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Anti-tumour immune response in GL261 glioblastoma generated by Temozolamide Immune-Enhancing Metronomic Schedule monitored with MRSI-based nosological images

Anti-tumour immune response in GL261 glioblastoma generated by Temozolomide Immune-Enhancing Metronomic Schedule monitored with MRSI-based nosological images

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MRSI-based response follow-up in TMZ-treated GL261 glioblastoma

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ABSTRACT:

Glioblastomas (GB) are brain tumours with poor prognosis even after aggressive therapy. Improvements in both therapeutic and follow-up strategies are urgently needed. In previous work we described an oscillatory pattern of response to Temozolomide (TMZ) using a standard administration protocol, detected through MRSI-based machine learning approaches. In the present work, we have introduced the Immune-Enhancing Metronomic Schedule (IMS) with an every 6-days TMZ administration at 60mg/kg, and investigated the consistence of such oscillatory behaviour.

A total of n=17 GL261 GB tumour-bearing C57BL/6j mice were studied with MRI/MRSI every 2 days, and the oscillatory behaviour (6.2±1.5 days period from the TMZ administration day) was confirmed during response. Furthermore, IMS-TMZ produced significant improvement in mice survival (22.5±3.0 days for controls vs 135.8±78.2 for TMZ-treated), outperforming standard TMZ treatment.

Histopathological correlation was investigated in selected tumour samples (n=6) analyzing control and responding fields. Significant differences were found for CD3+ cells (lymphocytes, 3.3±2.5 vs 4.8±2.9 respectively) and Iba-1 immunostained area (microglia/macrophages, 16.8±9.7% and 21.9±11.4% respectively).

Unexpectedly, during IMS-TMZ treatment, tumours from some mice (n=6) fully regressed and remained undetectable without further treatment for one month. These animals were considered "cured" and a GL261 re-challenge experiment performed, with no tumour reappearance in 5 out of 6 cases.

Heterogeneous therapy response outcomes were detected in tumour-bearing mice, and a selected group was investigated (n=3 non-responders, n=6 relapsing tumours, n=3 controls). PD-L1 content was found ca. 3-fold increased in the relapsing group when comparing with control and non-responding groups, suggesting that increased lymphocyte inhibition could be associated to IMS-TMZ failure. Overall, data suggest that host immune response has a relevant paper in therapy response/escape in GL261 tumours under IMS-TMZ therapy. This is associated to changes in the metabolomics pattern, oscillating every 6 days, in agreement with immune cycle length, which is being sampled by MRSI-derived nosological images.

KEY WORDS: glioma; orthotopic tumours; immune response; metronomic therapy; immune memory, TMZ; PD-L1

Abbreviations used: **3DiCSI**, 3D Interactive Chemical Shift Imaging; **BTDP**, below threshold detection period; **CPA**, cyclophosphamide; **CTLs**, cytotoxic T lymphocytes; **DAMPs**, damage-associated molecular patterns; **DCs**, dendritic cells; **DMSO**, dimethyl sulfoxide; **DMPM**, dynamic MRSI processing module; **ETL**, echo train length; **FASTMAP**, fast automatic shimming technique by mapping along projections; **FOV**, field of view; **GB**, Glioblastoma; **HIF**, hypoxia-inducible factor; **IMS**, immune-enhancing metronomic schedule; **IT**, inter-slice thickness; **MDSCs**, myeloid-derived

suppressor cells; **MTX**, matrix size; **MRI**, magnetic resonance imaging; **MRS**, magnetic resonance spectroscopy; **MRSI**, magnetic resonance spectroscopic imaging; **NA**, number of averages; **NMF**, non-negative matrix factorization; **NS**, number of slices; **PD**, progressive disease; **PD-1**, programmed death-1; **PD-L1**, programmed death-ligand 1; **PR**, pattern recognition; **PRe**, partial response; **PRESS**, point-resolved spectroscopy; **PVDF**, polyvinylidene fluoride; **RANO**, response assessment in neuro-oncology; **RARE**, rapid acquisition with relaxation enhancement; **RECIST**, response evaluation criteria in solid tumours; **ROIs**, regions of interest; **SDi**, stable disease; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **ST**, slice thickness; **SW**, sweep width; **T2w**, T2 weighted image; **TAT**, total acquisition time; **TE**, echo time; **TEeff**, effective echo time; **TMZ**, temozolomide; **TR**, repetition time; **TRI**, tumour responding index; **TV**, tumour volume; **VAPOR**, variable power and optimized relaxation delay; **VOI**, volume of interest; **WB**, western blot; **wt**, wild-type

1 | INTRODUCTION

Glioblastoma (GB) is the most frequent type of primary central nervous system malignancy in adults with very poor prognosis, which has not significantly improved despite the development of new diagnostic strategies and innovative therapies.[1] Combination chemotherapy after surgery such as Temozolomide (TMZ) with radiotherapy have been used as standard adjuvant therapeutic choice producing an average survival of 14.6 months.[2]

For therapy response assessment in GB, the most common monitoring approach is magnetic resonance imaging (MRI), which is the preferred modality to provide information about tumour size and local tumour tissue extension.[3] Radiological and clinical guidelines are used to evaluate GB response to therapy, particularly through the application of the response assessment in neuro-oncology (RANO)[4] and Response Evaluation Criteria in Solid Tumours (RECIST) criteria.[5] However, MRI information has been shown to be inaccurate due to the pseudoresponse and pseudoprogression phenomena occurring in some cases.[6] Functional and molecular imaging can provide more accurate information and has attracted a lot of attention in the last decade. Among functional methods, magnetic resonance spectroscopy (MRS) may provide information about the metabolomic profile of the investigated lesion.[7,8] Compared to MRS merely acquiring a single spectrum from a certain volume, magnetic resonance spectroscopic imaging (MRSI) is a technique that can provide metabolomic information superimposed to anatomical information, making it possible to gather this type of information from different regions of the studied tissue.[9,10] In this sense, MRSI

might be an interesting approach to complement MRI information in evaluating tumour response to therapy.

Furthermore, the MRSI signal obtained from different types of tissue can be automatically categorized by pattern recognition (PR) techniques creating nosological maps potentially useful to detect and characterize therapy response in a noninvasive way.[11–13] In this respect, the tumour responding index (TRI) as an evaluation parameter has been put forward to measure the extent of response to treatment using the obtained nosological images.[14] In previous studies from our group, the multi-slice MRSI technique has allowed us to observe TRI oscillations (ca. 6 days period) in GL261-tumour bearing mice treated with TMZ.[14] Moreover, other authors have described that treatment with alkylating agents (e.g., TMZ) trigger the host immune system recruitment, eventually leading to tumour cell death. [15] Still, it has been described that the whole immune cycle in mice brain usually requires around six days[16], which agrees with the oscillation period found in previous studies.[14] Beyond that, it was described that administering chemotherapy with consecutive cycles every 6 days could trigger immune system activation[17] and we coined the expression "Immune-Enhancing Metronomic Schedule" (IMS) for this type of administration protocol[18]. In the late steps of each immune cycle, activated T cells traffic to and infiltrate the tumour bed, specifically recognizing and binding cancer cells and killing their target.[19] In addition, microglial and macrophage cells, which can contribute up to 30% of a brain tumour mass, play a role in GB growth control.[20]

Programmed death-1 (PD-1) and its ligand (PD-L1) play a key role in tumour immune escape and the modulation of tumour microenvironment, closely related with tumour generation and development.[21] Chemotherapy can modify this tumour microenvironment. Thus, a recent study on 30 patients with thymic epithelial tumours revealed that PD-L1 expression increased in patient's tumours after chemotherapy.[22] Similar findings have been communicated in other types of tumour such as rectal adenocarcinoma and breast cancer.[23,24] These studies reveal a potential link between chemotherapy and tumour immunoresistance. However, little is known about how PD-L1 content changes in GB after chemotherapy, neither preclinical nor clinical.

Consequently, in this work, our goal was to observe the evolution of TRI in GL261 tumour-bearing mice treated with IMS-TMZ which was expected to enhance immune system participation in response to therapy. Furthermore, the tumour-associated immune population was characterized by histopathology studies, the contribution of PD-L1 to tumour relapse was investigated by western blot (WB) analysis, and the long-

term antitumour immune memory was examined by re-challenging TMZ-cured mice with GL261 glioma cells.

2 | MATERIALS AND METHODS

2.1 | GL261 cells

GL261 mouse glioma cells were obtained from the Tumour Bank Repository at the National Cancer Institute (Frederick, MD, USA) and were grown as previously described.[25] Cells were checked for mouse STR profile and interspecies contamination, as well as PCR evaluation to discard Mycoplasma and virus contamination.

2.2 | Animal model

All studies were approved by the local ethics committee (*Comissió d'Ètica en Experimentació Animal i Humana* (CEEAH, https://www.uab.cat/web/experimentacio-amb-animals/presentacio-1345713724929.html), according to the regional and state legislation (protocol CEA-OH-9685 / CEEAH-3665).

A total of 37 C57BL/6 female wild-type (wt) mice weighing 22.4±2.2 g were used in this study. Six out of 37 C57BL/6 GL261 tumour bearing mice were generated in a previous study[14] and further analyzed by histopathology studies in the present work. Thirty-one C57BL/6 GL261 tumour bearing additional mice were generated for this work (See Figure 1 and Table 1). They were obtained from Charles River Laboratories (l'Abresle, France) and housed in the animal facility of the *Universitat Autònoma de Barcelona*. Tumours were induced in C57BL/6 mice by intracranial stereotactic injection of 10^5 GL261 cells as previously described by us.[26] Mice were weighed every other day and tumour volumes were followed twice a week using T2 weighted image (T2w) MRI acquisitions. Mice with the most homogeneous weights and tumour sizes were randomly chosen before therapy start to make experimental groups.

2.3 | Animal treatment

For *in vivo* experiments, TMZ (Sigma-Aldrich, Madrid, Spain) was dissolved in 10% dimethyl sulfoxide (DMSO) in saline solution (0.9% NaCl). IMS-TMZ 60 mg/kg was administered to n=19 tumour-bearing mice using an oral gavage, every 6 days, from day 11 post inoculation (p.i.), while control mice received 10% DMSO vehicle. After treatment, animals meeting endpoint criteria were euthanized by cervical dislocation according to animal welfare protocol advice for ethical reasons, the brain was removed and tumour resected. Animals cured by the IMS-TMZ therapy were followed up and a re-challenge experiment was carried out (see section 2.4 for further details).

Regarding the histopathological analysis, six mice were used, four treated with TMZ

and two controls from [14]. For these cases the TMZ administration scheme was the one described in previous studies, shown in Figure S1A. Still, control animals from [14] did not receive 10% DMSO vehicle.

2.4 | Tumour-specific Immune memory studies

2.4.1 |Criteria for cured mice

When GL261 tumours were reduced after IMS-TMZ treatment until abnormal mass detection by MRI was no longer possible, or its volume remained stable (usually below 2 mm^3) during at least 2 weeks, TMZ administration was halted. Then, MR images were acquired twice a week. Whenever the volume of the residual/abnormal mass was stable or decreased for one month, mice were transiently declared "cured" as in[17].

2.4.2 |Re-challenge experiment

Mice cured by the IMS-TMZ treatment (n=8) were inoculated with GL261 cells again, symmetric but contralateral to the initial injection site. For re-implantation, three C57BL/6 female wt mice were also implanted as controls, to check for consistency and growth rate in contralateral side. All mice were followed-up (weight + welfare parameters) twice a week and volumetric T2w MRI was acquired once a week.

2.5 | In vivo MRI and MRSI studies

2.5.1 | Data acquisition

In vivo MRI/MRSI studies were performed at the joint nuclear magnetic resonance facility of the *Universitat Autònoma de Barcelona* and *Centro de Investigación Biomédica en Red-Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN)* (Cerdanyola del Vallès, Spain), Unit 25 of NANBIOSIS (www.nanbiosis.es). The 7T Bruker BioSpec 70/30 USR spectrometer (Bruker BioSpin GmbH, Ettlingen, Germany) equipped with a mini-imaging gradient set (400 mT/m) was used. A 72 mm inner diameter linear volume coil was used as transmitter, and a mouse brain surface coil as a receiver for brain MRI studies.

Mice were positioned in a bed, which allowed delivery of anaesthesia (isoflurane, 1.5-2.0% in O_2 at 1 L/min), with an integrated heating water circuit for body temperature regulation. Respiratory frequency was monitored with a pressure probe and kept between 60–80 breaths/min.

MRI studies

GL261 tumour-bearing mice were screened by acquiring high resolution coronal T2w images using a Rapid Acquisition with Relaxation Enhancement (RARE) sequence to

detect brain tumour presence and to monitor its evolution stage with repetition time (TR)/effective echo time (TEeff) = 4200/36 ms. The whole set of parameters used in MRI acquisitions can be found in the supplementary file. MRI data were acquired and processed on a Linux computer using ParaVision 5.1 software (Bruker BioSpin GmbH, Ettlingen, Germany).

MRSI studies

The MRSI acquisitions were performed every two days with mice in the longitudinal group, starting at day 15-17 post-implantation.

Consecutive 14 ms echo time (TE) MRSI with point-resolved spectroscopy (PRESS) localization grids were acquired individually across the tumour, using as a reference T2w high resolution images, as described in previous work.[14] Tumour was covered by 3-4 MRSI grids with matrix size of 10x10 or 12x12 (technical details of MRSI acquisitions can be found in the supplementary file). In order to ensure quality of the acquired data, shimming was performed individually for each MRSI grid. MRSI grids were spatially located such that the volume of interest (VOI) included most of the tumoural mass as well as normal/peritumoural brain parenchyma.

Water suppression was performed with Variable Power and Optimized Relaxation Delay (VAPOR), using a 300 Hz bandwidth. Linear and second order shims were automatically adjusted with Fast Automatic Shimming Technique by Mapping Along Projections (FASTMAP) and six saturation slices (ST, 10 mm; sech-shaped pulses: 1.0 ms/20250 Hz) were positioned around the VOI to minimize outer volume contamination in the signals obtained.

2.5.2 | MRI and MRSI Processing and Post Processing

Tumour volume calculation

Manual segmentation of abnormal brain mass in T2w images was performed and tumour volumes were calculated from T2w high resolution horizontal images using the equation:

$$TV\ (mm^3) = [(AS_1 \times ST) + [(AS_2 + (\ldots) + AS_{10}) \times (ST + IT)]] \times 0.075^2 \qquad \text{(Equation 1)}$$

Where TV is the tumour volume, AS is the number of pixels contained in the region of interest in each slice of the MRI sequence, ST is the slice thickness, IT the inter-slice thickness and 0.075^2 the individual pixel surface area in mm^2. The tumour area was calculated from pixels in each slice with ParaVision 5.1 software. The inter-slice volume was estimated adding the inter-slice thickness to the corresponding slice thickness in Equation 1.

Brain MRSI post‐processing and pattern recognition strategies

MRSI data were post-processed essentially as described in.[25] Briefly, data were initially pre-processed at the MR workstation with ParaVision 5.1, and then post-processed with 3D Interactive Chemical Shift Imaging (3DiCSI) software package version 1.9.17 (Courtesy of Truman Brown, Ph.D., Columbia University, New York, NY, USA) for line broadening adjustment (Lorentzian filter, 4 Hz), zero-order phase correction and exporting the data in ASCII format. Dynamic MRSI processing Module (DMPM), running over MatLab 2013a (The MathWorks Inc., Natick, MA, USA) was used to align all spectra within each MRSI matrix, using the choline signal as reference, 3.21 ppm). The 0–4.5 ppm region of each spectrum in the MRSI matrix was normalized to unit length and exported in ASCII format for performing the PR analysis. No baseline correction was performed in these spectra.

After that, the non-negative matrix factorization (NMF) semi-supervised methodology [27,28] was applied for the extraction of meaningful source signals from the MRSI investigated tumours. From the biochemical viewpoint, the source extraction technique to classify MRS data assumes that in each voxel there is a mixture of heterogeneous tissues and their metabolites from which the contribution of each source can be obtained. A previously described semi-supervised approach[29], which relies on Convex-NMF for the final source extraction, was used for classifying pixels into normal brain parenchyma, actively proliferating tumour and tumour responding to treatment; and for calculating nosologic maps representing the spatial response to treatment. Green colour is used when the GB responding to treatment source contributes the most, blue for normal brain parenchyma, red for actively proliferating GB and black for undetermined tissue. A more detailed explanation on NMF can be found in the Supplementary file.

2.5.3 |Tumour Responding Index (TRI) calculations

In order to measure the extent of response to treatment using the obtained nosological images, a numerical parameter named TRI was calculated (Equation 2) [14].

$$\mathrm{TRI} = \frac{\text{Tumour responding pixels}}{\text{Total tumour pixels}} \times 100 \qquad \text{(Equation 2)}$$

TRI is stated as the percentage of green, responding tumour pixels of all grids over the total tumour pixels of all recorded grids. Then, tentative ranges of TRI categories were established to classify the different response to treatment levels observed in the studied animals, taking into account both TRI percentage and volumetric data from MRI measurements according to RECIST criteria[5]. An adapted set of RECIST criteria was used to classify cases into Progressive disease (PD), Partial response (PRe) or Stable disease (SDi). See the Supplementary file for the adapted RECIST criteria.

In this work, a TRI cycle was defined provided a change between maximum and minimum TRI values was above 10%. Values below this threshold were attributed to possible experimental variability and were not counted as 'cycles'.

2.6 | Antibodies

For histopathology studies, Anti-CD3 antibody (dil. 1:300, rabbit, Ref A0452) was purchased from Dako (Santa Clara, CA, USA) and Anti-Iba-1 antibody (dil. 1:300, goat, Ref ab5076) was purchased from Abcam (Cambridge, UK).
For PD-L1 WB studies, Anti-PD-L1 antibody (dil. 1:400, rabbit, Ref ab233482) was purchased from Abcam (Cambridge, UK), β-tubulin antibody (dil. 1:1000, rabbit, Ref 2146) was purchased from Cell Signalling Technology (Beverly, MA, USA) and secondary antibody towards rabbit IgG (dil.1:2000), conjugated to horseradish peroxidase, was purchased from Bio-Rad (Richmond, CA, USA).

2.7 | Histopathology studies

TMZ-treated cases used for histopathological validation were the following: C1100 (TRI = 61.8%), C1108 (TRI = 70.3%), C971 (TRI = 44.1%) and C1022 (TRI = 46.5%). In addition, two control (non-treated) tumour-bearing mice were also explored (C1110 and C1111). MRI/MRSI data, nosological images and TRI calculations are described in.[14]
Fixed brains were embedded in paraffin and serial horizontal sections were cut, taking into account MRSI slices in order to ensure spatial correlation. Brain was split in half and each half was cut in 20 sections of 5 µm. The upper half of the brain corresponded to MRSI Grids 1 and 2, while the lower half of the brain corresponded to MRSI Grids 3 and 4.
One section corresponding to each MRSI Grid position was selected for CD3+ immunohistochemical staining for detecting the presence of T lymphocytes and Iba-1 immunohistochemical staining for detecting the presence of activated macrophages/microglia in treated and control tumours.
After immunostaining, the preparations were digitized for quantification using a Nanozoomer 2.0HT (Hamamatsu Photonics France, Massy, France). Then, CD3+ positive cells and percentage of Iba-1 positive immunostained cellular areas were counted in each section in fields that corresponded to green or red tumour areas in nosological images. From each chosen area, 5 fields of 0.025 mm^2 were randomly selected at 40x magnification, using NDPview 1.2.53 software (Hamamatsu Photonics France SARL, Massy, France). Fields were placed in the more highly cellular areas, avoiding poor or acellular areas. Exceptionally, in some samples, fewer fields were counted if the corresponding area of the nosological image was not large enough to

evaluate 5 fields.

2.8 | PD-L1 expression by Western blotting

Cases used for PD-L1 expression were those with the following codes: fast growth tumours escaping IMS-TMZ therapy right from the start, defined as “non-responding” (C1288, C1319 and C1356, n=3); cases showing transient tumour shrinkage but eventually relapsing, defined as “relapsing” (C1290, C1292, C1341, C1342, C1343 and C1349, n=6). Control cases received 10% DMSO vehicle with the same schedule (C1266, C1300 and C1317, n=3).

Tissue Homogenization and Protein Extraction. Whole GL261 tumours obtained after euthanization at endpoint were weighted and protein extracted as described in [30], details can be found in Supplementary file. Protein concentration was determined by Bradford assay (Bio-Rad) and equal amounts of protein (80 µg) were loaded on 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), blotted on polyvinylidene fluoride (PVDF) Immobilon-P membranes (Millipore, Darmstadt, Germany), and immunodetected with the corresponding antibodies using a chemiluminescent detection method (Clarity™ Western ECL Blotting Substrates, Bio-Rad, California, U.S.A.). Chemiluminescent signal was obtained with Chemidoc MP Image System and quantified with the ImageJ software.

2.9 | Statistical analysis

Variance homogeneity was assessed with the Levene’s test. Sample distribution was assessed with the Kolmogorov-Smirnov test. A two-tailed Student’s t-test for independent measurements was used for comparisons, for samples of equal or different variances (depending on the Levene’s test result). Comparisons of survival rates were performed with the Log-Rank test. The significance level for all tests was $p<0.05$.

3 | RESULTS

Table 1 summarizes the cases described in this study and the analyses performed with each one.

3.1 | IMS-TMZ treatment and follow up in GL261 tumour-bearing mice

In this part of the study 19 mice were used. Among them, 13 mice were treated with TMZ using an IMS protocol and 6 were administered with vehicle, also in an IMS administration (see Table 1 for individual codes). The IMS administration scheme can

be found in Figure S1A. All mice were followed-up according to the supervision parameters for animal health status and weighted (Figure S1C). The tumour volume at therapy starting (day 11 p.i.) in this section was 5.4 ± 2.6 mm^3, close to tumour volumes in previous work with similar TMZ schedule (6.0 ± 1.2 mm^3)[30]. Tumour volume evolution of vehicle and TMZ-treated mice is shown in Figure S1B. As described before,[31] TMZ treatment with the IMS administration has a positive impact in the survival of GL261 tumour-bearing mice (Figure S1D). Thus, the average survival rate for control mice was 22.5 ± 3.0 days whereas the TMZ-treated animals survived significantly more, 135.8 ± 78.2 days ($p<0.05$ according to Log Rank Test), being 61.5% of TMZ treated mice alive at day 150 p.i. and some individuals still alive at 464 days p.i.

3.2 | Multi-slice MRSI-based volumetric analysis of therapy response assessment under IMS-TMZ treatment

A longitudinal study was performed with eleven TMZ-treated GL261 tumour-bearing mice and six vehicle treated mice (see Table 1 for individual codes). Mice were studied every two days (see Figure S2A) until endpoint. The start of MRSI explorations was conditioned by the measured tumour volume. According to our experience and data described by our group[31], tumour volumes below 10-20 mm^3 are not properly segmented by MRSI, and this was the cut-off point to start MRSI acquisitions. An example can be seen in Figure S2B. Results are summarized below.

3.2.1| TMZ-treated mice

The relationship between TRI and tumour volume evolution, as well as the corresponding nosological images of three most representative cases of TMZ treated mice are shown in Figure 2 (for the remaining cases, refer to Figures S3 and S4).

Several TRI oscillations were observed in the TMZ-treated mice and these were generally coincident with SDi and PRe tumour stages (Table S1), according to the adapted RECIST criteria. Oscillations were observed between days 17 and 39 p.i. depending on the case observed. It is worth mentioning that in the first MRSI time points (days 15-19 depending on the case), where the evolution tumour would be still classified as PD, TRI oscillations can already be seen.

In five cases (C1263, C1264, C1270, C1380 and C1383) tumour escaped from therapy and met PD criteria, leading to the euthanization of the mice. During this relapse, which started between days 35-41, either TRI cycles were not found anymore, or this value was no longer reliable. When the slope of the tumour growth increased dramatically or tumour relapsed beyond volumes of ca. 50 mm^3, TRI do not seem to suggest response, even if oscillating features are seen. Moreover, the combination of

high tumour volume (>70mm^3) and relatively low TRI (e.g. C1270, TRI <40%) resulted in bad survival outcome in comparison with other cases, and tumour grew uncontrolled until the second TRI oscillation appearance.

Regarding cases with complete follow-up, a total of 48 doses of TMZ were given, resulting in 26 TRI oscillation peaks, 21 of them corresponding with tumour growth arrest or volume decrease, whereas 5 TRI peaks were no longer able to coincide with controlled tumour growth (almost exclusively at relapsing time points). In addition, 8 TMZ doses corresponded to periods in which tumour volume were below the minimum volume for nosological imaging segmentation while 2 TMZ doses corresponded to endpoint with no further explorations made.

It is worth mentioning that TRI peak appear ca. 6-8 days after the first therapy administration period. The only case which delayed this appearance – 12 days, but only 6 days after the second therapy dose, C1383– could be due to segmentation problems due to borderline tumour volume between therapy cycles one and two. For all followed tumours, the first TRI peak agreed with a certain decrease in the tumour growth slope.

Spectra acquired were of overall good quality and examples of chosen MRSI spectra for case C1263 are shown in Figure S2A.

3.2.2| Additional cases from cured mice

For mice with full MRI/MRSI follow-up, six of the TMZ treated mice analyzed (see Table 1 for individual codes) had tumours which disappeared after IMS-TMZ treatment, which obviously prevents the generation of nosological images. Details about their tumour volume and TRI evolution can be found in the supplementary material (Figure S3).

As in other TMZ-treated mice, TRI oscillations or punctual increases could be found for most cases. For example, in the first days of MRSI monitoring, TRI cycles were found in four out of six cases. After that time, the relationship between tumour volume evolution and TRI changes was different in every case.

The maximum volume achieved in this group was 37.4 mm^3, and tumour volume decrease was found around the second TMZ therapy dose, lasting until ca. day 30 p.i. For cases with enough volume for nosological imaging segmentation, oscillations were seen during active response. For example, case C1276 (Figure S3A) showed TRI oscillations from days 17 till 27 p.i., when the tumour was classified either as SDi or PRe. Case C1382 presented TRI oscillations at days 17 and 21 p.i., followed by a gradual decrease which concurred with tumour disappearance.

Cured mice had tumours with a trend to smaller volumes at therapy start (average

4.3±1.4 mm^3 vs 6.9±3.4 mm^3 in non-cured mice from this work), although this difference was not statistically significant.

3.2.3| Vehicle-treated mice

Six vehicle-treated mice were analysed for comparison purposes (Figures S5 and S6). The tumour evolution in this group was classified as PD throughout the period of MRSI analysis (Table S2), as expected. The TRI oscillatory pattern is not observed in any of the cases, except in the C1258, where a possible TRI cycle is observed (Figure S5), although tumour growth slope did not decrease. In the remaining 5 cases, no TRI cycles were observed, indicating that clear TRI oscillations are only present when tumour-bearing mice respond to IMS-TMZ treatment.

3.3 | Histopahology validation

Six mice were used for histopathological analysis, 4 treated with TMZ and two non-treated. Comparison of responsive and unresponsive zones (i.e. green and red areas of the nosological images) was performed (see Figure 3). Responsive zones presented significantly higher CD3+ positive cell counting and higher percentage of Iba-1 immunostained area in comparison with unresponsive zones. Values for CD3+ were 4.8 ± 2.9 and 3.3 ± 2.5 CD3+ positive cells in responsive and unresponsive zones, respectively (n=53 and n=94 fields). For Iba-1, the percentage of positive immunostained area was 21.9 ± 11.4% for responsive and 16.8 ± 9.7% for unresponsive zones (n=53 and n=95 fields). Individual fields from responsive zones could achieve values up to 42% of area occupancy by Iba-1 positive cells while unresponsive zones could reach values as low as 1.4%. Although it could be contributing to the MRSI-detected spectral pattern changes, there is variability, and differences between regions within the same tumour are not so clear cut in certain individual cases.

3.4 | PD-L1 content in GL261 tumours

PD-L1 content was analyzed in 12 frozen dissected GL261 tumours: 9 from IMS-TMZ treated mice and 3 control, with growth curves shown in Figure S7. WB results and quantification are shown in Figure 4. After normalization to Tubulin, PD-L1 band intensity was 0.23 ± 0.04 in the GL261 control group whereas it was 0.64 ± 0.14 in the relapsing GL261 group and 0.19 ± 0.02 in non-responding group. The PD-L1 content was found significantly higher ($p<0.001$) in the relapsing group when comparing with control group (2.8 fold) and non-responding group (3.4 fold), whereas no differences were found for PD-L1 content between non-responding and control tumours.

3.5 | IMS-TMZ activates Tumour-specific immune memory

We were also interested in a preliminary evaluation of whether IMS-TMZ treatment could induce long-term antitumour immunity in the cured C57BL/6 mice (tumour growth curves shown in *Figure S8*). For that, the re-challenge experiment described in section 2.4.2 was performed. Three wt mice were implanted in parallel as controls. Control tumours grew normally and no significant differences were found with standard GL261 tumour doubling time of 2.4 ± 0.3 days[32]. Regarding the re-challenged mice, only one of re-implanted tumours in the TMZ-cured mice (case C1286) grew after 10 days while the rest remained tumour-free until present time (range 150-464 days post-implantation). With respect to case C1286, TMZ was administered as usual in an IMS protocol and tumour disappeared after only one TMZ dose. After being cured again, this mouse has been followed-up (weight + welfare parameters) twice a week and MRI acquisitions were acquired once a week, for the rest of its lifetime (until 175 days post-implantation) and no tumour mass has been detected in its brain.
This high rate (100 %) of tumour rejection (*Table 2*) suggests that the cured mice seem to be acquiring antitumour immune memory to GL261.

The tumour volume and TRI graph of a representative case (C1276) displaying immune memory is shown in *Figure 5.* For this specific case, tumour reached a maximum volume of 18.2 mm^3 at day 17 p.i., decreasing to 1.5 mm^3 after 7 TMZ doses. Two TRI cycles were observed during tumour-shrinking (Figure S4). The scar/residual abnormal mass caused by tumour growth was stable for one month; therefore, the mouse was declared as cured (see section 2.4.1). On day 74 p.i this cured mouse was re-implanted on the other side of the brain parenchyma with GL261 cells. No tumour mass was detected in the cured mouse brain within 3 months after tumour re-implantation

4 | DISCUSSION

4.1 | IMS-TMZ therapy triggering a repeated oscillatory TRI behavior

During this work, eleven TMZ-treated and six vehicle treated tumour-bearing mice were analyzed by MRSI-based nosological images (representative cases shown in Figures 1, S3-S6). Results confirmed the oscillatory response level (i.e. TRI values) of TMZ-treated mice due to changes in the metabolomic spectral pattern. This was already shown in a previous study with the standard 5-2-2 TMZ administration protocol in a smaller cohort of animals [14] but its reproducibility with the IMS-TMZ protocol had not yet been tested. Multi-slice MRSI acquisitions allowed us not only to monitor TRI oscillations along the period of transient response to therapy, but also to

sample intratumour heterogeneity. The TMZ administration seems to be the responsible of "setting" cycles, since the period between therapy administration and appearance of a TRI peak is consistent along different cases, ranging 4-8 days during regular transient response periods. Also, it has been shown that, prior or during tumour relapse, these oscillations disappeared, with a different TRI behavior: it either assumed an increasing, non-oscillating trend or only showed small, incipient increases. This confirms that consistent TRI oscillations are related to metabolomic changes due to tumour response, but mostly absent during tumour relapse. The average value for time lapse recorded between TMZ administration and TRI peak appearance (being TRI peak average 66.8 ± 22.3 % in all studied cases, n = 11) was 6.2 ± 1.5 days.

In control cases (Figures S5-S6), no TRI oscillations were observed, with the possible exception of case C1258, although no coherent tumour decrease was seen at possible TRI peak in C1258. This suggests that the lack of response to therapy leads to absence of clear TRI oscillation. In such cases, an increase in TRI could suggest local metabolomics changes resembling the ones observed during therapy (e.g. spontaneous host immune response), although these are clearly not enough to arrest tumour growth.

Results from TMZ-treated mice suggest that TMZ administration "resets" the immune system cycle clock, since TRI maximum peaks appear in average 6.2 ± 1.5 days after therapy administration (Figures 1, S3 and S4). This value is in agreement with work previously published by us with a non-metronomic TMZ administration schedule (distance between TRI maxima, 6.3 ±1.3 days, n=4, [14]) and also with calculations approached in single-slice acquired cases (distance between TRI maxima, 6.2 ± 2.0 days, calculated for n=3 cases from [29]). This value is also in line with the length of immune cycle in mice brain, described to be of ca. 6 days [33]. Moreover, taking into account that TMZ has already been described to behave as an immunogenic drug, which triggers the exposure and release of immunogenic signals [34,35], we hypothesize that each TMZ cycle is triggering a new 'turn' of the immune cycle leading to an arrival of new sub-clones of immune system cells within tumours ca. 6 days later. This would contribute to metabolomic pattern changes sampled by MRSI acquisitions, leading to changes in nosological images (see figure 6 for a schematic explanation).

Still, it is worth noting that TRI peak maxima are also accompanied, in some instances, by a reduction of the tumour volume characterizing either a partial response case (see Figure 2, case C1263 and C1270) or stable disease (case C1264). This reproducible behavior may underlie that a high TRI is indicating an active anti-tumoural response triggered by the immunogenic potential of TMZ.

The monitoring of TRI evolution could enable the design of personalized therapeutic schemes, adapting the TMZ therapy schedule in order to obtain an optimal anti-

tumour effect. Once fully validated that the 'green pattern' is associated with a productive action of the host immune system against the tumour, its presence would indicate that no further TMZ doses are needed until TRI starts decreasing, which would mean that a new, resistant clone of tumour cells is replacing cells killed by immune system action. The new TMZ administration would trigger a new turn in the immune system cycle, with priming and amplification of lymphocytes enabling them to kill this new tumour cells sub-clone.

One of the handicaps of the MRSI-based nosological image calculation technique is that small sized tumours do not produce confident segmentation (e.g. case C1263 in which the tumour zone was mislabeled as normal brain parenchyma, Figure S2B). We named this period "below threshold detection period" (BTDP) since the semi-supervised source analysis software was not able to properly segment the tumour. However, since small tumours have a trend to better survival or either cure, the metabolomics signature would not be as relevant as in tumours with larger volumes, in which this information is relevant to assess the efficacy of a therapeutic strategy.

These results demonstrate how non-invasive methods based on PR analysis of MRSI acquisition can be applied in order to improve therapeutic success, making it possible to develop enhanced and personalized therapies based on metabolomic information, which takes place before or in the absence of MRI volume-related changes.

Finally, if the relationship between TRI cycles and efficient host immune system attraction for tumour fighting is fully confirmed, we should be also able to evaluate and quantify the response level in combined treatments (e.g. synergistic TMZ+ anti-PD-1 or TMZ + CX-4945, an ATP-competitive CK2 protein kinase inhibitor described for preclinical GB treatment[30]), as well as to refine administration timing. Our surrogate biomarker for therapy response, coded in nosological images, was developed with TMZ-treated cases [29] and one of the fundamental questions then was whether it would be useful with other therapeutic agents. Indeed, it was also proven robust to detect tumour response in cyclophosphamide (CPA) treated mice[31], suggesting that the changes sampled are not restricted to TMZ, but rather being related to local changes during different treatments. Those would be probably related to immune system action against tumours, which could be an extremely valuable biomarker in tuning therapy administration to obtain maximal effectiveness and improve outcome.

4.2 | Histopathology results

Our working hypothesis is that the recorded TRI oscillations could be at least partially due to local metabolic changes resulting from immune system action within the tumour tissue. This hypothesis is supported by histopathological findings (section 3.3 of this manuscript), obtained from samples reported in [14]: TMZ-treated samples

presented a higher number of lymphocyte-like cells in comparison with untreated samples. This was further confirmed with immunostaining for CD3+ (T lymphocytes marker) and Iba-1 (microglia/macrophages marker). Values were significantly higher in tumour zones classified as "responding", in comparison with control cases or "unresponsive" areas. Moreover, the TRI imaging biomarker was proven to be correlated with the proliferation index Ki67 [14,29], also reflecting that metabolomics changes could be related to the proliferation status of tumour cells.

Results obtained in the present work reinforce the concept of IMS-TMZ as a good alternative for GB treatment. This hypothesis needs to be further validated in future work by assessing the recruitment of the host immune system elements through histopathological validation during TRI maximum and minimum values: in addition to CD3 and Iba-1, other markers such as FoxP3 (regulatory T lymphocytes) or specific markers for different polarization extremes of macrophages (M1 and M2 subtypes) will be useful. Importantly, this may be of relevance due to the fact that macrophages/microglia intimately interact with tumour cells and may contribute, depending on the cellular subtype, to tumour growth, migration and invasion of tumour cell, destruction of the extracellular matrix, neoangiogenesis and an immunosuppressive microenvironment. [36] Also, their quantification at different time points along therapy protocols would be of help to understand better their potential contribution to the metabolomics pattern of responding and non- responding tumours.

4.3 | A possible explanation for resistance to TMZ therapy in GL261 tumours: PD-L1 content increase

Immune evasion is one of the features of cancer[37], and overexpression of PD-L1 on the surface of the cancer cells is one way of escaping the immune system attack.[38] Several studies reveal that up-regulated expression of PD-L1 on tumour cells could be a potential link between chemotherapy and tumour immunoresistance [22–24]. In some cases studied in this work, we observed a transient therapy response to IMS-TMZ followed by fast tumour regrowth. WB results shows that increased PD-L1 content was found in late relapsing tumours when comparing to vehicle-treated tumours, which could provide a possible explanation for relapse in these mice.

In this respect, other authors have already described that treatment with alkylating agents (e.g., TMZ) trigger host immune system recruitment.[15] Our hypothesis is that one of the reasons contributing to GL261 tumour regrowth during IMS-TMZ would be the appearance of tumour cell sub-clones which up-regulate PD-L1. These PD-L1 over expressing tumour cells would be protected from productive T-lymphocyte attack and, consequently, would replace the tumour cell population not overexpressing PD-L1. This would finally lead to tumour escape due to the immunosuppressive effect of

increased PD-L1 content. With respect to control tumours, they should barely trigger the production of immunogenic signals due to the lack of damage caused by TMZ, thus resulting in less T cell infiltration into tumour tissue. Accordingly, no selection of tumour cells with increased PD-L1 expression for clonal expansion would happen.
In the case of "non-responding" GL261 tumours, results show that the expression level of PD-L1 is equivalent to vehicle-treated tumours, and lower than in late relapsing ones. Our hypothesis to explain this difference is that "non-responding" tumours would escape therapy by a different mechanism, not yet clarified, rather than "late relapsing" tumours, which could use high PD-L1 content for this. Initial tumour mass duplication time and, eventually, overall tumour mass could probably hamper on its own immune system capability of the host[39], then, no positive selection for PD-L1 high expression clones would be required in this instance for tumours to grow unencumbered. This result brings us significant insight for future research in preclinical GL261 GB, indicating that anti- PD-1 immunotherapy should be more effective as second line treatment in late relapsing GL261 tumours, while combined TMZ and kinase inhibitors such as CX-4945 may be best for fast growth, "non-responding" GL261 tumours. In addition, kinase inhibitors could contribute an added value through another pathway, impairing hypoxia-inducible factor (HIF) stabilization which in turn could lead to decrease of PD-L1 content.[40] These hypotheses are amenable to test in future work and can lead to further improvement of the outcome of tumour-bearing mice.

4.4 | IMS-TMZ therapy may establish long-term specific anti-tumour immunity.

Alkylating agents such as CPA and TMZ have been described to induce the immunogenic cell death of tumour cells by activating the immune system through the exposure and emission of damage-associated molecular patterns (DAMPs), triggering the recruitment of the host immune system [15,34,41,42] . However, as relevant as eliciting the immune system is avoiding to impair its amplification, and here the IMS gains importance. In the GL261 mouse model, TMZ produces a significantly better survival rate when administered in a 6-day cycle [30] instead of in daily schedule of 5 consecutive days [43]. Alkylating agents, including TMZ, are known to induce side effects related to the immune system such as leukopenia and neutropenia, when administered daily [44]. If TMZ is administered in a continuous schedule, the anti-tumour immune cycle may be hampered due to the inhibition of the proliferation of immune cells, such as primed CD8+ T lymphocytes. On the other hand, in a metronomic administration schedule, TMZ would not interfere with the proliferation of immune cells (time period in Figure 6C), since the next TMZ dose is administered after completion of the amplification step. This would be of benefit to establishing long-term specific anti-tumour immunity. Whereas all control, vehicle-treated mice developed rapidly growing GL261 tumours,

IMS-TMZ-cured mice resisted secondary re-challenge tumour development. Our preliminary findings in this suggest that antitumour immunological memory is established by the host immune system of cured mice. The efficiency to trigger antitumour immunity may be supported by the induction of immunogenic cell death by the TMZ effect on GL261 tumour cells[18]. In this respect, we cannot ignore that GL261 is a moderately immunogenic cell line[45]. Thus, a basal part of the response to therapies may be helped by this basal immunogenicity, like in "hot" tumours[46]. Nonetheless, it seems clear that this basal immunogenicity alone is not able to make C57BL/6 mice resistant to GL261 GB growth, in the absence of IMS-TMZ therapy (Figure S1B). Accordingly, this IMS-TMZ may promote long-term therapeutic responses through generation of immunological memory with concomitant prevention of tumour relapse. Wu and Waxman[17] found an increased number of CD8+ T cells and decreased number of circulating macrophages and myeloid-derived suppressive cells (MDSCs) populations in cured mice treated with "metronomic" IMS-like CPA treatment, which rejected the GL261 tumour on re-challenge[17]. In this sense, further work will be needed to explore the actual mechanism of antitumour immune memory in our system; thus, assessment of different immune populations could be performed in mice that rejected the re-implanted GL261 tumours compared to control tumours. Animal models of cancer are used to produce results of relevance for translation to human patients, eventually. In this respect, one relevant corollary of the described work from the imaging part should be that MRS/MRSI studies of glioblastoma patients during treatment should consider whether oscillatory variations comparable to the ones described in this work for GL261 GB under therapy may be happening, and exercise due caution interpreting single datapoint results to predict response to therapeutic strategies being evaluated.

In addition to the beneficial effects related to immune system, the IMS administration schedule also allows us to reduce the cumulative amount of administered TMZ and avoid consequent development of other tumours due to the mutagenic effects of TMZ, such as lymphomas[31]. Moreover, if a translational protocol reaches the clinical pipeline, the reduction of TMZ dosage should also decrease undesired side effects in patients. The curative effect of the IMS-TMZ protocol alone in 38% of the overall investigated mice, indicates that similar protocols, avoiding damage to the amplification phase of the immune cycle, could be of relevance to patients

5 | CONCLUSIONS

Results in this work confirmed that the IMS-TMZ protocol produced the expected oscillatory changes in the metabolomic pattern sampled by MRSI-based nosological images. This oscillatory pattern could act as a biomarker of the effective attack of the

host immune system onto the GL261 tumour during therapy monitoring, especially during transient response. TMZ administered using the Immune-Enhancing Metronomic Schedule produces significantly better survival rate than non-IMS protocols and may even establish long-term specific anti-tumour immunity in cured mice. With regard to GL261 GB tumours showing transient response to therapy followed by tumour regrowth, PD-L1 content increase in the tumour tissue could be a possible explanation for the recurrence. Further studies will be needed to assess the presence, type and amount of recruited host immune system elements at the point of maximum and minimum TRI values. This will help us to understand better their potential contributions to the metabolomic pattern changes detected. Finally, whenever the relationship between TRI oscillations and productive host immune system attraction for tumour fighting is fully demonstrated, we should be also able to use this to evaluate and quantify the extent of response in combination therapies, thereby further improving therapy scheduling and allowing for its personalization.

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Figure 1. Experimental schedule of the 37 mice harboring a GL261 GB included in this work, corresponding to the contents of Table 1.

Figure 2. Nosological images and graphical representation of the tumour volume evolution for the tumour region in the cases (A) C1263, (B) C1264 and (C) 1270. Tumour volume in mm^3 (black line, left axis) and the percentage of green, responding pixels (TRI) obtained taking into account total pixels counting (green line, right axis). In the upper part of every image, chosen time points show the evolution of the nosological images in four rows of colour-coded grids superimposed to the T2w-MRI for each slice. Vertical arrows indicate days of therapy administration. In the graph below, green shaded columns indicate TMZ administration days. TRI cycle duration (therapy administration to next peak maxima) are highlighted in every image. In (A), from days 31 to 37 it was not possible to evaluate TRI evolution because tumour volume was below the limit for confident MRSI segmentation, which we called below detection threshold period (BDTP).

*Figure 3. Boxplot showing **A)** $CD3^+$ positive cells/field (n=147) and **B)** % of Iba-1 positive immunostained cellular areas/field (n=148) in red and green areas of each MRSI grid of TMZ treated and control cases. Significant differences were observed for $CD3^+$ positive cell counting (p = 0.0009) and for Iba-1 positive immunostained cellular areas (p = 0.0011) with unpaired Student's t-test. The limits of the box represent quartiles 1 (Q1) and 3 (Q3) of the distribution, the central line corresponds to the median (quartile 2). The whiskers symbolize the maximum and minimum values in each distribution. Examples of **C)** $CD3^+$ and **D)** Iba-1 immunostaining in different analyzed fields of case C971 corresponding with green (responsive) and red (unresponsive zones). Nosological images obtained from Grid 1 of the case C971 superimposed to the T2w-MRI. Both green and red zones could be distinguished within the tumour, showing a heterogeneous pattern of response. The red and green areas from the nosological image have been manually drawn over the tumour (shown in red and green lines). One representative field has been selected in each area (red and green circle have the same area). In the circular fields shown, cell count was 6 and 3 positive cells for CD3+ and 15.54% and 10.49% of positive immunostained cellular areas for Iba-1, for green and red areas, respectively. Arrows point to positive cells. Magnification (40×).*

*Figure 4. Frozen dissected tumours from IMS-TMZ treated mice (A) WB for tumour total protein homogenate (80 µg) from different mice treated with IMS-TMZ (non-responding and relapsing tumours), n=9, compared with vehicle treated mice, n=3. PD-L1 and Tubulin proteins were analyzed. (B) Quantification of WB result including the non-responding, relapsing and control tumour samples, PD-L1 band intensity (after normalization to Tubulin) are 0.19 ± 0.02 in the non-responding group, 0.64 ± 0.14 in the relapsing group and 0.23 ± 0.04 in the control group. ***=p <0.001 for Student's t-test for the comparison among non-responding, relapsing and control group.*

Figure 5. Graphical representation of the tumour volume evolution, TRI cycles and re-challenge time point of cured mouse (C1276). T2w images shows that the tumour reached the maximum volume, 18.2 mm^3 at day 17 p.i., then tumour was ablated after 7 doses of IMS-TMZ therapy, and the scar

caused by tumour growth was stable for one month (cured). On day 74 p.i cured mouse was re-implanted on the other side of the brain parenchyma with GL261 cells, no tumour mass was detected in the cured mouse brain within 3 months (90 days post-rechallenge) after tumour cells re-injection.

Figure 6. Hypothetic schema of the cycle for immune response against a preclinical GB tumour after two therapy cycles and resulting nosological images, using as example images from case C1270. The whole cycle is thought to last 6-7 days in mouse brain. When treated with TMZ at day 0 (A), tumour cells release and expose immunogenic signals which attract dendritic cells (DCs) and macrophages to the tumour site. Initially (day 1-2), the immune system is not especially active against these particular tumour cell clones and the nosological images correspond mostly to actively proliferating tumour, thus TRI is low (B). At days 3-4, DCs migrate to the lymph nodes and prime naïve CD8+ effector T cells, which start to proliferate. It is important that TMZ (or any antiproliferative agent) not to be administered in this period because it would impair lymphocytes proliferation and hamper proper immune response. TRI may start increasing between day 3-4 (allowing for inter-subject variability) partially due to innate immune system action against tumour or to primed lymphocytes from a previous therapy cycle attacking the tumour again(C). At days 5-6 of the cycle, a new wave of effector T cells arrive at the tumour site and jointly with macrophages efficiently attack the tumour. In this period, we may observe a TRI peak maximum and, in some instances, even reduction in tumour volume (D).

Group	Longitudinal follow-up			Validation set				
				Western blot			Histopathology	
Treatment	IMS-TMZ		IMS-Vehicle	IMS-TMZ		IMS-Vehicle	5+2+2 TMZ	Non-treated
Mice code	C1263 C1264 C1270 C1380 C1383	C1276# C1281# C1284# C1285# C1286# C1382#	C1258 C1260 C1261 C1359 C1360 C1361	C1288 C1319 C1356 C1290 C1292 C1341 C1342	C1343 C1349	C1300 C1317 C1266	C1100 C1108 C971 C1022	C1110 C1111
Number of animals	17			12			6	
MR data	MRSI/MRI			MRI			MRSI/MRI	

Table 1. Table illustrating animal distribution in different groups investigated in this work. # means cured cases (see figure S3 for representative cases and section 2.4.1 for the "cured mice" criteria). Total "n" (35) is different from the n stated in section 2.2 and 3.1 (37) because 2 cases (C1345 and C1351) did not have full longitudinal follow-up with MRI and MRSI, but still, were followed up only with MRI (see section 2.4.1).

	Primary tumour implantation wt control mice	Tumour re-challenge IMS-TMZ cured mice
Mice with growing tumour	3	0
Mice rejecting tumour	0	8
Tumour rejection rate	0%	100%

Table 2. Comparison of GL261 tumour take rates between primary tumour implantation, and GL261 re-challenge in IMS-TMZ-cured mice.