

Article

Low-intensity Pulsed Ultrasound-mediated Blood-brain Barrier Opening Increases Anti-programmed Death-ligand 1 Delivery and Efficacy in GL261 Mouse Model

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Abstract: Therapeutic antibodies targeting immune checkpoints have shown limited efficacy in clinical trials in glioblastoma (GBM) patients. Ultrasound-mediated blood-brain barrier opening (UMBO) using low-intensity pulsed ultrasound improved drug delivery to the brain. We explored the safety and the efficacy of UMBO plus immune checkpoint inhibitors in preclinical models of GBM. A Blood-brain barrier (BBB) opening was performed using a 1 MHz preclinical ultrasound system in combination with 10 μ l/g microbubbles. Brain penetration of immune checkpoint inhibitors was determined, and immune cell populations were evaluated using flow cytometry. The impact of repeated treatments on survival was determined. In syngeneic GL261-bearing immunocompetent mice, we showed that UMBO safely and repeatedly open the BBB. BBB opening was confirmed visually and microscopically using Evans's blue dye and magnetic resonance imaging. UMBO plus anti-PDL-1 was associated with a significant improvement of the overall survival compared to anti-PD-L1 alone. Using mass spectroscopy, we showed that the penetration of therapeutic antibodies can be increased when delivered intravenously compared to non-sonicated brains. Furthermore, we observed an enhancement of activated microglia percentage when combined with anti-PD-L1. Here, we report that the combination of UMBO and anti-PD-L1 dramatically increases GL261-bearing mice's survival compared to their counterparts treated with anti-PD-L1 alone. Our study highlights the BBB as a limitation to overcome to increase the efficacy of anti-PD-L1 in GBM and supports clinical trials combining UMBO and in GBM patients.

Keywords: SonoCloud; GL261 mouse model; ultrasound-mediated drug delivery; glioblastoma; immune checkpoint inhibitors

Introduction

GBM is the most malignant primary brain tumor in adults, with a median overall survival of less than 18 months after initial diagnosis¹. Despite significant efforts in the neuro-oncology field to develop new therapeutic alternatives, temozolomide (approved in 2005) remains the gold standard chemotherapy in GBM treatment². For over five decades, research has been focused on developing new anti-cancer therapies for GBM,

including anti-neoplastic agents³, molecular targeted drugs⁴, immunotherapeutic approaches⁵, and angiogenesis inhibiting compounds⁶; however, the prognosis of patients has hardly improved⁷. The existence of the blood-brain barrier (BBB), which is specific to the blood vessels in the central nervous system (CNS), prevents most systemic therapeutic compounds from reaching the brain parenchyma and GBM cells⁸ although it is disrupted in some areas (*i.e.*, blood-tumor barrier).

Several innovative strategies have been studied to enhance the delivery of chemotherapeutic agents and antibodies to the brain⁸. UMBO using low-intensity pulsed ultrasound (LIPU) has now been studied in preclinical⁹ and clinical settings¹⁰. LIPU is delivered to the brain simultaneously with an I.V. of micron-sized bubbles for a few minutes, allowing the microbubbles to oscillate. Microbubble oscillation produces a mechanical stretching on vessel walls that allows a transient BBB opening¹¹. UMBO has shown a good safety profile for BBB opening in recurrent GBM patients^{10,12} and is now being studied in dozens of clinical trials using a range of transcranial¹³ or implantable ultrasound devices¹⁴ for treating both primary and secondary brain tumors as well as neurodegenerative diseases¹⁵.

The choice of therapeutic agents to deliver after UMBO is crucial and remains a point of discussion among researchers. Direct stimulation of the immune system with immune checkpoint inhibitors (ICI, *e.g.*, PD-1/PD-L1) showed promising effects alone or with other chemotherapies in multiple cancers. PD-L1 proteins are expressed as surface molecules by cancerous cells such as GBM cells¹⁶ and provide a tumor escape mechanism when bound to PD-1 proteins at the surface of activated T-lymphocytes leading to their exhaustion¹⁷. Despite their promise in other cancers, nivolumab (anti-PD-1) has shown no additional efficacy over bevacizumab in phase III clinical trials in recurrent GBM patients¹⁸. Similarly, Avelumab (anti-PD-L1) in combination with molecular targeted drugs did not improve the outcome of GBM patients¹⁹. In the present study, we evaluated the effect of anti-PD-L1 and anti-CTLA-4 alone and in combination with UMBO in syngeneic GL261 mouse models.

Materials and Methods

Low-intensity pulsed ultrasound preclinical device

The pre-clinical ultrasound system (CarThera, Paris, France) was identical to that described in other studies (Supplementary Figure 1-A)⁹. The system consisted of a 1 MHz, 10-mm diameter acoustic transducer that was coupled to the head of the mouse at 15-mm from the transducer. Sonication was performed for 120 seconds using a 25,000-cycle burst at a 1 Hz pulse repetition frequency and an acoustic pressure of 0.3 MPa as measured in water.

Calibration of low-intensity pulsed ultrasound device:

The ultrasound transducer (CarThera®) used in this study was calibrated in regular basis (Figure 1-A, B). The aim was to map the ultrasound field and to determine the electrical set point that the generator uses during the experiments to obtain the targeted acoustic pressure in situ. The calibration was performed using degassed water at room temperature with a 200 μ m needle hydrophone (HNC0200, ONDA). A 2D acoustic field was scanned at 5 mm from the transducer surface with a 3-axis computer-controlled motorized positioning system (UMS, Precision Acoustics, U.K.). A transducer velocity pattern of 1.05 MHz was then obtained by acoustic holography. The 3D acoustic field was computed from this pattern with the Rayleigh integral. The hydrophone was positioned at the spatial peak pressure determined from the 3D acoustic field. The ratio between the active electrical power drawn by the transducer measured with an oscilloscope and the square of the spatial peak acoustic pressure was measured. This ratio is used as a calibration coefficient by the generator during subsequent experiments: the active electrical power needed to obtain the targeted pressure is calculated by the generator using this coefficient at the

beginning of manipulation, and the generator adjusts its set point to obtain the specified active electrical power measured internally.

Figure 1

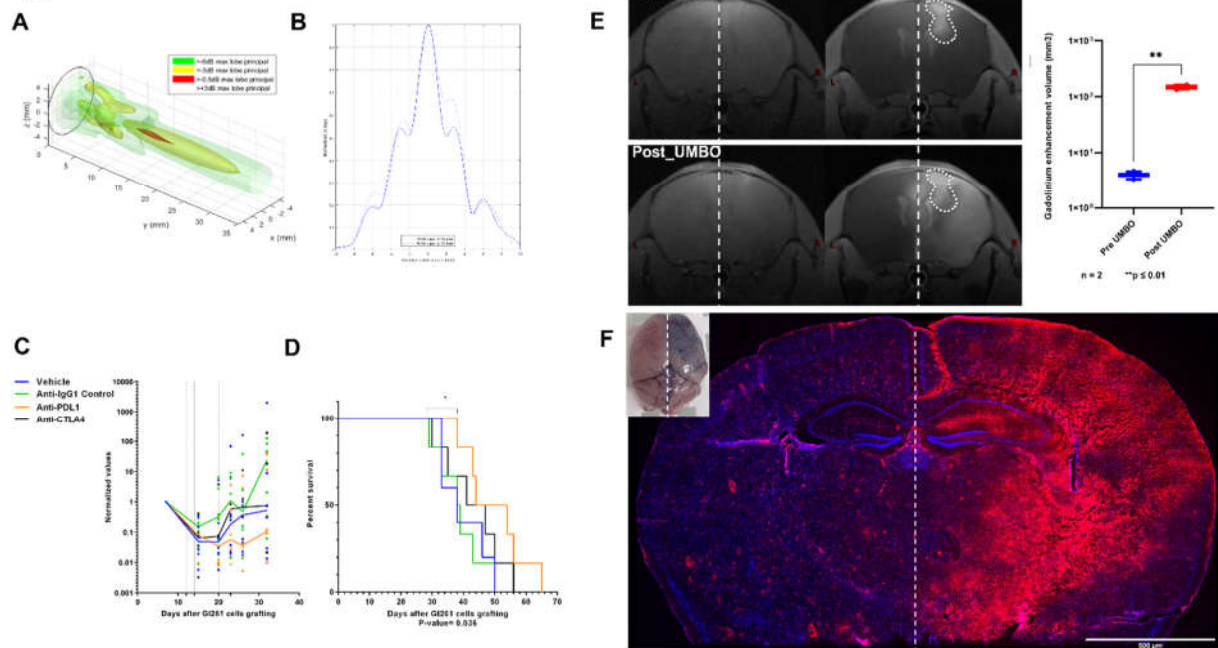


Figure 1. UMBO parameters are safe and anti-PD-L1 increased survival of GL261. Panel A-B: 3D structure of the ultrasound waves generated by the calibrated low-intensity ultrasound system. Panel C: Animals were treated with anti-PDL-1, IgG1, anti-CTL-4, and vehicle for four doses. bioluminescence measures normalized to the first measured value performed on day 7 after cell inoculation in GL261-bearing mice model. Each dot represents values for one animal and the line represents the median value for the group. Bioluminescence signal was measured weekly. Dotted lines represent the days of treatments. Panel D: Kaplan Meier curves in GL261-bearing mice. Panel F: Evans blue staining was enhanced in sonicated brain hemisphere compared to the control hemisphere visually and by fluorescence (Panel F; Evans blue in red, DAPI in blue). Panel E: T1W MRI showed a marked Gd contrast enhancement within an hour following UMBO. A significant enhancement of Gd following UMBO in mice (n=2, **p ≤ 0.01). The lower two T1-MRI images were obtained after UMBO (pre- Gd left MRIs; and post-Gd UMBO is safe and effective in C57BL/6 mice. right MRIs).

Ultrasound-mediated blood-brain barrier opening (UMBO)

UMBO was delivered to both UMBO and UMBO plus anti-PD-L1 groups. Anti-PD-L1 (6E11 Genentech) was administered intraperitoneally in two independent experiments (n=8) at a dose of 200 µg sixty minutes before UMBO application. Mice were maintained under anesthesia with isoflurane (2%, 2L/min O₂). For each UMBO application, 10 ml/kg SonoVue® was injected through the intravenous route less than 10 seconds before the start of the ultrasound application. For each session, UMBO was validated using an additional control mouse. Each control mouse was injected intravenously with a solution of 2.7% Evans blue (Sigma, E2129) in phosphate buffer saline (PBS) at a dose of 4 mL/kg ten minutes post-sonication. All mice received 10 mL/kg warm saline injection in each treatment protocol before anesthesia to prevent any possible hypovolemia or hypothermia effect. I.P injection of anti-PD-L1 injection was given 60 min before sonication to ensure anti-PD-L1 absorption (Supplementary Figure 1-B) UMBO test mice were sacrificed 15 minutes following Evans' blue injection, and their brain was harvested. The passage of Evans blue was assessed both visually and by ZEISS Axio-Scan fluorescence imaging of cryo-sectioned brains.

Cell culture and in vivo studies

GL261 cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. Cells were passaged twice weekly according to their confluence. The animal ethics committee at the Ministry of Higher Education and Research in Paris approved all protocols involving live mice (protocol #17503 and #26137). C57BL/6 mice were purchased from Charles River and were given a week of acclimation before starting any experiment.

GL261 was transduced with a *luciferase/mKate2* vector as described before²⁰ GL261-luciferase (1.4×10^5 cells/2 μ L) were inoculated into the right caudate nucleus-putamen (AP +1 mm, DV -0.25 mm, ML -0.15 mm) of 7-8 weeks old C57BL/6 females using a stereotactic injection frame (David Kopf Instruments, CA). Mice were imaged using the IVIS Spectrum (PerkinElmer) 10 minutes following a 2 mg subcutaneous injection of luciferin (Sigma, L9504). The growth of GL261-luciferase cells was confirmed by two IVIS imaging one week apart of intracranial cell injection. We observed that mice with bioluminescence values lower than 5.00×10^5 photon/second would not develop GBM tumors during the characterization of tumor growth in our mouse models. Therefore, we have included mice with bioluminescence values over 5.00×10^5 photon/second. Mice were randomly placed into treatment arms once they passed the bioluminescence cutoff value.

Animals were treated with 200 μ g of anti-CTLA-4 (Bristol-Myers Squibb, G1-XAS-Ab), anti-PD-L1 (Genentech, 6E11), IgG1 (BXCELL, BE0083) and InVivoPure pH 6.5 Dilution Buffer (BXCELL, IP0065) for four doses (Supplementary Figure 1-B). Unless stated otherwise, animals were sacrificed when they showed signs of tumor-associated illness (20% body weight loss or changes in behavior or posture).

Pharmacokinetic (PK) analysis of therapeutic antibodies with and without UMBO

The PK analysis was performed using an identical molecular weight with similar conformational structure IgG1 isoform. Thirty-six mice were used in the pharmacokinetic experiment. Mice were separated in control and UMBO groups. Six-time points were selected as follows: 0.15, 0.3, 3, 6, 24, 48 and 96 hours. Each mouse received a 200 μ g of nivolumab (Bristol-Meyers Squibb, New York, NY, USA) I.V injection 10 minutes following the BBB opening. 100 μ L of blood was collected through cardiac puncture using a pre-heparinized syringe. Serum was collected by centrifugation of the blood at 3500 rpm for 10 minutes. All samples (plasma and brain) were then analyzed using ultra-performance liquid chromatography (UPLC) system coupled to mass spectrometry (LC-MS/MS; MS-8060, Shimadzu, Japan). Peak integration and quantification were performed using LabSolutions Insight LC-MS software. Nivolumab was quantified with signature peptide ASGGITFSNSGMHWVR by nano-surface and molecular orientation limited proteolysis (Shimadzu, Japan)²¹.

MRI data acquisition

Two GL261-bearing mice were used in the experiment. Two sessions per mouse were completed in 2 consecutive days to decrease any distress effect of long isoflurane exposure. MRI acquisitions were performed using a preclinical 11.7 T MRI scanner (Biospec, Bruker BioSpin, Germany) equipped with a CryoProbe dedicated to mouse brain imaging (Biospec, Bruker BioSpin, Germany). Gd enhancement volume was estimated on T_{1w} MRI pre and post BBB opening manually on FSLeaves. Animals were anesthetized with 1% isoflurane in O₂ (2 L/min). Respiratory rate and body temperature were monitored while mice were restrained. For each animal, the protocol consisted in: (i) acquiring pre-gadolinium enhancement anatomical T₁-weighted (T_{1w}) images using a Multi-Slice Multi Echo (MSME) sequence with the following parameters: T.R. = 400 ms, T.E. = 5 ms (one single echo), four averages, 14 slices, and resolution = 60x60x500 μ m³, (ii) following injection of a total volume of 100 μ L of gadolinium (Gd; DOTAREM®, Guerbet, Aulnay-sous-Bois, France) at 0.5 mM and at physiological temperature in the tail vein of the mouse outside the MRI scanner, (iii) acquiring post-gadolinium T_{1w} images using the same sequence.

MGE sequence was acquired with the following parameters: T.R.= 80 ms, ten echoes ranging from T.E. = 2.7 ms to 35.1 ms (echo spacing = 3.6 ms), and isotropic resolution of 60x60x60 μm^3 .

mRNA sequencing

Six mice with a confirmed tumor of comparable sizes (as measured by bioluminescence imaging) were included in this experiment. Mice were divided into two groups (UMBO group and vehicle group). The vehicle group was treated with inVivoPure pH 6.5 Dilution Buffer (BXCELL, IP0065). Two treatment sessions (days 21 and 24) were applied in this experiment. Mice were sacrificed 24 hours after the last treatment by cervical dislocation, and the right hemisphere was stored in 5 mL RNALater (ThermoFisher AM7020). Lysing Matrix D (MBio, 6913050) was used to homogenize the collected brain tissues. mRNA was extracted using Maxwell RSC simply RNA automated RNA purification kit (Promega, AS1340). RNA quality was analyzed using high-sensitivity RNA chips. For RNA sequencing, NovaSeq 6000 sequencer (200 cycles, 800 million reads) and reagent kit. The reads (202 bp length, 100 million input reads) were mapped with the STAR v2.7.2a (default parameters) software to the reference genome (version GRCm38) on new junctions and known annotations. Mapping parameters were obtained from STAR outputs obtaining around 90% of unique mapped reads for all samples. Read counts from STAR were used as input for differential expression analysis using DESeq2. Furthermore, normalized counts were obtained using the variance-stabilizing transformation (VST) method from DESeq2 to be used as input for gene set variation analysis (GSVA)²² to evaluate signature enrichment of microglia expression (Slc2a5, Siglech, P2ry12, Gpr34, P2ry13, Olfml3, Tmem119, Fcrls)²³, microglia sensome (96 genes)²⁴ or antigen presentation related genes (Ciita, Psme2b, Erap1, Irf1, Tapbp, Psme2, Psme1, Pdia3, Psme3, Tap1, B2m, Calr, Tap2, Hspa1a, H2-Ab1k, H2-K1, H2-D1)²⁵. For heatmaps representation (ComplexHeatmap R package), VST gene expression values were first quantile normalized and log₂ transformed; then converted to Z-scores by subtracting the average expression value of gene *i* (G_i) of all samples from the gene expression the same G_i within sample $x(S_x)$, the resulting value was divided by the SD of G_i , the formula is: $Z\text{-score}_{G_i S_x} = (\text{Expression}_{G_i S_x} - \mu_{G_i}) / \sigma_{G_i}$

Immunohistochemistry (IHC)

A 150 KDa rat IgG2 antibody targeting PD-L1 was used in our IHC staining (BXCELL, #BE0101). A Goat anti-rat secondary IgG (H+L) antibody (BA-9400) was used to detect the anti-PD-L1. Iba1 protein was detected using 1:1000 (Abcam, #ab178846). Mouse brains were fixed overnight in 4% paraformaldehyde (PFA), then immersed in 30% sucrose overnight for cryoprotection. Next, brains were stored in Tissue-Tek® O.C.T and stored at -80° Celsius. 10 μm cryosections were harvested using Leica CM1950 cryostat. Slides were stored at -80°C until analysis.

Quantitative digital droplet polymerase chain reaction (ddPCR)

GL261 tumor-bearing mice four weeks following cell inoculation were used in the ddPCR experiment. A single UMBO treatment was completed, and 30 minutes later, blood (100 μL) was collected in heparinized tubes through cardiac puncture. Whole blood DNA was extracted automatically using Maxwell® Blood DNA Purification Kit (AS1010). QX200 ddPCR EvaGreen® was utilized to detect *mKate2* and *Luciferase* genes in the extracted DNA. Primer3Plus web interface was used to design *mKate2*, and *Luciferase* primers and primers were purchased from Life Technologies. The following forward (FR) and reverse (RV) primers were used: luciferase-FR, TCCACGATGAAGAAGTGCTC; luciferase-RV, AG-GCTACAAACGCTCTCATC; mKate2-FR, GGTGAGCGAGCTGATTAAGG; and mKate2-RV, GGGTGTGGTTGATGAAGTT.

Flow cytometry

Twenty mice with a confirmed tumor of comparable sizes were included in this experiment. Mice were separated into four groups: UMBO group, anti-PD-L1 (Genentech, 6E11) group, UMBO plus anti-PD-L1, and vehicle group (n=5/group). One treatment session was delivered in this experiment. Mice were perfused using cold distilled phosphate buffer saline (DPBS) ~16 hours after treatment. Tumor-bearing hemispheres were isolated immediately and stored in 2 mL ice-cold Hanks' balanced salt solution. According to the manufacturer's protocol, the right hemisphere was isolated and mixed in the enzyme mix solution from the adult brain dissociation kit (Miltenyi Biotec, #130-107-677). Cells gentleMACS® Octo Dissociator with Heaters (#130-096-427) and gentleMACS C Tubes (#130-093-237) were used to perform mice brain dissociation. The number of dissociated cells was calculated using Scepter® 3.0 Handheld Cell Counter. Flow cytometry were applied as described in (Supplementary Material and Methods)

Statistical tests

Statistical analysis was performed using Prism software (GraphPad). Data are shown as mean values plus and minus standard error of the mean (SEM). Statistical significance of differences between groups was verified using appropriate statistical tests. Significance level were denoted with asterisks: * for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$, and **** for $p \leq 0,0001$.

Results

Repeated UMBO is safe and effective in immunocompetent mice

Pilot study with no UMBO using GL261-luciferase orthotopic GBM mouse model was performed. This experiment aimed to determine the effect of anti-PD-L1 and anti-CTLA-4 in our GBM mouse model and select the best candidates to combine with UMBO. Anti-PD-L1 antibody alone showed a small regression in tumor growth (Figure 1-C) with a limited effect on survival of GL261-bearing (Figure 1-D), Anti-CTLA-4 treatment did not reduce tumor growth (Figure 1-C) or animal survival (Figure 1-D). UMBO parameters were previously optimized²⁶ and we evaluated UMBO parameters and treatment frequency in GL261 GBM mouse models. T₁w MRI also (Figure 1-E) showed a marked gadolinium contrast enhancement within an hour following the UMBO (Figure 1-E).

Biweekly UMBO (4 sonications in total without drug) was evaluated in the GL261-bearing mice. Mouse weight was unaffected (Figure 2-A) and no significant difference in the overall survival (OS) between UMBO and non-treated groups were observed (Figure 2-B). Overall, the UMBO parameters used for repeated BBB opening were safe and well-tolerated in GL261-bearing mice.

Furthermore, using immunofluorescence we were able to detect PD-L1 expression on the surface of GL261 cell line using anti-PD-L1 (Genentech, 6E11). RT-PCR was used to evaluate the quantitative expression of PD-L1 in GL261 cell line and we have used Nfpp10 cell line as a positive control. PD-L1 expression was significantly higher in the GL261 cell line compared to our control (Figure 2-H). Overall, this makes the anti-PD-L1 antibody the best candidate to combine UMBO in the GL261 GBM mouse model.

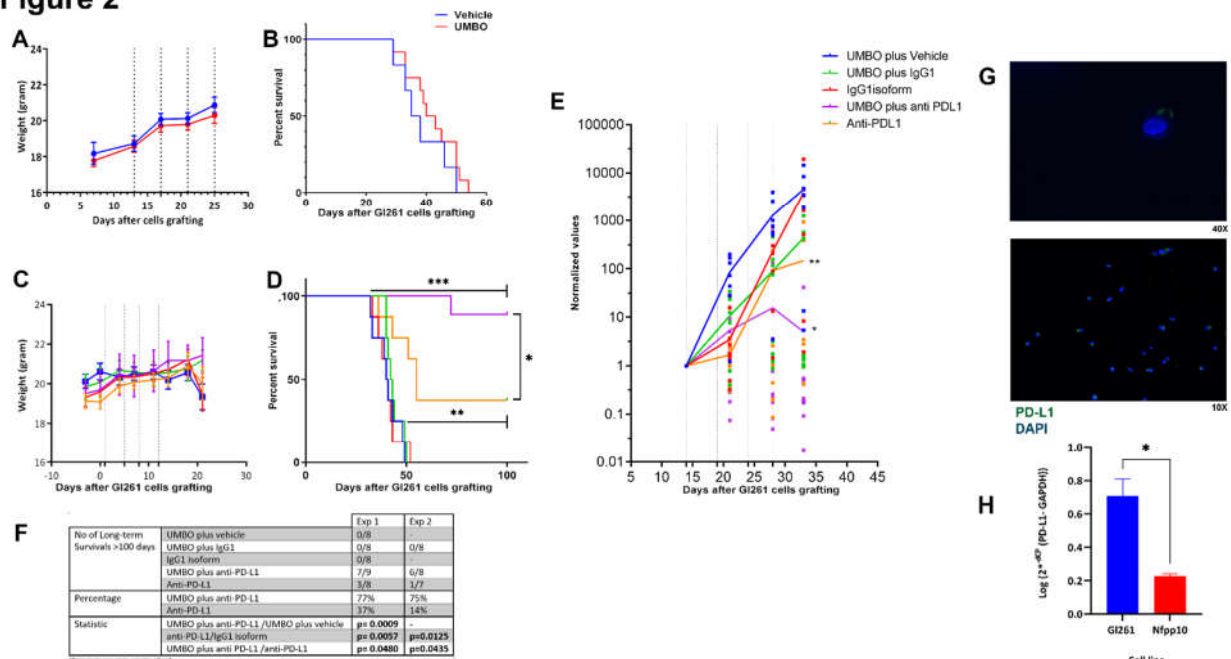
Figure 2

Figure 2. UMBO increased the efficacy of anti-PD-L1 in GL261-bearing mice. Panel A-B: Repeated UMBO alone does not affect OS (Panel B) or body weight in GL261-bearing mice compared to the non-treated (Panel A). Panel C: All treatments did not impact mice body weight. Panel D: UMBO plus anti-PD-L1 increased OS (* $p < 0.05$) compared to anti-PD-L1 alone and (** $p < 0.0001$) compared to control groups. Panel E: bioluminescence normalized to the first measured value after cell inoculation in GL261-bearing mice. Each dot represents values for one animal and the line represents the median value for the group. Bioluminescence signal was measured weekly. Dotted lines represent the days of treatments. Panel D: PD-L1 expression in GL261 and Nfpp10 cell lines (as positive control). Panel E: Immunofluorescence of PD-L1 expression in GL261 cell line.

UMBO dramatically increased the efficacy of anti-PD-L1 in GL261-bearing mice

We next investigated the combined effect of UMBO with anti-PD-L1 in the GL261-bearing mice. Mice with comparable bioluminescence values were divided into five groups: (i) UMBO group, (ii) anti-PD-L1 group, (iii) UMBO plus anti-PD-L1 group, (iv) IgG1 group, and (v) IgG1 plus UMBO group. Mice received an anti-PD-L1 antibody plus UMBO showed 76 % long-term survivors (13/17) compared to 26 % in anti-PD-L1 alone (4/15) and 0% in control groups (0/16). Kaplan-Meier estimate showed a significant difference in OS of UMBO's plus anti-PD-L1 treated mice versus anti-PD-L1 alone (Figure 2-D). Furthermore, a higher significance difference (Figure 2-D) was observed in UMBO plus anti-PD-L1 treated mice compared to the IgG1 plus UMBO treated mice in the GL261 GBM mouse model. Anti-PD-L1 antibody alone showed an early regression in tumor growth however a higher regression of tumor growth was observed among UMBO plus anti-PD-L1 treated group (Figure 2-D).

UMBO increased the penetration of anti-PD-1 and anti-PD-L1 antibodies into the brain parenchyma

IHC staining of anti-PD-L1 (BXCELL, BE0101) confirmed UMBO's trend to deliver anti-PD-L1 to the right hemisphere brain parenchyma (Figure 3-A). Furthermore, an already clinically optimized method²¹ was used to compare a size matched IgG1 antibody's pharmacokinetics with and without UMBO. Three C57BL/6 mice per time point (six-time points) per group were used in the analysis. We observed a comparable serum concentration of nivolumab in control and UMBO-treated mice.

Interestingly, higher concentrations of nivolumab were detected in mice brains treated with nivolumab plus UMBO. As expected, we detected a negligible concentration

($\leq 0.2 \mu\text{g}/200\text{mg}$ brain) of nivolumab in control mice brains (Figure 3-B). The maximum concentration (C_{max}) of nivolumab in normal brain tissue was detected at 24 hours and started to decline and reach a negligible concentration at 96 hours. Therefore, a regimen of a biweekly antibody administration was performed. Brain to plasma concentration ratio shows that UMBO enhanced the ratio of nivolumab passage across the BBB at 3, 24, 48 hours but not at 96 hours (Figure 3-C).

Figure 3

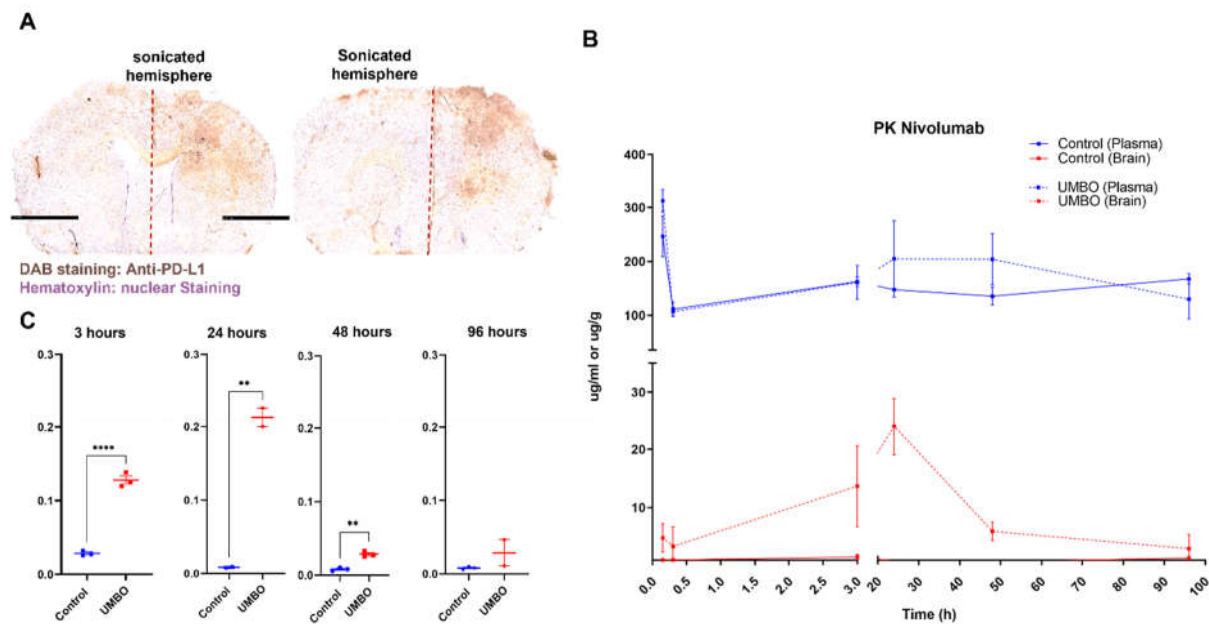


Figure 3. UMBO increased the delivery of ICIs to the brain parenchyma. Panel A: IHC staining of anti-PD-L1 (BXCELL) Panel B: PK of Nivolumab concentration in the C57BL/6 mice blood and brain. Panel C: Brain/plasma ratio of nivolumab concentration over time. UMBO enhanced the brain/plasma ratio of nivolumab compared to control mice.

UMBO plus anti-PD-L1 activates microglia and modulates microglial phenotype

We studied the immune cell populations in our treatment groups by flow cytometry. Interestingly, we found that UMBO plus anti-PD-L1 significantly enhanced the percentage of activated microglia compared to anti-PD-L1 treatment alone (Figure 4-B). UMBO alone was not associated with a significant enhancement of activated microglia percentage compared to the vehicle group however, a trend was observed ($p=0.150$). On the other hand, we did not observe any significant changes in the percentage of CD8^+ and CD4^+ T-lymphocytes or CD206^+ macrophages in all groups. Immunofluorescence staining of microglia in anti-PD-L1 plus UMBO treated group confirmed this finding and showed a phenotype of activated microglia. IHC showed a double nucleus staining of Iba1 in the UMBO plus anti-PD-L1 treated GL261-bearing mice suggesting a possible induction of microglia cell division (Figure 4-G).

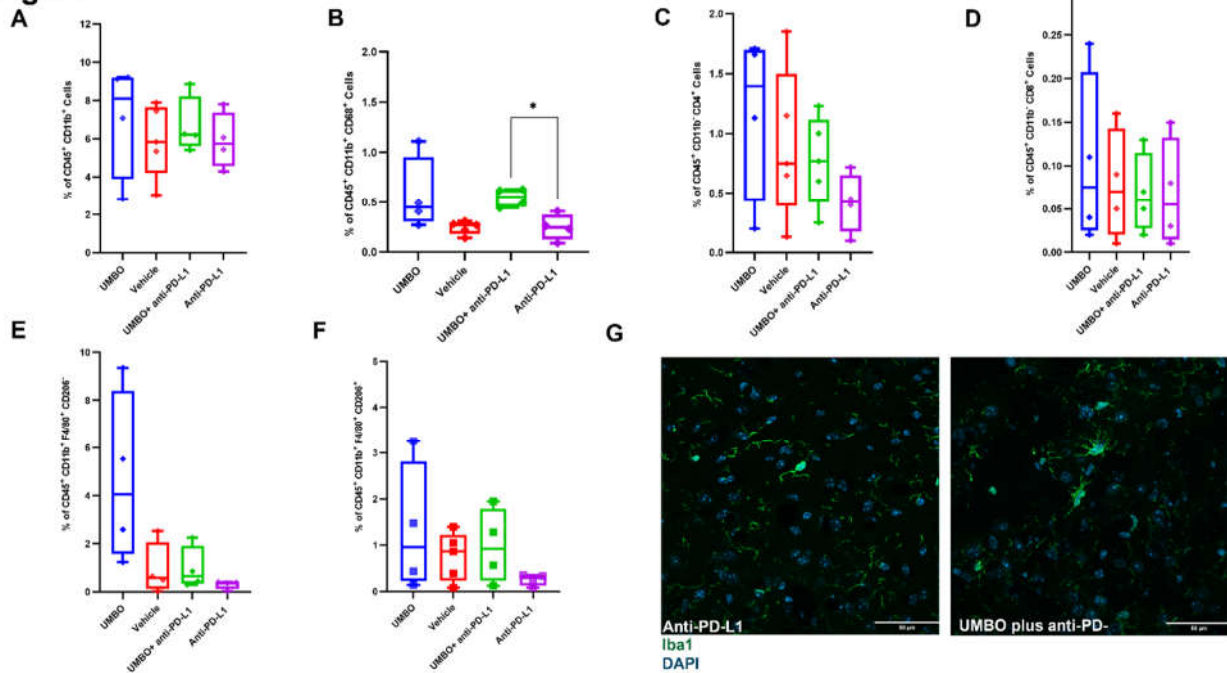
Figure 4

Figure 4. UMBO plus anti-PD-L1 activates microglia and modulates microglial phenotype. Panel A: Flow cytometry analysis of the percentage of total microglia in all different groups (n=4-5). Panel B: UMBO plus anti-PD-L1 significantly enhanced (*p<0.05) the percentage of CD68⁺ cells than anti-PD-L1 alone. Panel C-D: UMBO plus anti-PD-L1 did not influence CD4⁺ and CD8⁺ T-lymphocytes percentages compared to other groups. Panel E-F: No significant difference in CD206⁺ and CD206⁻ macrophages in all groups. UMBO plus anti-PD-L1 did not modulate macrophages' expression. Panel G: Green: Iba1 microglia/macrophages Blue: DAPI nuclear staining; microglia staining in anti-PD-L1 plus UMBO (right photos) treated group confirm a phenotype of activated microglia. Double nuclear staining in the UMBO plus anti-PD-L1 staining shows a possible induction of microglia cell division.

Using bulk RNA sequencing, compared whether UMBO modulates antigen presentation related genes compared to the vehicle group. UMBO did not influence antigen presentation (Figure 5-A) or affect microglia gene expression (Figure 5-B). Microglial ability to sense changes in the cellular environment was recently termed as microglia sensome²⁴. We used the same gene signature to evaluate microglial sensome with and without UMBO. Interestingly, UMBO significantly induced the expression of gene signatures for microglial sensome (Figure 5-C). Additionally, evaluated whether UMBO enhance circulating tumor DNA escape to the bloodstream. GL261-bearing mice with significant tumors were used in this experiment. We observed a significant elevation in the number of copies for both *luciferase* (Figure 5-E) and *mKate2* (Figure 5-F) in the UMBO treated group compared to the control.

Figure 5

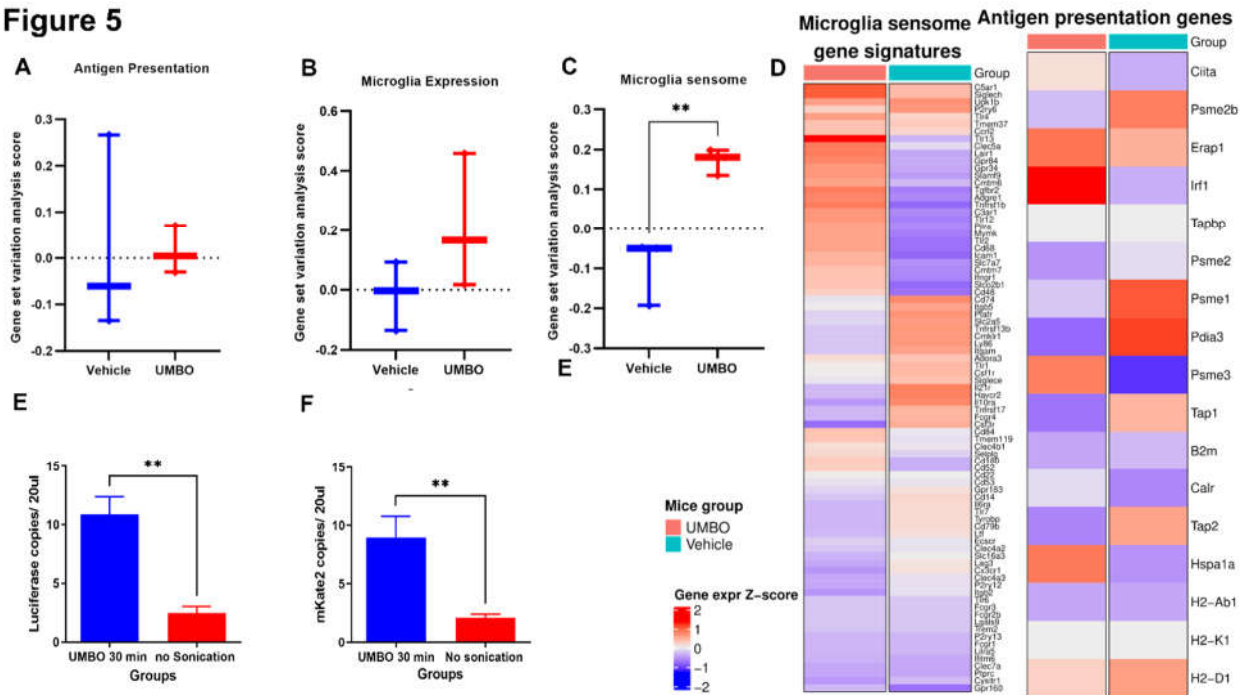


Figure 5. Repeated UMBO is associated with microglia's gene signature in the GL261 mouse model. Panel A: UMBO did not influence antigen presentation gene signature compared to the non-treated. Panel C: UMBO alone is associated with a significant (** $p < 0,01$) enrichment of microglia sensome gene signature (Panel D) compared to vehicle ($n=3$). No significant difference was observed in microglial gene expression (Panel B). Panel D: Heat maps of microglia gene signature and antigen presentation genes used in panels A and C. Panel E-F ddPCR analysis of *Luciferase* DNA (Panel F) and *mKate2* DNA (Panel E) in the blood 30 minutes following UMBO.

Discussion

UMBO and several innovative strategies continuously evolve to overcome the BBB by increasing drug delivery⁸. Immunotherapies, including ICIs and cell therapies, have revolutionized multiple solid tumors' treatments through activating the general anti-tumor immune response. The CheckMate-143 phase 3 clinical trial failed to demonstrate nivolumab demonstrate higher efficacy over bevacizumab and did not improve OS in GBM patients. Several reasons might explain the low efficacy of ICIs in GBM: (i) low tumor mutation load, (ii) lack of predictor of response and lack of selection of patients, (iii) low penetration of ICIs within the brain parenchyma, (iv) low peripheral priming, (v) local immunosuppression and (vi) low penetration of T-lymphocytes²⁷.

We explored the BBB as the limitation for antibody and lymphocytes penetration and priming and evaluated the potential of UMBO to deliver large therapeutics to the brain and the possible modulation of the immune microenvironment in GBM mouse model. We confirmed the limited efficacy of ICIs in the GL261-bearing and Nfpp10-bearing mouse models. Consistent with our data, Reardon et al. reported a limited efficacy of anti-PD-L1 and anti-CTLA-4 in GL261-bearing mice with a different treatment regimen²⁸.

To the best of our knowledge, this is the first research article that report a dramatic increase in the OS of GL261-bearing mice when treated with UMBO plus anti-PD-L1. Indeed, 76% of GL261-bearing mice treated with anti-PD-L1 plus UMBO survived longer than 100 days compared to 26% of mice treated with anti-PD-L1 alone. We investigated the mechanisms involved in the anti-tumor effect. We hypothesized that the BBB was responsible for the limited efficacy by blocking anti-CTLA-4 and anti-PD-L1 from reaching the GBM tumor. A recent study reported an enhanced efficacy of antibody conjugates following their delivery to GBM tumors²⁹. Furthermore, focused ultrasound enhanced the delivery of intranasal anti-PD-L1 but not OS in GL261-bearing mice³⁰ and a very recent

clinical study showed that UMBO enhanced the delivery of trastuzumab in brain metastases³¹.

In our setting, we reported that UMBO enhanced antibody concentration up to 28-fold compared to control. UMBO was optimized to disturb one hemisphere; however, in our PK analysis, we used a whole-brain homogenization method; therefore, local concentrations of nivolumab could have been higher. Consistent with our data, a study has shown that UMBO enhanced the delivery of bevacizumab³² ~149 KDa and anti-PD1³³ compared to non-sonicated brain in a glioma mouse model. UMBO plus 200 µg of the anti-PD-L1 biweekly treatment regimen was used to maintain the higher concentration of anti-PD-L1 within the brain parenchyma. Immune checkpoint blockade with anti-PD-L1 was performed on day 14 post-inoculation to allow for T-lymphocytes exhaustion³⁴.

UMBO stimulates a detectable peripheral circulation of GL261 DNA³⁵. Zhu et al., published article investigated the possibility of using UMBO for liquid biopsies in GBM models. They detected green fluorescent protein mRNA 20-mins following UMBO in the GL261-GFP expressing mouse model³⁶ supports the passage of tumor material from the brain to blood flow stream. In consistent with our timepoint, another research group described that UMBO increases cell-free DNA in a time-dependent manner³⁷. The priming effect of circulating DNA could activate naïve T-lymphocytes through their exposure to new antigens. BBB protects the tumor from T-lymphocytes infiltration and immune activation. Therefore, detecting GL261 tumors in the peripheral circulation might activate the global antitumor effect. Further functional analysis of lymphocyte activation should be performed to evaluate any priming effect of UMBO.

Our results showing microglia activation in the UMBO plus anti-PD-L1 treated GL261-bearing mice suggest a possible mechanism for the observed enhanced therapeutic efficacy of anti-PD-L1. Our flow cytometry analysis confirms published data that showed a higher ratio of Iba-1 staining in sonicated regions compared to the non-sonicated³⁸. PD-L1 is expressed on the cell surface of both GL261 and microglia³⁹. A possible effect on microglia phenotype might be related to the combined effect of UMBO and anti-PD-L1 delivery to the brain parenchyma. Activated microglia might have a cytotoxic effect against GL261 tumor cells⁴⁰ therefore further investigation of the activated microglia role in GBM should be addressed.

To date, there is no clear evidence on the effect of UMBO on T-lymphocytes passage to the brain. We did not observe any significant elevation in the percentage of CD8⁺ and CD4⁺ T-lymphocytes at one timepoint (~16 hours). This effect might be related to the timing of sample collection as we only evaluated our treatment regimen at one timepoint. We observed a delayed antitumor effect in UMBO and anti-PD-L1 group which can be related to a delayed effect on T-lymphocytes. Furthermore, we have not analyzed any subpopulations of CD8⁺ T lymphocytes *i.e.*, PD-1⁺ CD8⁺ T-lymphocytes.

Syngeneic mice models and especially the GL261 mouse model used in our experiments is a limitation of the current study. The GL261 mouse model: (i) has a high mutation load which is not consistent with GBM patients and (ii) a variability in terms of responses to ICIs *in vivo*³⁴. Another limitation is the inability to demonstrate functional analysis of the role of UMBO in priming naïve T-lymphocytes through their exposure to new antigens. Additional functional analysis on the effect of UMBO plus anti-PD-L1 would explain the dramatic effect on OS that was observed in our study.

Conclusions

Our study showed statistically significant increased brain penetration and efficacy of anti-PD-L1 in GL261-bearing mice when delivered by UMBO. We have also provided clear evidence of the possible safe and effective delivery of large therapeutic agents using UMBO. Further investigations are needed to confirm the impact of UMBO on brain penetration and efficacy of chemotherapeutic agents and anti-PD-L1 to overcome the resistance of GBM to the current treatments.

Acknowledgments: We acknowledge Genentech for providing anti-PD-L1 (Clone 6E11) antibody and we appreciate our valuable discussions with Dr. Michel Mallat and his comments regarding microglia. We acknowledge the Salk institute for biological studies for providing us with the Nfpp10 cells.

Funding: This work was supported by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement #766069 (GLIO-TRAIN).

Conflicts of interest: AI reports grants and travel funding from CarThera. A.C is a paid consultant of CarThera and have ownership interest in CarThera. C.S, G.B, M.C are employees of CarThera. All other authors have no conflict of interests.

Author Contributions: M.A: A.I, M.V conceived and planned the experiments. M.A, I.H N.L, E.Q, C.G, L.G, N.Z, M.S, L.M, A.P carried out the experiments. C.S, G.B, M.C, M.S, A.C provided significant technical and scientific support. M.A. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data availability statement: Raw data will be available upon request from the authors.

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