A new generation of DNA-based immunotherapy induces a potent immune response and increases the survival in different tumor models

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Supplementary methods

Cell lines

B16F10-OVA cells, which stably expresses OVA, were cultured in MEM supplemented with GlutaMAX, while GL261 were cultured in DMEM. Both media were supplemented with 10 % FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin (Gibco, Life Technologies, USA). Cells were subcultured in 75 cm^2 culture flasks (Corning® T-75, Sigma-Aldrich, USA) and incubated at 37°C and 5% CO_2. B16F10-OVA cells were obtained from Professor Johan Grooten (University of Ghent, Belgium). GL261 were provided by Professor Sophie Lucas (de Duve Institute, University of Louvain, Belgium).

Animals

Six-week-old C57BL/6NRj female mice were obtained from Janvier Labs (France) and housed in an air conditioned animal facility with ad libitum access to food and water. Temperature and humidity were monitored daily. For subcutaneous tumor implantation and electroporation, the mice were anaesthetized with a 150 to 200 µL intraperitoneal injection of 10 mg/mL ketamine and 1 mg/mL xylazine. For cranial surgeries, the ketamine/xylazine dose was 100 mg/kg and 13 mg/kg, respectively. Animals were sacrificed when the tumor volume was greater than 1500 mm^3 (subcutaneous models) or when they reached the end points (appearance or behaviour changes and clinical signs of distress: lack of grooming, paralysis, arched back, lack of movement plus 10% body weight loss and/or 20% body weight loss). Transgenic OT-I and OT-II mice were obtained from the University of Ghent (Belgium).

Subcutaneous tumor implantation and tumor measurement

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At day 0, a total of 1×10^5 B16F10-OVA cells diluted in 100 µL PBS were injected subcutaneously into the right flanks of C57BL/6 mice. A total of 2 x 10^6 GL261 cells diluted in 100 µl of PBS were injected subcutaneously into the right flank of C57Bl/6 mice. The tumor cells were inoculated before the plasmid treatment. Tumor size (for the subcutaneous models) was measured three times a week with an electronic digital calliper. Tumor volume was calculated as the length × width × height (in mm^3).

RT-PCR analysis

GL261 cells were analyzed by RT-PCR to verify the presence of TRP2 and gp100 antigens. Total RNA was isolated using TRIzol reagent (Thermo Fisher, Waltham, Massachusetts) and phenol separation. The quality and quantity of RNA were evaluated using a nanospectrophotometer (NanoDrop 2000, Thermo Fisher, Waltham, Massachusetts). One microgram of RNA was reverse transcribed using a first-strand synthesis system (SuperScriptTM, Thermo Fisher, Waltham, Massachusetts) and oligo(dT) primers according to the supplier's protocol. The resulting cDNA was used as template for 30 cycles of PCR amplification. The PCR products were subjected to electrophoresis on a SYBR Safe (Thermo Fisher Scientific) -stained 1.5% agarose gel.

Magnetic Resonance Imaging

MRI was performed was performed for all mice included in the study before the surgical resection of the tumor. MRI was performed using a 11.7 T Bruker Biospec MRI system (Bruker, Germany) equipped with a 1 H quadrature transmit/receive surface cryoprobe after anesthetizing animals with isoflurane mixed with air (2.5% for induction, 1% for maintenance). Tumor was visualized using rapid acquisition with relaxation enhancement (RARE) sequence (repetition time = 2500 ms; effective echo time = 30 ms; RARE factor = 8; field of view = 2 x 2 cm; matrix 256 x 256; Slice thickness = 0.3 mm; 25 contiguous slices were acquired, N average = 4).

Surgical resection of the GL261 tumor mass

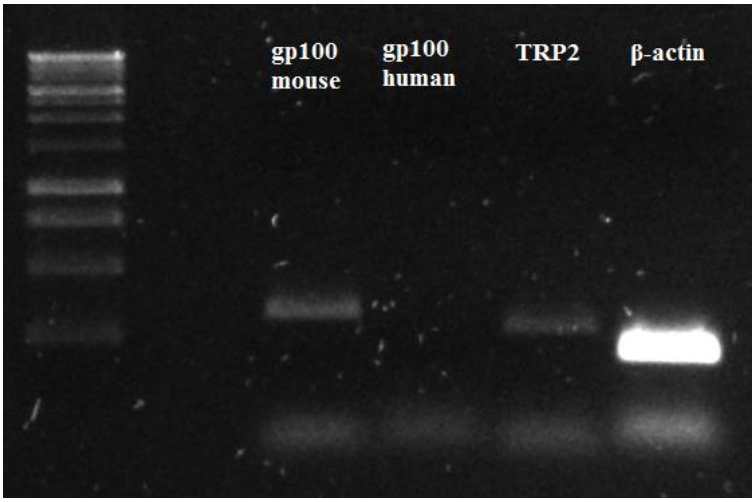
At day 17 post-tumor inoculation, the tumor mass was surgically removed by using the biopsy-punch resection technique. Briefly, animals were anaesthetized with ketamine/xylazine and immobilized in a stereotactic frame. An 8 mm incision was made in the midline along the previous surgical scar and a 2.1 mm diameter circular cranial window was created around the previous burr hole using fine tip tweezers (Dumont, Switzerland) to expose the brain. A 2 mm diameter biopsy punch (Kai Medical, Germany) was then inserted 3 mm deep and twisted for 15 s to cut the brain region surrounding the tumor. Once withdrawn, the tumor and brain tissues were aspired using a diaphgram vacuum pump (Vaccubrand GBMH+CO KG, Germany) connected to a Pasteur pipette and a 200 µl tip. Residual blood was removed from the surgical cavity using a haemostatic triangle (Fine Science Tools, Germany). The cranial window was then sealed with a 4 x 4 mm square piece of Neuro-Patch® (Aesculap, Germany) impregnated with a reconstituted fibrin hydrogel (25 mg/mL fibrin, 10 IU/mL thrombin, equal volumes; Baxter Innovations, Austria). [1]

Hematoxylin and eosin staining

At the first signs of pain and discomfort, tumor-bearing mice were sacrificed, and the brain was removed, fixed in 10% formalin solution (Merck, Germany) and embedded in paraffin days. 28 Then, it was sectioned (10 µm) using a MICROM 17M325 microtome (Thermo Fischer Scientific, USA) and collected on super-frost plus glass slide. For the histological analysis, the slides were stained with hematoxylin and eosin (H/E). Samples were processed using a Sakura DRS 601 automated slide stainer (Sakura Finetek Europe, The Netherlands).

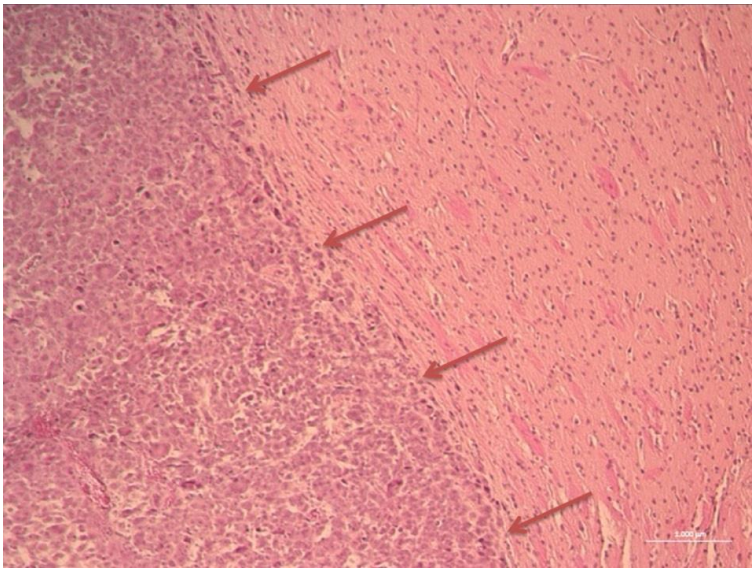
Supplementary figures and tables

Supplementary Figure 1

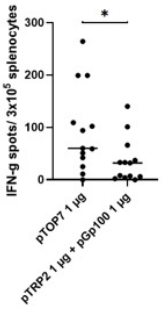


Supplementary Figure 1 – RT-PCR performed to verify the expression of murine gp100 and TRP2 antigens by the GL261 cells. Human gp100 and b-actin served as a negative control and as a housekeeping gene (positive control), respectively.

Supplementary Figure 2



Supplementary Figure 2 – Representative H/E image of the brain/tumor of an untreated (naïve) mouse sacrificed due to signs of discomfort and pain. The red arrows indicate the infiltrative margins between the tumor (left) and the brain (right).



Supplementary Figure3. Comparison of pTOP and conventional plasmids. The production of IFN by splenocytes stimulated by the TRP2 peptide was assessed by ELISpot. Mice were immunized by 1 g of pTOP7 or pTRP2 and pGp100 (pVAX plasmids encoding TRP2 or Gp100) at day 0, 7 and 14 and sacrificed at day 21.

Supplementary table 1

Supplementary table 1 – List of primers used for the study.

Gene	Primer sequence (5'→ 3')	Amplicon length
IL-6	For – CCGGAGAGGAGACTTCACAG Rev – TCCACGATTTCCCAGAGAAC	102 bp
IL-12	For – GGAAGCACGGCAGCAGAATA Rev – AACTTGAGGGAGAAGTAGGAATGG	180 bp
CCL2	For - GATGCAGTTAACGCCCCACT Rev - CCCATTCCTTCTTGGGGTCA	172 bp
TRP2	For – CCAGGATGACCGTGAGCAA Rev – GGGCAGTCAGGGAATGGAT	171 bp
Murine-gp100	For – GGAGCTTCCTTCCCGTGCTT Rev – GCTCCCATTGATGATGGTGT	321 bp
Human-gp100	For – ATAGGTGCTTTGCTGGCTGT Rev – ACCTGCCCATCTGGCAATAC	263 bp
b-actin	For – ACTCCTATGTGGGTGACGAG Rev – CATCTTTTCACGGTTGGCCTTAG	206 bp

References

1. Bianco, J, Bastiancich, C, Joudiou, N, Gallez, B, des Rieux, A, and Danhier, F (2017). Novel model of orthotopic U-87 MG glioblastoma resection in athymic nude mice. *Journal of Neuroscience Methods* **284**: 96-102.