Supplementary material

TG6050, an oncolytic vaccinia virus encoding interleukin-12 and anti-CTLA-4 antibody, favors tumor regression via profound immune remodeling of the tumor microenvironment.

Supplementary materials and methods

Transgene sequences.

- Anti-human CTLA-4 is a full length IgG1 that has very similar activities to those of ipilimumab and for which the sequence cannot be disclosed for the moment due to intellectual properties reasons.

Human single chain IL-12 (underlined: signal peptide and GS linker; bold p40 IL-12)
MCHQQLVISWFSLVFLASPLVA**IWELKKDVYVVELDWYPDAPGEMVVLTCD**
TPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLL
LLHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDL
TFSVKSSRGSSDPQGVTCGAATLSAERVRGDNKEYEYSVECQEDSACPAAE
ESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVEVS
WEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASI
SVRAQDRYYSSSWSEWASVPCSGGGGGGSRNLPVATPDPGMFPCLHHSQNLL
RAVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEACLPLELTKNESCLNSR
ETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIF
LDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTI
DRVMSYLNAS

- Anti-murine CTLA-4 9D9 is a full length IgG2a (underlined: signal peptides)
light
chainMKLPVRLLVLMFWIPASSSDVLMTQTPLSLPVSLGDQASISCRSSQSIVHS
NGNTYLEWYLQKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEA
EDLGVYYCFQGSHVPYTFGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVC
FLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEY
ERHNSYTCEATHKTSTSPIVKSFNRNEC

heavy chain
MERHWIFLFLFSVTAGVHSEVQLQQSGPVLVKPGASVKMSCKASGYTFTDYY
MNWVKQSHGKSLEWIGVINPYNGDTSYNQKFKGKATLTVDKSSSTAYMELN
SLTSEDSAVYYCARYYGSWFAYWGQGTLITVSTAKTTAPSVYPLAPVCGDTT
GSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSS

TWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPP
KIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNS
TLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVL
PPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGS
YFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK

Murine IL-12 (underlined: signal peptide and GS linker; bold p40 mIL-12)

MCPQKLTISWFAIVLLVSPLMA**MWELEKDVYVVEVDWTPDAPGETVNLTCDTPE
EDDITWTSDQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLLLHKKE
NGIWSTEILKNFKNKTFLKCEAPNYSGRFTCSWLVQRNMDLKFNIKSSSSSPDSR
AVTCGMASLSAEKVTLDQRDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNK
YENYSTSFFIRDIIKPDPPKNLQMKPLKNSQVEVSWEYPDSWSTPHSYFSLKFFVR
IQRKKEKMKETEEGCNQKGAFLVEKTSTEVQCKGGNVCVQAQDRYYNSSCSK
WACVPCRVRS**GGGGSGGGGSGGGGSRVIPVSGPARCLSQSRNLLKTTDDMVKTAR
EKLKHYSCTAEDIDHEDITRDQTSTLKTCLPLELHKNESCLATRETSSTTRGSCLPPQK
TSLMMTLCLGSIYEDLKMYQTEFQAINAALQNHNHQQIILDKGMLVAIDELMQSLNH
NGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTINRVMGYLSSA

Virus purification.

Viruses were amplified on Chicken Embryo Fibroblast (CEF) for 72 hours. The crude harvest containing infected cells and culture supernatant, was homogenized using a homogenizing mixer equipped with an in-line chamber (Silverson). The bulk was clarified by depth filtration using 5 µm filters (Sartopure) to minimize viral particles loss. The clarified viral suspension was subsequently concentrated and diafiltered with the formulation buffer (Tris 30mM, saccharose 100g/L, pH 7.6) by using tangential flow filtration and 0.2 µm pore size hollow fibers microfiltration column (Spectrumlabs). The intermediate purified bulk was then treated by three cycles of sonication/centrifugation to remove most of the residual of transgene's products that are generated along with the virus amplification. Finally, the purified virus was resuspended in formulation buffer, aliquoted and stored at -80°C until use.

Viral titer determination on Vero cells.

Viruses were titrated on Vero cells. The samples were 10-fold serially diluted in PBS supplemented with 1% Fetal Bovine Serum (FBS) and 1% cations (0.466 mM magnesium acetate, 0.68 mM calcium chloride), added to the cells and incubated for 30 min at room temperature. Then 2 ml/well of Vero medium supplemented with 10% FBS and 1% agarose

(Sigma; A9045) were added and incubated for 72 h at 37°C with 5% CO_2. A mix of complete medium with agarose containing neutral red (1 % agarose + 0.0132 % Neutral red) was added and incubated for 3 h at 37 °C with 5 % CO_2. Plaques corresponding to lytic areas were observed and counted and the infectious titers were calculated and expressed in PFU/ml.

Viral replication assay.

Tumor cells at 3E+05 cells/well were infected in suspension 30 min at 37°C with indicated viruses diluted in PBS supplemented with 1% FBS and 1% cations at Multiplicity of Infection (MOI) 0.001 (i.e. 1 virus for 1000 cells). Cells were grown in 6-well plates in triplicate and incubated at 37°C with 5% CO_2 for 24, 48 and 72 hours. At indicated time points, plates were frozen/thawed and infected cell suspensions were sonicated and released viruses titrated on Vero cells.

Oncolytic activity assay.

Tumor cells were infected, or not (mock), in suspension 30 min at 37°C with indicated viruses at different MOI and incubated at 37°C with 5% CO_2 for 5 days.
Cell viability was determined using cell titer blue viability assay according to the protocol provided by the manufacturer (Promega; G8081). Then, fluorescence was recorded at 590 nm (560 nm for excitation) using a fluorimeter (Spark®, TECAN). Finally, oncolytic activity, expressed as cell viability, is representative of the lytic activity of the tested viral samples on tumor cells. The oncolytic activity of each sample is expressed as a percentage of the mock-infected cell viability. The half maximal effective concentration (EC50) used to compare virus potencies, was calculated using GraphPad Prism 5.

Transgenes expression in the supernatants of infected cells.

Tumor cells were infected in triplicate with TG6050 or VV CTRL at MOI 0.01. After 3 days the culture supernatants were harvested, centrifuged at 18 000 g, and then filtered on 0.1 µm

filter to remove large cell debris and VACV. Supernatants of mock-infected cells and VV CTRL-infected cells were used as negative controls. For @CTLA-4 bioassays and N-Glycosylation analysis, clarified supernatants were concentrated 20 to 25-fold using Vivaspin 20, MWCO 30000, (Sartorius; VS2021).

@CTLA-4 concentrations were determined by ELISA. Medisorp microplates were coated with 100 µl of hCTLA4-Fc (R&D systems; 7268-CT) at 0.25 µg/ml. Standard curves were generated using purified @CTLA-4 from 10 pg/ml to 10 ng/ml. Anti-kappa light chain HRP conjugated antibody (Bethyl; A80-115P) diluted 1/10 000 and 3,3′,5,5′-Tetramethylbenzidine were used for development.

IL-12 concentrations were determined using Human IL-12 p70 DuoSet® ELISA kit (R&D Systems; DY1270-05) according to provider's instructions.

CTLA-4 blocking assay.

CTLA-4 blocking activity of @CTLA-4 present in culture media of infected tumor cells was evaluated using CTLA-4 Blockade Bioassay (Promega; JA3001) and following recommendations of the providers.

ADCC assay.

This assay uses recombinant modified effector and target cells to measure quantitively the ADCC. Effector cells were Jurkat-CD16 cells (Invivogen; ktl-nfat-cd16) which encode the luciferase reporter gene under the control of a native promoter which responds to FcγRIIIa (CD16a) engagement. Target cells were Raji-hCTLA-4 (Invivogen; raji-hctla4) stably transfected to express the human CTLA-4 at their surface. Briefly, 1E+05 cells/well of Raji-CTLA-4 were mixed with @CTLA-4 or culture supernatants of infected cells. Then, 2E+05 cells/well Jurkat-CD16 cells were added, and the plates were incubated 6 h at 37 °C with 5 % CO_2. Then, 20 µl of each well were transferred in white background plate followed by 50 µl

of Quanti Luc (Invivogen; rep-qlc1). The level of luminescence was measured with a luminometer (Spark® TECAN).

IL-12 functionality assessment using HEK-Blue™ IL-12 reporter cells.

IL-12 functionality was evaluated using 5E+04 cells/well HEK-Blue™ IL-12 (Invivogen; hkb-il12) reporter cells to which culture supernatants and IL-12 standard (concentrations ranging from 3 pg/ml to 100 ng/ml) were added. After 24 h of incubation at 37 °C, the enzyme activity of secreted alkaline phosphatase in the supernatant of HEK-Blue™ IL-12 cells was measured using QUANTI-Blue™ (Invivogen; rep-qbs) according to the provider protocol.

Purification of @CTLA-4 and N-glycosylation profile analysis.

@CTLA-4 was purified by incubating 0.8 to 1 ml of 20-fold concentrated culture supernatants with 100 µl of protein A magnetic beads (Cytiva; 28-9670-56), previously equilibrated in PBS (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 154 mM NaCl; pH 7.2), at 4°C for at least 3 h. Beads were washed with PBS and @CTLA-4 eluted by incubation with 100 µl glycine 100 mM pH 2.8 and elution neutralized immediately by addition of 10 µl of Tris 2 M pH 8. Protein concentration of elutions was measured by absorbance 280 nm using Nanodrop spectrophotometer and extinction coefficient at 280 nm of 1.53 $ml.mg^{-1}.cm^{-1}$ (determined from @CTLA-4 primary sequence and using Protparam software). Elution (~100 µl) of each purified mAb were sent to the Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO) for glycosylation analysis. Briefly, the mAbs were digested with trypsin protease at LSMBO after reduction and alkylation. The generated peptides were loaded on C18 nanocolumn coupled to quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive plus, Thermo Scientific, San Jose, CA). To each mass detected was attributed a state of N-glycosylation (i.e. glycoform) based on the masses of glycoside chains. The percentage of

each glycoform was calculated as follow: 100 x (peak intensity of the corresponding glycoform) / sum of peak intensity of all glycoforms.

Animal models and treatments.

Pharmacological models.

Mice were bought from Charles River and housed (up to five animals/cage) in a Specific Pathogen Free, single exclusive room. The Specific Pathogen Free status was checked twice per year by controls conducted by the Centre de Distribution, de Typage et Archivage Animal (Orléans).

Balb/cByJ and C57BL/6NCrl female 6-week-old mice were kept in accordance with policies on animal research at the Faculté de Pharmacie of Illkirch-Graffenstaden. *In vivo* projects were approved by the regulatory authorities for animal welfare. LLC1 and EMT6 experiments were performed at Oncodesign (Dijon, France). Ten mice par group were used. All animals underwent a clinical health inspection upon arrival. They were then acclimatized for 1 to 2 weeks before the start of the experiment. The mice are randomized using an internal R script developed at Transgene S.A. This involves sorting the tumor volumes of the animals in ascending order, and then randomly selecting them to form homogeneous groups. The number of animals selected aims to achieve reliable statistical power while minimizing the number of animals per group as much as possible. Based on a log-rank test, with alpha set at 5% and a power greater than 80%, and assuming 10% of mice are expected to survive after cell implantation in the control group, a difference of 50% (with 60% of mice alive in the experimental group) can be detected with ten mice per group. Blinding was not employed for all experiments due to the absence of internally validated procedures.

Mice were injected subcutaneously with prepared tumor cells either in the right flank or in both flanks. CT26 cells were implanted at 2E+05 cells/mouse, B16F10 at 3E+05 cells/mouse,

LLC1 and EMT6 at 1E+06 cells/mouse. For rechallenge experiment, mice were implanted with CT26 or Renca at 1E+06 cells/mouse. When tumors were palpable (~7 days post-implantation), animals were randomized and treated with 1E+07 PFU/injection (or otherwise specified on figure legend) intratumorally, three times with a two-day interval. For combination with anti-PD-1 mice were administered either with mAb anti-mouse PD-1 RMP1-14 (BioXCell; BE0146) or mAb rat IgG2a isotype control (BioXCell; BE0089) 250 µg/injection IP at D7, D11, D14, D17, D21 and D24 after cell implantation. Weight and tumor size were monitored at least twice a week. Mice were euthanized when the tumor volume reached 2000 mm^3 or when animals showed weight loss >10% of their previous weight. Tumor dimensions were measured with calipers, and their volumes (mm^3) were calculated using the formula ($\pi/6$) (length x $width^2$).

Depletion experiment.

Mice were treated before virus injection by intraperitoneal (IP) administrations of depleting antibodies. In order to deplete CD4+ or CD8+ T lymphocytes, 1 or 0.2 mg/injection of anti-CD4 (clone GK1.5, BioXCell; BE0003-1) or anti-CD8 (clone 2.43, BioXCell; BP0061) antibodies were injected at D-3 and D4 respectively (D0 = tumor cell implantation). The same dose of IgG2b isotype control (clone LTF-2, BioXCell; BE0090) was administered as a control for both antibodies. For NK cells depletion, 15 µl/injection Ultra-LEAF™ Purified anti-Asialo-GM1 antibody (BioLegend®; Poly21460) and IgG isotype control (BioXCell; BE0094) at 189 µg/injection were administered IP at D-3, D0 and D4.

Pharmacokinetics of mTG6050 and IL-12.

Mice bearing B16F10 tumors were treated as previously described. At D9 (before second injection), D11 (before third injection), D14 and D18, 3 mice/group were deeply anesthetized with Ketamine/Xylazine mixture. Then, the blood was collected by intracardiac puncture, one

part was added to Heparin tube and frozen at -80°C. Samples will be used to titer the virus. The other part was kept at +4°C until blood coagulation. Serum was collected by centrifugation and stored at -20°C until ELISA dosage. Tumors were harvested, weighted, and transferred in gentleMACS C tube (Miltenyi Biotec; 130-093-237) containing 1 ml of homogenization buffer, composed of PBS, BSA 1% (Sigma; A9647), EDTA 1mM (Sigma; E7889), Tween® 80 0.27g/l (Merck; 817061) and complete™, Mini EDTA-free Protease Inhibitor Cocktail (Roche; 11836170001). The gentleMACs program m_impTumor_01 was run. One part of the tumor homogenate was transferred into an Eppendorf tube and stored at -20°C for virus titration. The other part was centrifuged for 7 min at 500 g at 4°C. The supernatant was collected and recentrifuged for 15 min at 18000 g at 4°C. The supernatant was stored at -20°C until analysis. Finally, the supernatants were thawed on ice and centrifuged for 15 min at 18,000 g at 4°C before use.

ELISpot IFNγ experiment.

Lymphocytes isolation from mice spleens:

Spleens were collected and were crushed with a syringe plunger through a 70 µm cell strainer. The splenocyte suspension obtained was recovered in X-Vivo medium (LONZA; BE04-380Q) and laid over Lympholyte-M separation cell media (Cedarlane; CL5035) and centrifuged for 20 min at 1500 g at room temperature. The interphase containing lymphocytes was collected and washed with PBS (centrifugation 5 min at 400 g at room temperature). The red blood cells (RBC) were lysed by addition of BD Pharm Lyse™ solution 1X (BD; 555899) on the lymphocyte pellet and incubated at room temperature for 5 to 10 min. After one wash step in PBS (centrifugation 5 min at 400 g at room temperature), lymphocytes were resuspended in X-Vivo medium.

Lymphocytes restimulation either with peptides or killed cells.

ELISpot plates (Millipore; MSIPS4W10) were pre-treated with ethanol 35%, washed with sterile water, coated with anti-IFNγ purified mouse antibody at 15 µg/ml in PBS (Mabtech, (AN18; 3321-3-1000) and incubated overnight at +4 °C. The next day, plates were washed with sterile PBS and saturated with X-Vivo medium at 37 °C for 1 h. Peptide solution was added at a final concentration of 1 µg/ml in X-Vivo medium or X-Vivo medium alone was added as negative control (each condition was tested in quadruplicate). Peptides used for restimulation were CT26 AH1 (SPSYVYHQF), Vaccinia virus deoxyuridine 5'-triphosphate nucleotidohydrolase F2L (SPGAAGYDL), and irrelevant HPV16 E7 (RAHYNIVTF). Concanavalin A (ConA) at 5 µg/ml was added as a positive control. Then, 1E+06 or 3E+05 lymphocytes were added into each well. In case of restimulation by killed cells, CT26 cells were resuspended in X-Vivo medium containing mitomycin C (Sigma; MO503) at 25 µg/ml. Cells were then incubated 1 h at 37°C and washed 5 times in PBS. Finally, cells were resuspended in X-Vivo medium to obtain a suspension at 4E+05 cells/ml. Fifty µl of these cell suspensions were added by well instead of peptide. Plates were incubated at 37°C in 5 % CO2 for 20 h. After incubation, the cells were removed from the ELISpot plates. Plates were washed 5 times with PBS (200 µl/well) and incubated with 100 µl/well of biotinylated anti-mouse IFNγ mAb (Mabtech; R4-6A2; 3321-6-1000) at 1 µg/ml in PBS, 0.5 % FBS. Plates were incubated for 2 h at room temperature in the dark and then washed five times with PBS. Extravidin-Phosphatase alkaline (Sigma; E236, 1/5000 dilution in PBS, 0.5 % FBS) was added then phosphatase activity was measured by colorimetric detection by adding 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution (Sigma; B5655, 0.45 µm filtered). Plates were incubated at room temperature in the dark until distinct colored spots appeared in positive wells. Spots were counted with an ELISpot reader (CTL Immunospot reader, S5UV). Results were expressed as the mean number of spots forming units (SFU) per 1E+06 splenic lymphocytes. Each data corresponds to the mean of

quadruplicate subtracted by the mean of the SFU obtained without restimulation (i.e. medium alone).

Analysis on tumor infiltration by flow cytometry.

Tumors were dissociated to single cell suspension by using Tumor Dissociation Kit (mouse, Miltenyi Biotec; 130-096-730). Tumor dissociation was performed according to manufacturer's protocol. Briefly tumors were transferred into gentleMACS C tube (Miltenyi Biotec; 130-093-237) containing 2.35 ml medium (DMEM (ATCC®; 30-2002) for B16F10 tumor; RPMI (Sigma; R0883) for CT26 tumor) 10% FBS (Corning®; 35-070-CV) and 40µg/ml gentamycin (Sigma; G1272). Tissues were cut into small pieces. An enzyme mix of 100 µl of enzyme D, 10 µl of enzyme R and 12.5 µl of enzyme A was added to the tumor. Tubes were attached onto the gentleMACS dissociator. The gentleMACs program m_impTumor_02 was run. Tubes were detached and incubated for 40 min at 37°C. Tubes were attached for the second round onto the gentleMACS dissociator and the gentleMACs program m_impTumor_03 was run twice. Samples were detached from the gentleMACS dissociator and filtered using a 70 µm strainer (Miltenyi Biotec; 130-098-462). Filtered suspensions were washed with 10 ml corresponding medium, centrifuged at 300 g for 10 min. RBC were lysed with BD Pharm Lyse™ solution 1X (BD; 555899). Cell suspensions were washed with 10 ml Dulbecco's Phosphate Buffered Saline (DPBS, Sigma; D8537) and finally resuspended in DPBS. A cell sample was counted using the Vi Cell counter. Cells were transferred into 96-well plate for staining.

Immunophenotyping:

Cells were first incubated with mouse Fc Block (CD16/CD32 mAb, eBioscience™, Invitrogen; 14-0161-85) for 5 to 10 min at 4 °C and in a second time stained for viability with Live dead aqua (Invitrogen; L34957) for 15 min at 4 °C. Cells were washed with DPBS and

labeled with labeled-conjugated antibodies for 20 min at 4 °C. Three panels of antibodies were used:

1- Myeloid panel: CD45 anti-mouse, VioBlue (clone REA737, Miltenyi Biotec; 130-110-802), F4/80 anti-mouse, FITC (clone REA126, Miltenyi Biotec; 130-117-509), CD163 anti-mouse, PE (clone S15049I, BioLegend®; 155307), CD11b anti-mouse, PE-Vio 615 (clone REA592, Miltenyi Biotec; 130-113-807), CD3 anti-mouse, PerCP-Vio 700 (clone REA641, Miltenyi Biotec; 130-120-826), Ly-6C anti-mouse, PE-Vio 770 (clone REA796, Miltenyi Biotec; 130-111-918), PD-L1 anti-mouse, APC (clone 10F.9G2, BioLegend®; 124311) and CD38 anti-mouse, APC-Vio 700 (clone REA616, Miltenyi Biotec; 130-125-227).

2- T cell panel: CD45 anti-mouse, VioBlue (clone REA737, Miltenyi Biotec; 130-110-802), NK-1.1 anti-mouse, BV605 (clone PK136, BD Horizon™; 563220), CD127 anti-mouse, Vio Bright FITC (clone A7R 34, Miltenyi Biotec; 130-116-502), TIM-3 anti-mouse, PE (clone REA602, Miltenyi Biotec; 130-118-691), KLRG1 anti-mouse, PE-Vio 615 (clone REA1016, Miltenyi Biotec; 130-120-567), CD8a anti-mouse, PerCP-Vio 700 (clone 53-6.7, Miltenyi Biotec; 130-120-825), CD3 anti-mouse PE-Vio 770 (clone REA641, Miltenyi Biotec; 130-116-494), PD-1 (CD279) anti-mouse, APC (clone REA802, Miltenyi Biotec; 130-111-954) and CD4 anti-mouse, APC-Vio 770 (clone REA604, Miltenyi Biotec; 130-119-132).

3- Treg panel: LAG-3 (CD223) anti-mouse, Brilliant Violet 650 (clone C9B7W, BioLegend®; 125227), CD19 anti-mouse, Vio Bright 515 (clone REA749, Miltenyi Biotec; 130-112-040), FOXP3 anti-mouse/rat, PE (clone FJK-16s, eBioscience™; 72-5775-40), CD3 anti-mouse, PE/Dazzle 594 (clone 17A2, BioLegend®; 100245), CD45 anti-mouse, PerCP/Cyanine5.5 (clone I3/2.3, BioLegend®; 147705), CD25 anti-mouse, PE-Vio 770 (clone REA568, Miltenyi Biotec; 130-123-893), PD-1

(CD279) anti-mouse, APC (clone REA802, Miltenyi Biotec; 130-111-954) and CD4 anti-mouse, APC-Vio 770 (clone REA604, Miltenyi Biotec; 130-119-132).

Cells were washed twice with DPBS and resuspended in 100µl MACSQuant running buffer (Miltenyi Biotec, 130-092-747) for acquisition or fixed and permeabilized (anti-mouse/rat FOXP3 staining set, eBioscience™; 72-5775-40) for 30 min at 4°C before being stained with intracellular antibodies (Treg panel : mouse anti-TCF-7/TCF-1, BV421 (clone S33-966, BD Horizon™; 566692) and FOXP3 anti-mouse/rat, PE (clone FJK-16s, eBioscience™; 72-5775-40)) for 30 min at 4°C.

Data acquisition and analysis: Samples were acquired on a MACSQuant® Analyzer 16 Flow Cytometer (Miltenyi Biotec). Analyses were performed using Kaluza software.

3' RNA sequencing.

CT26 model: at D0, mice were implanted with CT26 cells and treated IT with vehicle, 1E+07 PFU VV CTRL or mTG6050 at D7, D9 and D11. Ten tumors per time point were harvested at D11 before third treatment, D15 and D17 and then stored at -80°C until processing. B16F10 model: same mode of operation was applied except that tumors were harvested at D11 before third treatment and D14. Tumors were weighted, cut into small pieces and total RNA were extracted using Qiagen RNeasy Plus Mini Kit (Qiagen; 74134). The quality of the samples was measured using Agilent RNA 6000 Nano Kit (Agilent; 5067-1511), and Agilent 2100 Bioanalyzer System, 2100 Expert Software. Twenty µL of 50 ng/µl of purified RNA (prepared by dilution in RNase Free water (Qiagen)) were dispatched in 96-wells plate and sent to IntegraGen SA (Evry, France) at -80°C for 3' RNA sequencing. Raw paired reads were first processed using a bioinformatic pipeline developed using the program Snakemake [1]. Briefly, 3 extra G added during library preparation were removed from read 2 using trimmomatic [2]. Unique Molecular Identifiers (UMIs) were then extracted from read 1 and

appended to the name of the paired read 2 (extract function from the UMI-tools package) [3]. Read 2 from each pair were then mapped with STAR [4] against a custom genome containing *Mus musculus* full genome (GRCm39 assembly) plus mTG6050 genome as an artificial chromosome. PCR duplicates were deduplicated with the program umi_tools dedup, using the method “directional” and readcount per cellular gene was then determined with the program htseq-count, mode “union” (union) [5]. Readcount data per sample was then normalized using DESeq2 [6] and a gene was considered differentially expressed if the fold change between two conditions was above 2, and the adjusted p-value below 0.1 (Benjamini-Hochberg correction for multiple testing). Gene Set Variation Analyses (GSVA) were performed with the R package GSVA [7] on the normalized readcount matrix, using the set of markers specific of immune pathways as reported [8].

Single cell RNA sequencing.

Balb/C mice bearing CT26 tumor were treated IT with vehicle or 1E+07 PFU mTG6050 at D7 and D9. Tumors were harvested at D11, pooled and transferred in GentleMACS C tubes containing 2.35 ml of RPMI-1640 (Sigma; R0883) media. First, tumors were dissociated mechanically using sterile scissors, then enzymatically using Tumor Dissociation Kit (mouse, Miltenyi Biotec; 130-096-730) in accordance with the provider’s protocol. Single cell suspensions viability was assessed using Vi Cell. Samples with cells viability >= 70 % and with an optimal concentration of 600-1000 cells/µl were processed.

Cells were isolated on a 10X Genomics Chromium Instrument, and barcoded scRNA-seq libraries were prepared and sequenced by the GenomeEast plateform at the IGBMC. Raw sequencing data was processed with 10X Genomics Cell Ranger 7.1.0 [9] using the same reference genome as for 3’ RNA sequencing. Quality control and basic data processing steps such as data normalization, integration, scaling, dimension reduction and clustering were performed using the R package Seurat [10]. Cell populations were manually assigned to

clusters using cell type markers. Signatures of M1 and M2 marker genes were analyzed in single cells with the R package AUCell [11], using the gene sets Stat1, Socs1 and Nos2 for M1 markers and Stat6, Socs2 and Arg1 for M2 markers selected as mice markers reported in both of the most polarized M1 or M2 macrophages reported in mice for the categories Fig. 1B in Murray et al. 2014 [12].

Toxicology study.

Toxicity of TG6050 was determined in cynomolgus monkeys under Good Laboratory Practices conditions. The cynomolgus monkey was selected as a relevant toxicology species due to a similar to human adaptive immune system. In addition, human IL-12 expressed by TG6050 is active in cynomolgus monkeys. Also, @CTLA-4 binds to non-human primate CTLA-4 and has pharmacologic activity only in primates.

TG6050 (Good Manufacturing Production batch) was administered IV to cynomolgus monkeys (3 males and 3 females per group) at 1E+07 and 5E+07 PFU/kg on days 1, 8, 15 and 22. These doses corresponded respectively to 6E+08 PFU and 3E+09 PFU human doses when scaled on 60 kg body weight. Control group (2 males and 2 females) received the formulation buffer. After the administration period, the animals were under observation for one week until the day of sacrifice to check the reversibility or progression of any treatment-related changes or delayed toxicity. The following endpoints/parameters were evaluated: clinical observations, body weight, body temperature, hematology, blood chemistry, coagulation, urine analysis, cytokine evolution, gross pathology, and histopathology. Cytokines (IL-12 and IFNγ) were analyzed via Luminex Platform (IL12p70IFNgCYPLVA) at regular timepoints over 29 days using a validated method with respective limits of quantification of 12.21 pg/ml (IL-12), and 2.44 pg/ml (IFNγ).

References

[1] Köster J, Rahmann S. Snakemake--a scalable bioinformatics workflow engine. Bioinformatics (Oxford, England) 2012;28:2520–2522.
[2] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics (Oxford, England) 2014;30:2114–2120.
[3] Smith T, Heger A, Sudbery I. UMI-tools: Modelling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy, 2016.
[4] Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. Bioinformatics (Oxford, England) 2013;29:15–21.
[5] Anders S, Pyl PT, Huber W. HTSeq - A Python framework to work with high-throughput sequencing data, 2014.
[6] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.
[7] Hänzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. BMC Bioinformatics 2013;14:7.
[8] Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell 2015;160:48–61.
[9] Zheng GXY, Terry JM, Belgrader P, Ryvkin P, Bent ZW, Wilson R, Ziraldo SB, Wheeler TD, McDermott GP, Zhu J, Gregory MT, Shuga J, Montesclaros L, Underwood JG, Masquelier DA, Nishimura SY, Schnall-Levin M, Wyatt PW, Hindson CM, Bharadwaj R, Wong A, Ness KD, Beppu LW, Deeg HJ, McFarland C, Loeb KR, Valente WJ, Ericson NG, Stevens EA, Radich JP, Mikkelsen TS, Hindson BJ, Bielas JH. Massively parallel digital transcriptional profiling of single cells. Nat Commun 2017;8:14049.
[10] Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, Hao Y, Stoeckius M, Smibert P, Satija R. Comprehensive Integration of Single-Cell Data. Cell 2019;177:1888-1902.e21.
[11] Sara Aibar, Stein Aerts. Laboratory of Computational Biology.VIB-KU Leuven Center for Brain & Disease Research. Leuven, Belgium. AUCell, Bioconductor, 2017.
[12] Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege J-L, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 2014;41:14–20.