasing the dose of injected antibody did not significantly improve intratumoral antibody penetration (Suppl. Fig. S2B-D).

We also noticed that injected mAb was mostly detected in filiform structures suggestive of blood vessels. Co-staining for CD146 confirmed that these structures were delineated by endothelial cells, suggesting that injected anti-PD-L1 antibody was trapped in the vicinity of blood vessels (Fig. 3A, left bottom panels). On the contrary, in mice treated with ACT of 5DXW-T61V-secreting OT-I T cells, nanobody detection was not restricted to $CD146^+$ structures. (Fig. 3A, right bottom panels).

To further quantify the enrichment of injected mAb or secreted nanobody on PD-L1-expressing cells in the tumor, signal intensity for PD-L1 staining and either injected mAb or secreted nanobody staining on all non-endothelial cells of multiple samples were computed using HALO software. As depicted in Fig. 3D (left panel), injected mAb was detected at higher levels on cells expressing low levels of PD-L1. On the contrary, secreted nanobody was detected in a proportionate manner on PD-L1-expressing cells (right panel). Pearson correlation calculation confirmed that the distribution of secreted nanobody on PD-L1-expressing cells in the TME was correlated to PD-L1 expression whereas the distribution of injected mAb was not (Fig. 3E). Importantly, poor antibody distribution was restricted to the

tumor, as the same analysis on spleen samples from anti-PD-L1 antibody-injected mice revealed an excellent correlation between injected mAb and PD-L1 detection (Suppl. Fig. S3A-B). These analyses demonstrate that besides being able to widely diffuse inside the tumor, secreted nanobody can also accumulate on cells with higher levels of PD-L1 expression.

Interestingly, although injected mAb was mostly detected on $CD146^+$ blood vessels structures, correlation between injected mAb and PD-L1 detection remained poor on these cells (Suppl. Fig. S3C left panel and S3D). This suggests that injected mAb is not enriched in the vicinity of blood vessels due to higher level of PD-L1 expression but rather because there is a barrier preventing deeper diffusion. In contrast, secreted nanobody detection correlated with PD-L1 expression on $CD146^+$ cells as it did for all other cells in the tumor (Suppl. Fig. S3C right panel and S3D).

Finally, we assessed whether tumor penetration would impact PD-L1 blocking capacity of anti-PD-L1 injected antibody or secreted nanobody at the tumor site. As shown previously, binding of rPD-1-PE to PD-L1-expressing cells is blocked *in vitro* by anti-PD-L1 injected mAb and secreted nanobody (Fig. 1D). Recombinant PD-1-PE staining was therefore applied to single cell suspensions from tumor samples of treated mice. Since tumor infiltrating-myeloid cells seem to be crucial PD-L1-expressing cells in the TME, we quantified rPD-1-PE binding on $CD11b^+F4/80^+$ and $CD11b^+Gr1^+$ cells. To normalize rPD-1-PE signal to the level of PD-L1 expression, each sample was also stained with an anti-PD-L1 antibody (clone MIH7) whose binding ability was not hampered by the injected mAb or the nanobody (Suppl. Fig. S4A-B). As shown in Fig. 3F, and as expected, a positive correlation between PD-L1 expression and rPD-1-PE signal was observed in all groups. However, in mice treated with ACT of 5DXW-T61V-secreting OT-I T cells, the slope angle of the correlation was decreased as compared to mice treated with ACT of control OT-I T cells, indicating a blockade of PD-L1 by the secreted nanobody. In contrast, the correlation remained unchanged in tumor samples from mice that received ACT of control OT-I T cells combined with injection of anti-PD-L1 antibody. These results suggest that injected mAb was not able to block PD-L1 in the TME, likely because of its inability to penetrate the tumor, as discussed above. The same observation was made in mice that did not receive ACT and were treated with PBS or anti-PD-L1 antibody alone (Suppl. Fig. S4C).

Nanobodies are known for their excellent tissue penetration ability, thanks to their small size allowing diffusion in the TME(27). When we injected tumor-bearing mice systemically with anti-PD-L1 nanobody at the same molar quantity as the anti-PD-L1 mAb, we observed a nice penetration of the injected nanobody into the tumor tissue (Suppl. Fig. S5A). Secreted nanobody was also found binding to PD-L1-expressing cells at a distance from the secreting T cells, confirming that secreted nanobody can indeed diffuse well within the TME (Suppl. Fig. S5B and S5C).

Altogether, delivery of anti-PD-L1 nanobody using tumor-specific T cells overcame the barrier of intratumoral penetration and improved PD-L1 blocking at the tumor site as compared to injected anti-PD-L1 antibody. This presumably underlies the better therapeutic activity of our targeted delivery approach.

PD-L1 occupancy with injected antibody happens mostly in the periphery

Since we observed a low intratumoral penetration of anti-PD-L1 antibody, we next compared PD-L1 occupancy achieved by anti-PD-L1 antibody in the periphery and in the tumor. Recombinant PD-1-PE staining could not be used in that aim, since no binding of r-PD1 was found on cells from secondary lymphoid organs, possibly because of the lower level of PD-L1 expression in those organs as compared to the tumor site (Suppl. Fig. S6).

We used flow cytometry to evaluate the proportion of antibody-bound or free PD-L1 molecules upon anti-PD-L1 antibody injection by staining with the same antibody clone labeled with PE fluorochrome. *In vitro* assays confirmed that staining with anti-PD-L1 clone 10F.9G2 coupled to PE ('anti-PD-L1-PE') was prevented upon preincubation with unlabeled antibody, in a dose-dependent manner. This allowed to estimate the proportion of PD-L1 molecules free from antibody binding (Fig. 4A-B). We translated this concept *in vivo* and estimated the proportion of "free PD-L1" on different cell populations in MC38-OVA tumor-bearing mice by comparing anti-PD-L1-PE staining between mice treated with anti-PD-L1 injected mAb and mice that did not receive the antibody (Fig. 4C-D). We focused our analysis on myeloid cells from the tumor and from secondary lymphoid organs. As shown in Fig. 4C-D, the percentage of free PD-L1 on myeloid cells in secondary lymphoid organs was low, suggesting a high coverage by the injected anti-PD-L1 antibody. On the contrary, the majority of PD-L1 molecules appeared free from antibody binding at the tumor site (Fig. 4D). When evaluating the presence of PD-L1 blocking therapy in the sera of treated mice as the ability to block rPD-1 binding on PD-L1-expressing tumor cells, we found that the sera from mice treated with anti-PD-L1 antibody prevented the binding of rPD-1-PE, revealing the presence of the blocking antibody (Fig. 4E). Systemically injected anti-PD-L1 antibody exhibits therefore a detrimental distribution pattern, leading to massive PD-L1 occupancy in the periphery but not in the TME. This phenomenon might underlie the suboptimal therapeutic efficacy of anti-PD-L1 antibody as well as the triggering of irAEs.

Targeted nanobody delivery reduces systemic exposure to PD-L1 blocking therapy

We next evaluated whether targeted delivery of anti-PD-L1 nanobody would reduce systemic exposure to PD-L1 blocking therapy. In that aim, we first analyzed the distribution of transferred T cells in MC38-OVA or MC38 tumor-bearing mice treated with ACT of 5DXW-T61V-secreting OT-I T cells. By staining for intracellular HA Tag in CD8 T cells, we observed that 5DXW-T61V-secreting OT-I T cells were highly enriched in MC38-OVA tumors, but not in MC38 tumors (Fig. 5A). We next evaluated the percentage of myeloid cells bound by the nanobody in the tumor and in secondary lymphoid organs. As shown in Fig. 5B, the secreted nanobody was detected on different PD-L1^{+} immune cells at the tumor site, but not in the secondary lymphoid organs in MC38-OVA tumor-bearing mice. Furthermore, in contrast to MC38-OVA tumor-bearing mice, the secreted nanobody was barely detectable in MC38 tumors by flow cytometry (Fig. 5B), or by IF (Fig 5C). These results confirmed the antigen-specificity of T-cell-mediated nanobody delivery to the tumor site. This was not dependent on the high-affinity OVA antigen recognized by OT-1 T cells, as we also observed tumor-specific delivery of the anti-PD-L1 nanobody in mice bearing melanoma T429.18, which expresses the natural MAGE-type antigen P1A, after adoptive transfer of TCRP1A CD8 T cells transduced with the 5DXW-T61V nanobody expression construct (Fig. 5D and 5E).

Altogether, these results demonstrate that nanobody-secreting T cells migrate preferentially to the tumor and therefore allow specific intratumoral delivery of anti-PD-L1

nanobody. Similarly, when using IF to estimate nanobody distribution in the spleens of mice treated with ACT of 5DXW-T61V-secreting OT-I T cells, nanobody detection could not be differentiated from background staining, suggesting that nanobody amounts were very low in the spleen as compared to the tumor in these mice (Suppl. Fig. S7A). In sharp contrast, we observed a wide distribution of injected mAb on PD-L1^{+} cells in the spleen of antibody-treated mice (Suppl. Fig. S7B), strongly correlating with PD-L1 expression (Suppl. Fig. S3A-B) and in accordance with our flow cytometry results. Furthermore, no PD-1 blocking activity was observed in the sera of mice treated with ACT of 5DXW-T61V-secreting OT-I T cells (Fig. 4E), indicating that the secreted anti-PD-L1 nanobody was not circulating in the blood.

Besides targeted delivery, the short half-life of nanobodies in the blood may further contribute to the reduced systemic exposure to secreted nanobody. As shown in Suppl. Fig. 8, when systemically injecting purified 5DXW-T61V nanobody into mice, a wide distribution of injected nanobody was found after one hour in the tumor, lymph nodes, spleen, and serum. However, after 24 hours, the injected nanobody could no longer be detected (Suppl. Fig. S8A-D).

Collectively, these data confirmed that targeted delivery of anti-PD-L1 nanobody using tumor-specific T cells leads mostly to nanobody secretion at the tumor site. This strategy therefore reduces systemic exposure to PD-L1 blockade as compared to the systemic injection of anti-PD-L1 antibody and might minimize the risk of developing irAEs.

Discussion

The current report demonstrates that local delivery of anti-PD-L1 nanobodies using tumor-specific T cells enhances the potential of immune checkpoint blockade therapy by deeply reaching PD-L1 at the tumor site. The use of engineered T cells to deliver immunotherapeutic agents has mainly been described in the context of chimeric antigen receptor (CAR)-T cells(15-17). Secreting CAR-T cells were proven to be effective, but these studies were mostly focusing on improving CAR-T cells function and persistence in the tumor. Whether engineered T cells could be more potent than systemic treatment to deliver immune checkpoint blockade at the tumor site has not been evaluated so far. Furthermore, in these reports, CAR-T cells were engineered to secrete anti-PD-L1 antibody(15) or anti-PD-1 single-chain fragment variable (scFv)(16, 17), which might not be ideal tools for local secretion. ScFv represents the association of the variable light chain and variable heavy chain of an antibody, resulting in a molecular weight of around 30 kDa. However, it was shown that monomeric nanobodies (15 kDa) had better tumor penetration capacities than dimeric nanobodies (30 kDa)(27). We therefore proposed to secrete nanobodies to obtain a better intratumoral penetration. We chose the non-glycosylated mutant of the anti-PD-L1 blocking nanobody 5DXW, as this nanobody could be produced as a 15 kDa protein. Another advantage of nanobody secretion is their modularity: since coding sequences are short, it is possible to combine multiple VHH sequences in the same viral vector(28). Application of CAR-T technology to solid tumors is currently limited by numerous factors(29). We would favor the engineering of tumor infiltrating lymphocytes (TILs) for secreting anti-PD-L1 nanobodies. ACT of TILs has proven its efficacy in melanoma and lung cancer patients(30, 31), and viral transduction of human TILs is feasible(32, 33).

We observed a wide distribution of secreted nanobody in the tumor bed. This was likely related to the ability of the nanobody to diffuse in the TME, as good intratumoral distribution was also observed after systemic injection of the nanobody. It has been shown that the tumor penetration ability was superior for nanobodies than full antibodies(27). Whether nanobody penetration was further increased by the cytotoxic activity of secreting T cells is a question deserving further investigations.

Although the use of nanobodies raise the concern of their immunogenicity in humans, camelid and human immunoglobulin heavy chain variable genes display a high sequence identity, and nanobodies can be further humanized to reduce their immunogenicity(34, 35). From the current experience accumulated in early phase clinical trials, occurrence of anti-drug antibodies (ADA) is comparable between humanized nanobodies and humanized or fully human immunoglobulins(35). Anti-PD-L1 antibodies used in the clinic are not exempt of immunogenicity, and cancer patients treated with the humanized anti-PD-L1 antibody Atezolizumab developed ADA at high frequency, ranging from 13 to 54% of patients across trials(36). In conclusion, although experience of nanobody use in human remains scarce as compared to antibodies, immunogenicity does not appear as a major limitation for their clinical application.

Importantly, our observations shed light on the poor tumor penetration of anti-PD-L1 antibody. It was demonstrated that anti-PD-L1 antibody distributes mostly to non-tumor tissues(37). Deng et al. further suggested that the antibody could not accumulate in the tumor parenchyma and was primarily associated with vasculature at the tumor site(38). This poor tumor uptake seems particularly detrimental since PD-L1 itself is expressed at higher levels on immune cells, such as macrophages or dendritic cells, in the tumor compared to non-tumor tissue(39). Importantly, these observations have also been confirmed in humans by Bensch *et al*., who tracked radiolabeled Atezolizumab in cancer patients. They observed antibody uptake in several organs and notably target-specific binding on PD-L1 in the spleen and in lymph nodes. On a few biopsies, they also noticed a poor colocalization between radiolabeled antibody and PD-L1 expression in the tumor at the microscopic level(40). Collectively, and further suggested by this report, it seems very likely that the penetration of anti-PD-L1 antibodies in the tumors of cancer patients is therefore heterogeneous. Poor intratumoral penetration has also been observed in mice when an antibody targeting epidermal growth factor receptor (EGFR) on cancer cells. These findings have recently been confirmed in patients with head and neck cancer(41, 42). Furthermore, it was shown in mice that poor antibody penetration limited efficacy when targeting EGFR or HER2(43, 44). Antibody penetration should therefore be regarded as a key barrier to efficacy of PD-L1 blocking therapy and this deserves further evaluation in cancer patients.

Anti-PD-L1 systemic therapy is also limited by the emergence of immune-related adverse events (irAEs) that are most of the time manageable but still lead to death in a small fraction of patients. It was demonstrated in cancer patients treated with the anti-CTLA-4 antibody ipilimumab that systemic concentration of ipilimumab correlated with the rate of irAEs(45). Concerning anti-PD-1/L1 agents, a link between drug exposure and toxicity is less clear, but the rate of irAEs is globally lower than with anti-CTLA4 antibody(46, 47). Nevertheless, it is generally accepted that reducing systemic exposure to immune checkpoint blockade might decrease the risk of developing irAEs. In mouse models, intratumoral or peri-tumoral

administration of ICI antibodies was suggested as a strategy to reduce toxicity(13, 14). We demonstrated that injected anti-PD-L1 antibody was massively occupying PD-L1 in secondary lymphoid organs, which might trigger the activation of T cells directed to self-antigens. On the contrary, the use of nanobody-producing tumor-specific T cells allowed tumor-specific nanobody delivery, thanks to the accumulation of transferred T cells at the tumor site. This favorable distribution pattern was not observed with systemic nanobody injection. Furthermore, while the short half-life of nanobodies in the blood stream limits sustainable tumor exposure after systemic injection, it likely contributes to the weak systemic exposure with our strategy. Indeed, we observed no PD-L1 blocking activity in the serum of mice treated with ACT of 5DXW-T61V-secreting T cells. Our strategy therefore minimizes the risk of irAEs as compared to the injection of anti-PD-L1 antibody.

It has been described that PD-L1 was expressed by endothelial cells in the tumor and that it suppressed the activity of antigen-specific T cells in mice(48) and correlated with poor CD8 T cell infiltration in humans(49). By immunofluorescence, we also observed PD-L1 expression on endothelial cells in the tumor. Both injected anti-PD-L1 antibody and secreted nanobody were found on endothelial cells. These two strategies must therefore be able to block the potential immunosuppressive role of PD-L1 on endothelial cells in the tumor. However, whereas secreted nanobody bound PD-L1 on endothelial cells in a proportionate way, injected antibody accumulated on these cells independently of the level of PD-L1 expression, suggesting that its accumulation on these cells is related to its inability to penetrate deeper into the tumor.

In conclusion, we have shown that targeted delivery of anti-PD-L1 nanobody could improve tumor control and reduce systemic exposure to PD-L1 blockade compared to injected anti-PD-L1 antibody *in vivo*. We demonstrated that locally secreted nanobody efficiently reached PD-L1 at the tumor site and left peripheral PD-L1 untouched, while anti-PD-L1 antibody had a detrimental distribution pattern, massively occupying PD-L1 in the periphery but not in the tumor. Our report suggests that tumor penetration is a limiting factor for the efficacy of ICI antibodies and propose the use of nanobody-secreting T cells as a promising strategy to overcome this limitation.

Acknowledgements

We thank Phuong Minh Le and Loubna Boudhan for excellent technical help , Vincent Stroobant for TSA reagents production, the LAF animal facility for mice production, Professor T. Michiels and Professor M. Moser for kindly providing plasmids and cell lines, and Isabelle Grisse for editorial assistance. This work was supported by Ludwig Cancer Research, de Duve Institute (Belgium), and Université catholique de Louvain (Belgium). This work was also supported by grants from the following: Le Fonds de la Recherche Fondamentale Stratégique–WELBIO (Walloon Excellence in Life Sciences and Biotechnology), Belgium (Grant WELBIO-CR-2019C-05); Fonds pour la Recherche Scientifique (FNRS), Belgium (Grants EOS O000518F and PDR T.0091.18); Fondation contre le Cancer, Belgium (Grant 2018-090).

References

1 Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat Immunol* **2013**; 14: 1212-1218.

2 Herbst RS, Giaccone G, de Marinis F, Reinmuth N, Vergnenegre A, Barrios CH *et al*. Atezolizumab for First-Line Treatment of PD-L1-Selected Patients with NSCLC. *N Engl J Med* **2020**; 383: 1328-1339.

3 Powles T, Park SH, Voog E, Caserta C, Valderrama BP, Gurney H *et al*. Avelumab Maintenance Therapy for Advanced or Metastatic Urothelial Carcinoma. *N Engl J Med* **2020**; 383: 1218-1230.

4 Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H *et al*. Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. *N Engl J Med* **2018**; 379: 2108-2121.

5 Motzer RJ, Penkov K, Haanen J, Rini B, Albiges L, Campbell MT *et al*. Avelumab plus Axitinib versus Sunitinib for Advanced Renal-Cell Carcinoma. *N Engl J Med* **2019**; 380: 1103-1115.

6 Gutzmer R, Stroyakovskiy D, Gogas H, Robert C, Lewis K, Protsenko S *et al*. Atezolizumab, vemurafenib, and cobimetinib as first-line treatment for unresectable advanced BRAF(V600) mutation-positive melanoma (IMspire150): primary analysis of the randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* **2020**; 395: 1835-1844.

7 Jenkins RW, Barbie DA, Flaherty KT. Mechanisms of resistance to immune checkpoint inhibitors. *Br J Cancer* **2018**; 118: 9-16.

8 Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science* **2015**; 348: 69-74.

9 Thommen DS, Schumacher TN. T Cell Dysfunction in Cancer. *Cancer Cell* **2018**; 33: 547-562.

10 Pitt JM, Marabelle A, Eggermont A, Soria JC, Kroemer G, Zitvogel L. Targeting the tumor microenvironment: removing obstruction to anticancer immune responses and immunotherapy. *Ann Oncol* **2016**; 27: 1482-1492.

11 Berner F, Bomze D, Diem S, Ali OH, Fassler M, Ring S *et al*. Association of Checkpoint Inhibitor-Induced Toxic Effects With Shared Cancer and Tissue Antigens in Non-Small Cell Lung Cancer. *JAMA Oncol* **2019**; 5: 1043-1047.

12 Wang DY, Salem JE, Cohen JV, Chandra S, Menzer C, Ye F *et al*. Fatal Toxic Effects Associated With Immune Checkpoint Inhibitors: A Systematic Review and Meta-analysis. *JAMA Oncol* **2018**; 4: 1721-1728.

13 Francis DM, Manspeaker MP, Schudel A, Sestito LF, O'Melia MJ, Kissick HT *et al*. Blockade of immune checkpoints in lymph nodes through locoregional delivery augments cancer immunotherapy. *Sci Transl Med* **2020**; 12.

14 Fransen MF, van der Sluis TC, Ossendorp F, Arens R, Melief CJ. Controlled local delivery of CTLA-4 blocking antibody induces CD8+ T-cell-dependent tumor eradication and decreases risk of toxic side effects. *Clin Cancer Res* **2013**; 19: 5381-5389.

15 Suarez ER, Chang de K, Sun J, Sui J, Freeman GJ, Signoretti S *et al*. Chimeric antigen receptor T cells secreting anti-PD-L1 antibodies more effectively regress renal cell carcinoma in a humanized mouse model. *Oncotarget* **2016**; 7: 34341-34355.

16 Rafiq S, Yeku OO, Jackson HJ, Purdon TJ, van Leeuwen DG, Drakes DJ *et al*. Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo. *Nat Biotechnol* **2018**; 36: 847-856.

17 Li S, Siriwon N, Zhang X, Yang S, Jin T, He F *et al*. Enhanced Cancer Immunotherapy by Chimeric Antigen Receptor-Modified T Cells Engineered to Secrete Checkpoint Inhibitors. *Clin Cancer Res* **2017**; 23: 6982-6992.

18 Nguyen VK, Muyldermans S, Hamers R. The specific variable domain of camel heavy-chain antibodies is encoded in the germline. *J Mol Biol* **1998**; 275: 413-418.

19 Hu Y, Liu C, Muyldermans S. Nanobody-Based Delivery Systems for Diagnosis and Targeted Tumor Therapy. *Front Immunol* **2017**; 8: 1442.

20 De Vos J, Devoogdt N, Lahoutte T, Muyldermans S. Camelid single-domain antibody-fragment engineering for (pre)clinical in vivo molecular imaging applications: adjusting the bullet to its target. *Expert Opin Biol Ther* **2013**; 13: 1149-1160.

21 Diebold SS, Cotten M, Koch N, Zenke M. MHC class II presentation of endogenously expressed antigens by transfected dendritic cells. *Gene Ther* **2001**; 8: 487-493.

22 Zhu J, Powis de Tenbossche CG, Cane S, Colau D, van Baren N, Lurquin C *et al*. Resistance to cancer immunotherapy mediated by apoptosis of tumor-infiltrating lymphocytes. *Nat Commun* **2017**; 8: 1404.

23 Shanker A, Auphan-Anezin N, Chomez P, Giraudo L, Van den Eynde B, Schmitt-Verhulst AM. Thymocyte-intrinsic genetic factors influence CD8 T cell lineage commitment and affect selection of a tumor-reactive TCR. *J Immunol* **2004**; 172: 5069-5077.

24 Broos K, Keyaerts M, Lecocq Q, Renmans D, Nguyen T, Escors D *et al*. Non-invasive assessment of murine PD-L1 levels in syngeneic tumor models by nuclear imaging with nanobody tracers. *Oncotarget* **2017**; 8: 41932-41946.

25 Fedorov AA, Fedorov, E.V., Samanta, D., Almo, S.C., Ploegh, H., Ingram, J., Dougan, M. Crystal structure of mouse PD-L1 nanobody: RCSB Protein Data Bank, 2015.

26 Oh SA, Wu D-C, Cheung J, Navarro A, Xiong H, Cubas R *et al*. PD-L1 expression by dendritic cells is a key regulator of T-cell immunity in cancer. *Nature Cancer* **2020**; 1: 681-691.

27 Debie P, Lafont C, Defrise M, Hansen I, van Willigen DM, van Leeuwen FWB *et al*. Size and affinity kinetics of nanobodies influence targeting and penetration of solid tumours. *J Control Release* **2020**; 317: 34-42.

28 Xie YJ, Dougan M, Ingram JR, Pishesha N, Fang T, Momin N *et al*. Improved Antitumor Efficacy of Chimeric Antigen Receptor T Cells that Secrete Single-Domain Antibody Fragments. *Cancer Immunol Res* **2020**; 8: 518-529.

29 Wagner J, Wickman E, DeRenzo C, Gottschalk S. CAR T Cell Therapy for Solid Tumors: Bright Future or Dark Reality? *Mol Ther* **2020**; 28: 2320-2339.

30 Creelan BC, Wang C, Teer JK, Toloza EM, Yao J, Kim S *et al*. Tumor-infiltrating lymphocyte treatment for anti-PD-1-resistant metastatic lung cancer: a phase 1 trial. *Nat Med* **2021**; 27: 1410-1418.

31 Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. *Curr Opin Immunol* **2009**; 21: 233-240.

32 Zhang L, Morgan RA, Beane JD, Zheng Z, Dudley ME, Kassim SH *et al*. Tumor-infiltrating lymphocytes genetically engineered with an inducible gene encoding interleukin-12 for the immunotherapy of metastatic melanoma. *Clin Cancer Res* **2015**; 21: 2278-2288.

33 Forget MA, Tavera RJ, Haymaker C, Ramachandran R, Malu S, Zhang M *et al*. A Novel Method to Generate and Expand Clinical-Grade, Genetically Modified, Tumor-Infiltrating Lymphocytes. *Front Immunol* **2017**; 8: 908.

34 Klarenbeek A, El Mazouari K, Desmyter A, Blanchetot C, Hultberg A, de Jonge N *et al*. Camelid Ig V genes reveal significant human homology not seen in therapeutic target genes, providing for a powerful therapeutic antibody platform. *MAbs* **2015**; 7: 693-706.

35 Rossotti MA, Belanger K, Henry KA, Tanha J. Immunogenicity and humanization of single-domain antibodies. *FEBS J* **2021**.

36 Enrico D, Paci A, Chaput N, Karamouza E, Besse B. Antidrug Antibodies Against Immune Checkpoint Blockers: Impairment of Drug Efficacy or Indication of Immune Activation? *Clin Cancer Res* **2020**; 26: 787-792.

37 Kurino T, Matsuda R, Terui A, Suzuki H, Kokubo T, Uehara T *et al*. Poor outcome with anti-programmed death-ligand 1 (PD-L1) antibody due to poor pharmacokinetic properties in PD-1/PD-L1 blockade-sensitive mouse models. *J Immunother Cancer* **2020**; 8.

38 Deng R, Bumbaca D, Pastuskovas CV, Boswell CA, West D, Cowan KJ *et al*. Preclinical pharmacokinetics, pharmacodynamics, tissue distribution, and tumor penetration of anti-PD-L1 monoclonal antibody, an immune checkpoint inhibitor. *MAbs* **2016**; 8: 593-603.

39 Tang H, Liang Y, Anders RA, Taube JM, Qiu X, Mulgaonkar A *et al*. PD-L1 on host cells is essential for PD-L1 blockade-mediated tumor regression. *J Clin Invest* **2018**; 128: 580-588.

40 Bensch F, van der Veen EL, Lub-de Hooge MN, Jorritsma-Smit A, Boellaard R, Kok IC *et al*. (89)Zr-atezolizumab imaging as a non-invasive approach to assess clinical response to PD-L1 blockade in cancer. *Nat Med* **2018**; 24: 1852-1858.

41 Lu G, Fakurnejad S, Martin BA, van den Berg NS, van Keulen S, Nishio N *et al*. Predicting Therapeutic Antibody Delivery into Human Head and Neck Cancers. *Clin Cancer Res* **2020**; 26: 2582-2594.

42 Lee CM, Tannock IF. The distribution of the therapeutic monoclonal antibodies cetuximab and trastuzumab within solid tumors. *BMC Cancer* **2010**; 10: 255.

43 Shin TH, Sung ES, Kim YJ, Kim KS, Kim SH, Kim SK *et al*. Enhancement of the tumor penetration of monoclonal antibody by fusion of a neuropilin-targeting peptide improves the antitumor efficacy. *Mol Cancer Ther* **2014**; 13: 651-661.

44 Bordeau BM, Yang Y, Balthasar JP. Transient Competitive Inhibition Bypasses the Binding Site Barrier to Improve Tumor Penetration of Trastuzumab and Enhance T-DM1 Efficacy. *Cancer Res* **2021**; 81: 4145-4154.

45 Feng Y, Roy A, Masson E, Chen TT, Humphrey R, Weber JS. Exposure-response relationships of the efficacy and safety of ipilimumab in patients with advanced melanoma. *Clin Cancer Res* **2013**; 19: 3977-3986.

46 Centanni M, Moes D, Troconiz IF, Ciccolini J, van Hasselt JGC. Clinical Pharmacokinetics and Pharmacodynamics of Immune Checkpoint Inhibitors. *Clin Pharmacokinet* **2019**; 58: 835-857.

47 Wang Y, Zhou S, Yang F, Qi X, Wang X, Guan X *et al*. Treatment-Related Adverse Events of PD-1 and PD-L1 Inhibitors in Clinical Trials: A Systematic Review and Meta-analysis. *JAMA Oncol* **2019**; 5: 1008-1019.

48 Taguchi K, Onoe T, Yoshida T, Yamashita Y, Tanaka Y, Ohdan H. Tumor Endothelial Cell-Mediated Antigen-Specific T-cell Suppression via the PD-1/PD-L1 Pathway. *Mol Cancer Res* **2020**; 18: 1427-1440.

49 Liu S, Qin T, Liu Z, Wang J, Jia Y, Feng Y *et al*. anlotinib alters tumor immune microenvironment by downregulating PD-L1 expression on vascular endothelial cells. *Cell Death Dis* **2020**; 11: 309.

Figure legend:

Figure 1. Engineered OT-I T cells secrete anti-PD-L1 nanobody *in vitro* and *in vivo*

(A) Schematic of the empty control vector and the vector for HA-tagged nanobody expression. Murine Stem Cell Virus (MSCV) promoter is used to drive expression. Link refers to a short sequence coding for a "G-G-G-G-S" linker. **(B)** Representative flow cytometry plots demonstrating nanobody expression in 5DXW-T61V-secreting OT-I T cells, detected by intracellular staining for HA Tag. The data shown are representative of 3 experiments. **(C)** Western blot analysis on whole cell lysates and supernatant from transduced OT-I T cells. The data shown are representative of 3 experiments. **(D)** Secreted nanobody blocks recombinant PD-1 binding on MC38-OVA PD-L1^{+} tumor cells after 24h of co-culture with 5DXW-T61V transduced OT-I T cells. Cells co-cultured with OT-I control T cells plus anti-PD-L1 antibody 10F.9G2 was used as a positive control. The data shown are mean ± SEM, n=3 per condition. An unpaired t-test was used to determine statistical significance. ****, p<0.0001. The data shown are representative of 3 experiments. **(E**) Representative flow cytometry plots demonstrating secreted nanobody detection on tumor macrophages (upper panel) and tumor cells (lower panel). MC38-OVA tumor-bearing mice were treated with ACT of 5DXW-T61V-secreting or control OT-I T cells, and tumor samples were collected 7 days after ACT. Histograms represent surface staining with anti-HA Tag-PE or anti-PD-L1-PE antibody, gating on CD45^{+}CD11b^{+}F4/80^{+} cells (macrophages) and CD45^{-} (tumor cells) cells in the tumor.

Figure 2. ACT of anti-PD-L1 nanobody-secreting T cells improves tumor control *in vivo*

(A) Schematic of the experimental design. C57BL/6 mice were inoculated subcutaneously (SC) with 1.5 x 10^6 MC38-OVA tumor cells. After ten days, mice were randomized and treated either with anti-PD-L1 monoclonal antibody (clone 10F.9G2, 100 µg *i.p.*) alone or in combination with ACT of control OT-I T cells (10 x 10^6 cells), ACT of control OT-I T cells (10 x 10^6 cells), ACT of 5DXW-T61V-secreting OT-I T cells (10 x 10^6 cells) or PBS control (100 µL *i.p.*). Anti-PD-L1 and PBS injection were repeated for 4 doses in total. Tumor size was recorded every 2-3 days. **(B)** Average tumor growth for each treatment group. The data shown are mean ± SEM and are pooled from 2 independent experiments. Two-way ANOVA was used to determine statistical significance. ACT OT-I 5DXW-T61V vs ACT OT-I control, **** p<0.0001; ACT OT-I control + anti-PD-L1 mAb vs ACT OT-I control, ** p=0.0080; ACT OT-I 5DXW-T61V vs ACT OT-I control+ anti-PD-L1 mAb, * p=0.023; PBS vs anti-PD-L1 mAb, p=0.0001; PBS vs ACT OT-I control, p=0.0001. **(C)** Individual tumor growth curve for each treatment group. Mice with a tumor size <1300 mm^3 (Partial response,PR) or ≤ 20 mm^3 (Complete response, CR) at day 18 are highlighted as red dots.

Figure 3. Secreted nanobody exhibits enhanced tumor penetration compared to injected antibody

C57BL/6 mice were inoculated subcutaneously with MC38-OVA tumor cells. When tumors reached around 200 mm^3, mice were treated with anti-PD-L1 monoclonal antibody (clone 10F.9G2, 'injected mAb', 100 µg *i.p.* day 0 and day 4), ACT of 5DXW-T61V-secreting OT-I T cells or ACT of control OT-I T cells (10 x 10^6 cells, intravenous injection at day 0). Mice were sacrificed after 7 days, and tumors were collected for immunofluorescence (IF) analysis. **(A)** Colocalization (in yellow) of PD-L1 staining and injected mAb staining (left panel) or PD-L1 staining and nanobody staining (right panel), detected and visualized with HALO software after IF staining of tumor samples. Areas where only PD-L1 was detected appear in red, and

areas where only injected mAb (left panel) or nanobody (right panel) was detected appear in green. Bottom panels: IF images of the same tumors at higher magnification. Anti-CD146 antibody staining of endothelial cells appears in white. The data shown are representative of 6 samples per group. **(B)** Comparison of PD-L1/injected mAb and PD-L1/nanobody colocalization areas in the tumor from mice received 100 µg of injected mAb or ACT of 5DXW-T61V secreting OT-I T cells. The data shown are calculated as a percentage of co-stained surface area over total PD-L1-stained surface area for each tumor sample. PD-L1 expression was not significantly different accross treatment groups **(C)**. **(B-C)** Data shown are mean ± SEM, n=6. An unpaired t-test was used to determine statistical significance, *** p=0.0009, ns p=0.23. **(D)** Kernel density estimation plots representing the correlation between PD-L1 and injected mAb staining intensities or between PD-L1 and nanobody staining intensities on individual non-endothelial cells ($CD146^-$) for all biological replicates. 1,456,477 cells were identified from 6 tumors of mice injected with anti-PD-L1 antibody (left panel), and 1,308,210 cells were identified from 6 tumors of mice treated with ACT of OT-I 5DXW-T61V (right panel). **(E)** Boxplot representing the Pearson correlation between PD-L1 staining intensity and injected mAb or nanobody staining intensity over all non-endothelial cells from tumor samples of indicated treatment groups. Pairwise contrasts between correlations were calculated using Welch's t-test, *** p=0.00015. **(B-E)** The data shown are pooled from 2 experiments. **(F)** Evaluation of PD-L1 blocking capacity at the tumor site. Cell suspensions from tumor samples of mice treated with indicated ACT modalities were split in two, and stained either with rPD-1-PE or anti-PD-L1-PE antibody (clone MIH7). Correlation between rPD-1-PE and anti-PD-L1-PE staining intensities on $CD11b^+F4/80^+$ or $CD11b^+$ $Gr1^+$ cells based on flow cytometry analysis. Each dot represents one of the two myeloid populations from one sample. n=6-10, samples were pooled from two independent experiments. Linear regression slopes were compared with ANCOVA to determine significance. ACT OT-I control vs ACT OT-I 5DXW-T61V, * p=0.0,034; ACT OT-I control + anti-PD-L1 antibody vs ACT OT-I 5DXW-T61V, *** p = 0.0001; ACT OT-I control vs ACT OT-I control + anti-PD-L1 antibody, ns p=0,397.

Figure 4. Injected anti-PD-L1 antibody occupies PD-L1 in the periphery but not in the tumor

(A) Schematic of experimental design. **(B)** Injected mAb prevents the binding of anti-PD-L1-PE to $PD\text{-}L1^+$ tumor cells. MC38-OVA $PD\text{-}L1^+$ tumor cells were incubated with increasing concentrations of anti-PD-L1 antibody clone 10F.9G2 ('injected mAb') and then stained with anti-PD-L1 antibody clone 10F.9G2 coupled to PE (anti-PD-L1-PE). Percentage of free PD-L1 was calculated based on mean fluorescence intensity (MFI) of anti-PD-L1-PE staining of cells pre-incubated with anti-PD-L1 antibody clone 10F.9G2 (injected mAb) as a percentage of MFI from control tumor cells. The data shown are mean ± SEM, n=2. Representative of 2 experiments. **(C-D)** Occupancy of PD-L1 by injected mAb in treated mice. MC38-OVA tumor-bearing mice were treated with PBS or anti-PD-L1 antibody (clone 10F.9G2, 100 µg *i.p.* day 0 and day 4). Tumor, spleen and lymph nodes were collected after 7 days. **(C)** Flow cytometry plots representing anti-PD-L1-PE staining on $CD11b^+$ $CD11c^+$ cells in the spleen and $CD11b^+$ $CD11c^+$ $F4/80^+$ in the tumor from one anti-PD-L1 mAb-treated mouse or one PBS-treated mouse. Mouse IgG1 PE antibody was used as a staining control (upper panel). **(D)** Percentage of free PD-L1 on different myeloid populations in anti-PD-L1 mAb-treated mice. The calculation was based on mean fluorescence intensity (MFI) of anti-PD-L1-PE staining of cells from anti-PD-L1 mAb-treated mice as a percentage of MFI from PBS-injected mice. PBS-treated mice, n=10. Anti-PD-L1-injected mice, n=7-10 depending on sample type. Paired t-

test was used to determine significance. $CD11b^+$ $CD11c^+$ in spleen or lymph nodes vs $CD11b^+$ $CD11c^+$ $F4/80^+$ in the tumor, **** $p<0.0001$. $CD11b^+Gr1^+$ cells in the spleen vs in the tumor, **** $p<0.0001$. The data shown are representative of two experiments. **E)** Abundance of anti-PD-L1 mAb in the serum of treated mice. MC38-OVA tumor-bearing mice were treated with PBS, ACT of OT-I 5DXW-T61V, or injected mAb, as depicted in Fig.2A. Mice were sacrificed after 7 days and serum from each mouse was collected. B16-OVA $PD\text{-}L1^+$ tumor cells were incubated with sera from treated mice and then stained with recombinant-PD-1-PE (rPD-1-PE). rPD-1 blocking activity is calculated as the reduction of rPD-1-PE median fluorescence intensity with sera from treated mice as compared to sera from PBS-treated mice. The data shown are mean ± SEM, representative of 2 experiments. Mice treated with PBS, n=5; mice treated with anti-PD-L1 mAb, n=11; mice treated with ACT of OT-I 5DXW-T61V, n=7.

Figure 5. Specific tumor-targeted delivery of PD-L1 blocking therapy by nanobody-secreting T cells

(A-B) MC38 or MC38-OVA tumor-bearing mice were treated with ACT of 5DXW-T61V-secreting OT-I T cells. Tumor, spleen, blood, and lymph nodes samples were collected 7 days after ACT. **(A)** Percentage of nanobody-secreting T cells among total CD8 T cell population across organs, as detected by intracellular anti-HA Tag staining by flow cytometry in MC38 or MC38-OVA tumor-bearing mice treated with ACT of 5DXW-T61V-secreting OT-I T cells. **(B)** Flow cytometry analysis of the percentage of cells bound by anti-PD-L1 nanobody among different cell populations in the lymph nodes, spleen and tumor of MC38 or MC38-OVA tumor-bearing mice treated with ACT of 5DXW-T61V-secreting OT-I T cells. **(C)** Colocalization (in yellow) of PD-L1 staining and nanobody staining in MC38 tumors **(C, left panel)** or MC38-OVA tumors **(C, right panel)**. The data shown are mean ± SEM, n=4-9. **(D-E)** T429.18 tumor-bearing mice were treated with ACT of control TCRP1A CD8 T cells or 5DXW-T61V-secreting TCRP1A CD8 T cells. **(D)** Percentage of $CD45^+$ or $CD45^-$ cells bound by anti-PD-L1 nanobody in the lymph nodes, spleen, and tumor of T429.18 tumor-bearing mice treated with ACT of 5DXW-T61V-secreting or control TCRP1A CD8 T cells. The data shown are mean ± SEM, n=4-5. **(E)** Colocalization (in yellow) of PD-L1 staining and nanobody staining in T429.18 tumors was visualized with HALO software after IF processing of tumor samples. Areas where only PD-L1 was detected appear in red and areas where only nanobody was detected appear in green. Right panels: IF image of the same tumor at higher magnification. Anti-CD146 antibody staining of endothelial cells appears in white.