Wu, S, Calero-Pérez, P, Villamañan, L, Arias-Ramos, N, Pumarola, M, Ortega Martorell, S, Julià-Sapé, M, Arús, C and Candiota, AP

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

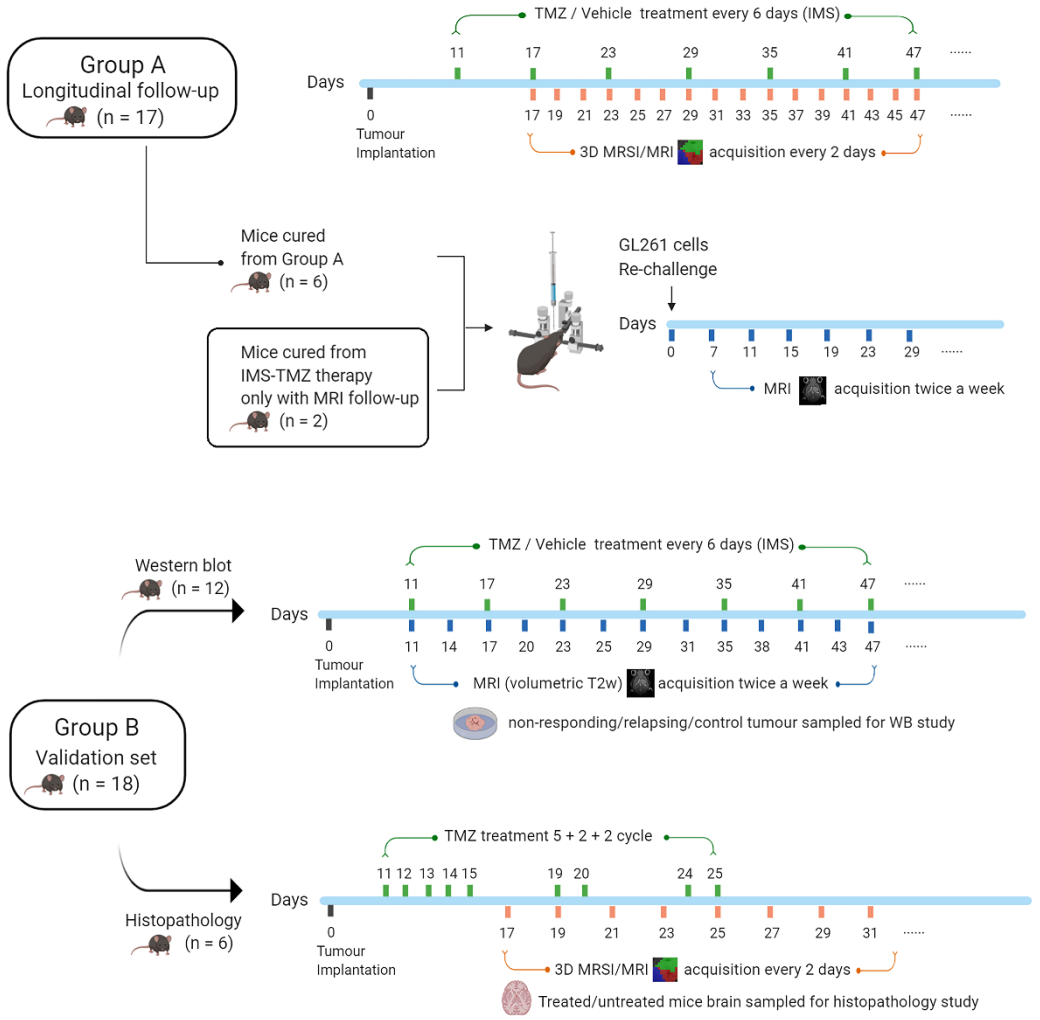

Figure 1. Experimental schedule of the 37 mice harbouring a GL261 GB included in this work, corresponding to the contents of Table 1.

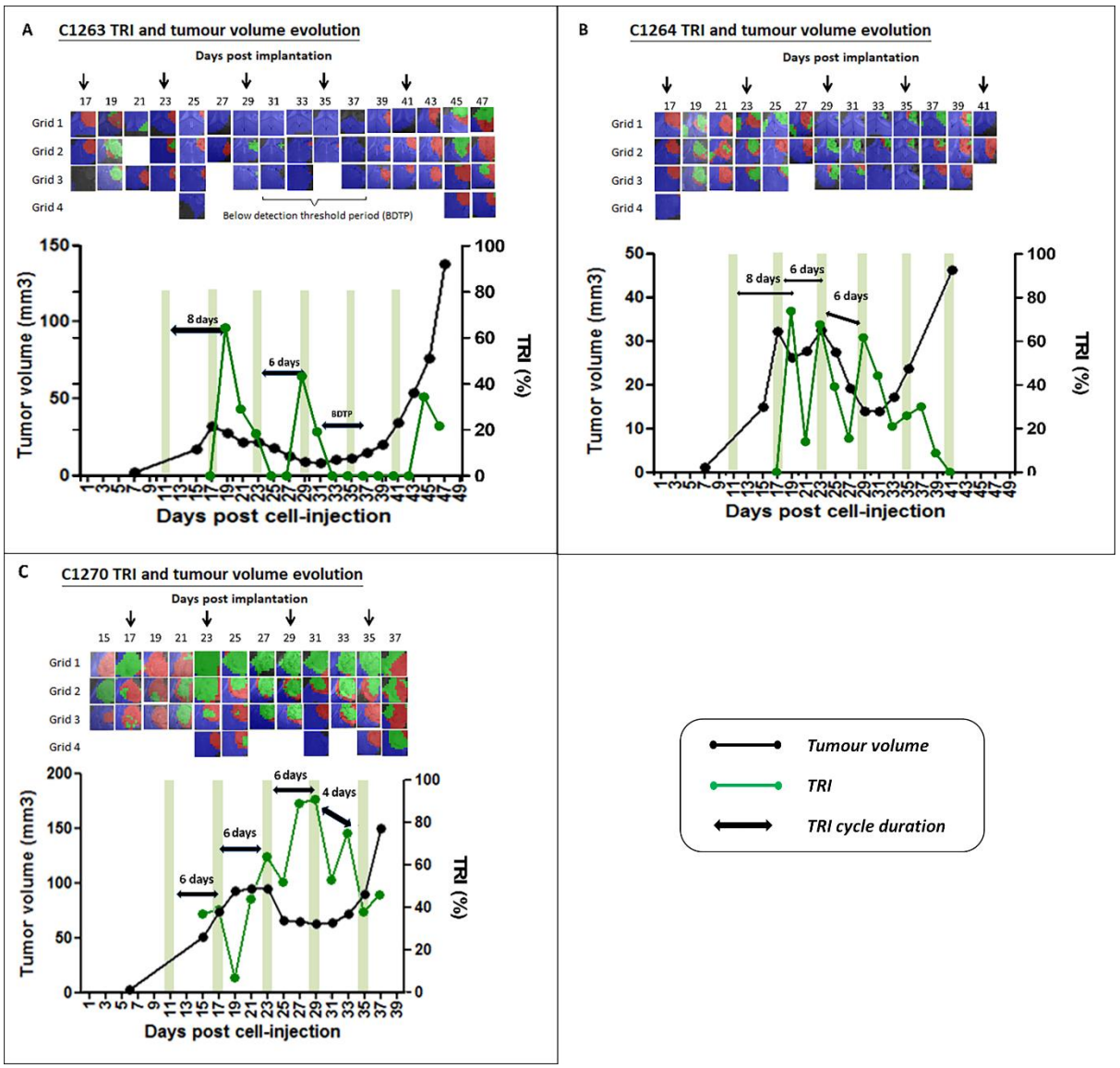

Figure 2. Nosological images and graphical representation of the tumour volume evolution for the tumour region in the cases (A) C1263, (B) C1264 and (C) 1270. Tumour volume in mm^3 (black line, left axis) and the percentage of green, responding pixels (TRI) obtained taking into account total pixels counting (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). TRI peaks appear after TMZ administration time points with a frequency of 6.4 ±1.3 days (n =3 mice with n = 9 total cycles counted).

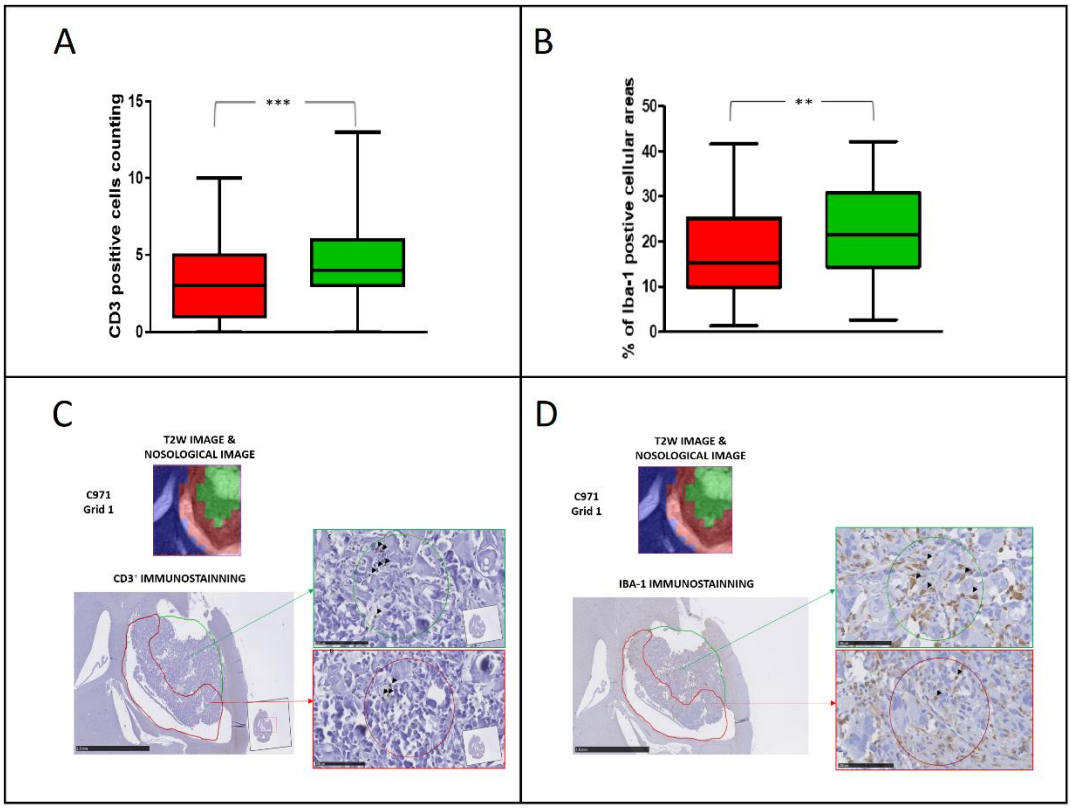

*Figure 3. Boxplot showing **A)** CD3⁺ positive cells/field (n=147) and **B)** % of Iba-1 positive immunostained cellular areas/field (n=148) in red and green areas of each MRSI grid of TMZ treated and control cases. Significant differences were observed for CD3⁺ positive cell counting (p = 0.0009) and 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×). （Data arises from control tumour n =2, and treated tumour n =4）*

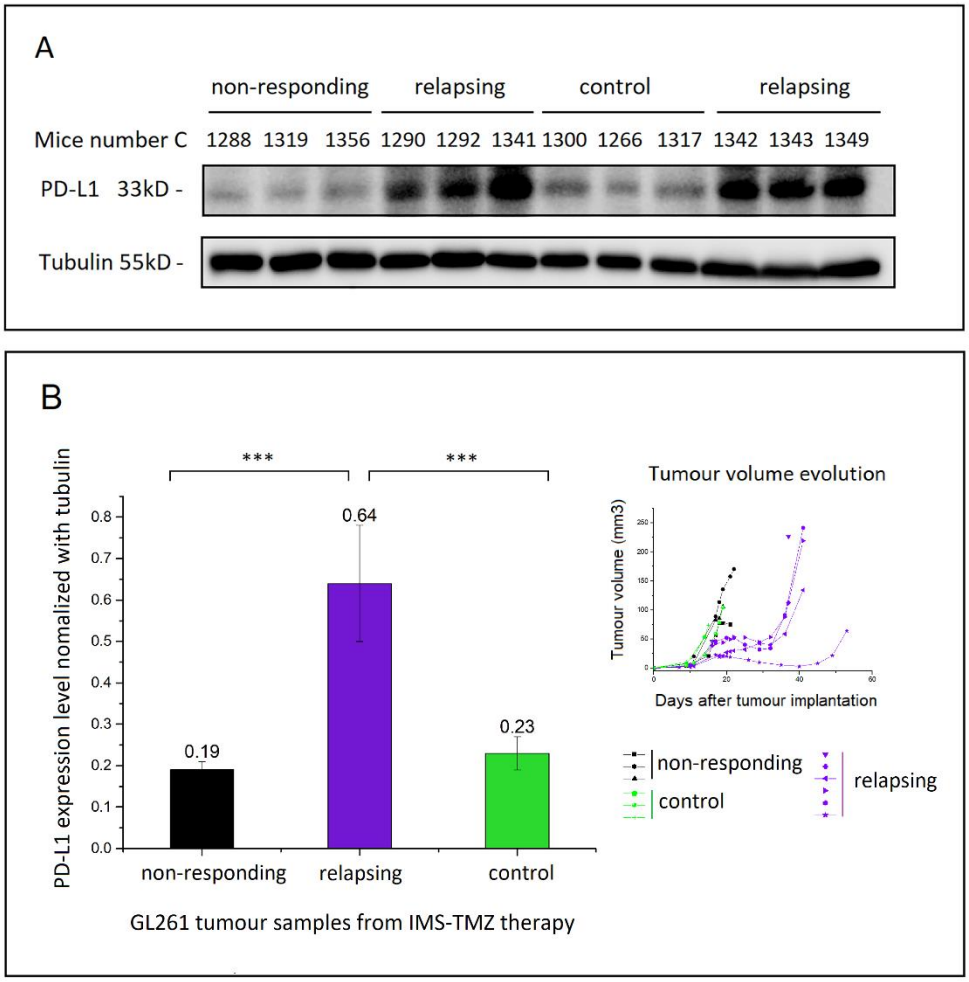

*Figure 4. Frozen dissected tumours from IMS-TMZ treated mice (A) WB for tumour total protein homogenate (80 µg) from different mice treated with IMS-TMZ (non-responding, n=3 and relapsing tumours, n=6), compared with vehicle treated mice, n=3. PD-L1 and Tubulin proteins were analyzed. (B) Quantification 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.*

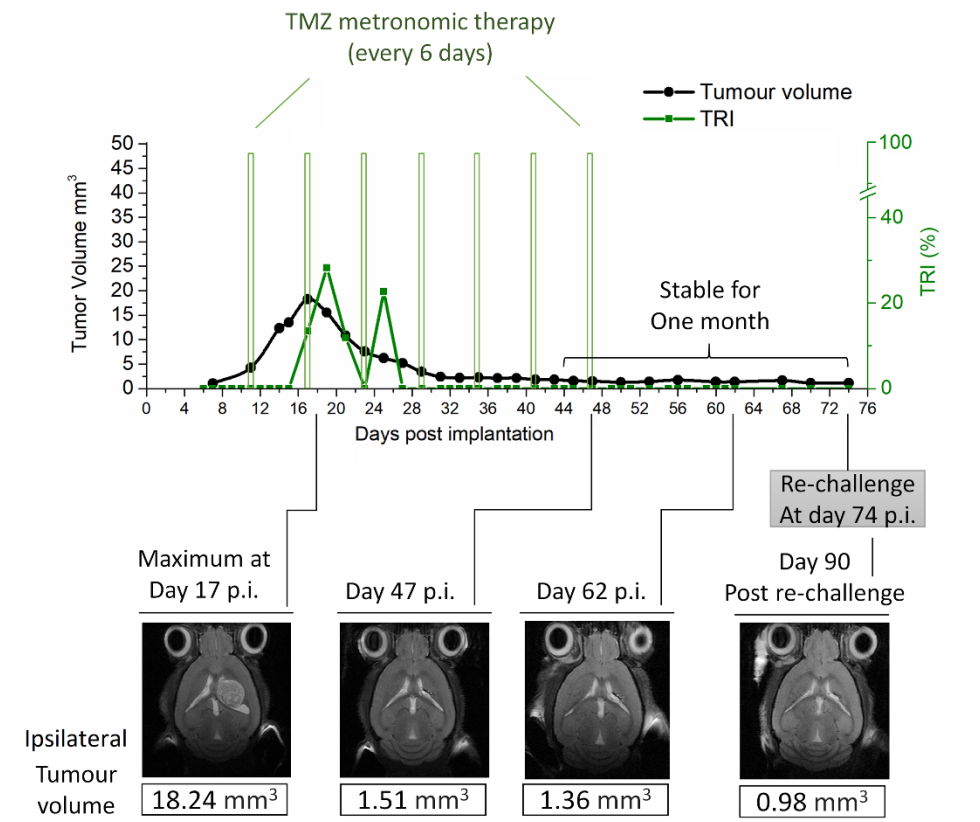

Figure 5. Graphical representation of the tumour volume evolution, TRI cycles and re-challenge time point of cured mouse (C1276). T2w images show that the tumour reached the maximum volume, 18.2 mm^3 at day 17 p.i., then tumour was ablated after 7 doses of IMS-TMZ therapy, and the scar caused by tumour growth was stable for one month (cured). On day 74 p.i the cured C1276 mouse was re-implanted on the other side of the brain parenchyma with GL261 cells, no tumour mass was detected in its brain within 3 months (90 days post-rechallenge) after tumour cells re-injection.

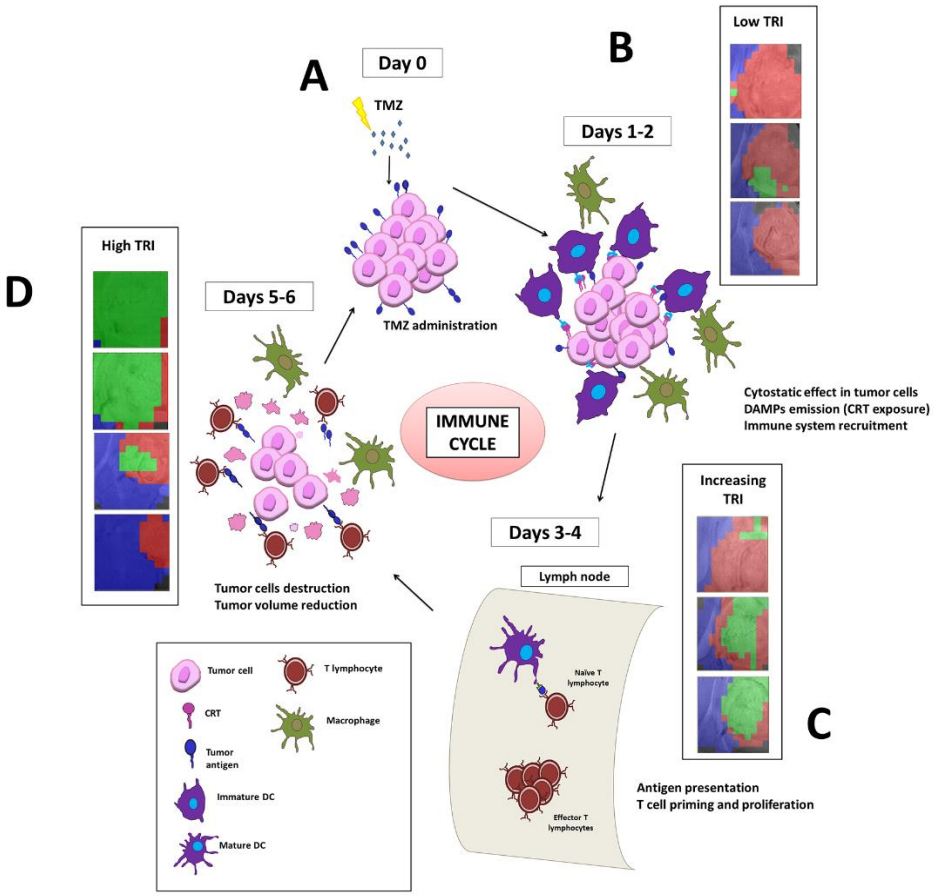

Figure 6. Hypothetic schema of the cycle for immune response against a preclinical GB tumour after two therapy cycles and resulting nosological images, using as example images from case C1270. The whole cycle is thought to last 6-7 days in mouse brain. When treated with TMZ at day 0 (A), tumour cells 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).