Agent-based modelling reveals the role of the tumour microenvironment on the short-term success of combination temozolomide/immune checkpoint blockade to treat glioblastoma

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LIST OF ABBREVIATIONS
ABM Agent-based model
ICB Immune checkpoint blockade
IMC Imaging mass cytometry
PCF Pair correlation function
PD-1 Programmed death protein-1
PD-L1 Programmed death ligand-1
TMZ Temozolomide

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ABSTRACT: Glioblastoma is the most common and deadly primary brain tumour in adults. All glioblastoma patients receiving standard-of-care surgery-radiotherapy-chemotherapy (i.e., temozolomide-TMZ) recur, with an average survival time of only 15 months. New approaches to the treatment of glioblastoma, including immune checkpoint blockade and oncolytic viruses, offer the possibility of improving glioblastoma outcomes and have as such been under intense study. Unfortunately, these treatment modalities have thus far failed to achieve approval. Recently, in an attempt to bolster efficacy and improve patient outcomes, regimens combining chemotherapy and immune checkpoint inhibitors have been tested in trials. Unfortunately, these efforts have not resulted in significant increases to patient survival. To better understand the various factors impacting treatment outcomes of combined TMZ and immune checkpoint blockade, we developed a systems-level, computational model that describes the interplay between glioblastoma, immune, and stromal cells with this combination treatment. Initializing our model to spatial resection patient samples labelled using imaging mass cytometry, our model’s predictions show how the localization of glioblastoma cells, influence therapeutic success. We further validated these predictions in samples of brain metastases from patients given they generally respond better to checkpoint blockade compared to primary glioblastoma. Ultimately, our model provides novel insights into the mechanisms of therapeutic success of immune checkpoint inhibitors in brain tumours and delineates strategies to translate combination immunotherapy regimens more effectively into the clinic.

SIGNIFICANCE STATEMENT: Extending survival times for glioblastoma patients remains a critical challenge. Although immunotherapies in combination with chemotherapy hold promise, clinical trials have not shown much success. Here, systems models calibrated to and validated against patient samples can improve preclinical and clinical studies by shedding light on the factors distinguishing responses/failures. By initializing our model with imaging mass cytometry visualization of patient samples, we elucidate how factors such as localization of glioblastoma cells and CD8+ T cell infiltration impact treatment outcomes.
KEYWORDS: glioblastoma, quantitative systems pharmacology, immune checkpoint blockade, temozolomide, agent-based models, computational immunology, imaging mass cytometry

INTRODUCTION
Glioblastoma is a rare but lethal brain cancer (Alifieris and Trafalis, 2015). Standard of care (SOC) for glioblastoma is maximal surgical resection, followed by radiation and chemotherapy with temozolomide (TMZ) (Stupp et al., 2005). If left untreated, most patients succumb to glioblastoma within three months after their initial diagnosis (Becker and Yu, 2012). SOC improves survival (Barker et al., 2012), but due to treatment resistance and despite its intensive treatment plan, the average survival time is only 15 months (Thakkar et al., 2014).

Immunotherapies such as immune checkpoint blockade (ICB) (McGranahan et al., 2019) have shown remarkable success in treating various cancers (Bausart et al., 2022). Immune checkpoints modulate the strength of the immune response and maintain self-tolerance (Ribas and Wolchok, 2018), but tumours exploit them to evade immune pressure. For example, programmed death ligand-1 (PD-L1) receptors on glioblastoma cells bind to checkpoint proteins (programmed death protein-1 (PD-1)) on CD8+ T cells to suppress the immune response (Pardoll, 2012; Han et al., 2020). ICB works by blocking PD-L1/PD-1 interaction, reactivating CD8+ T cell-mediated antitumoral immune responses. While successful against non-central nervous system cancers (Hazarika et al., 2017), recent clinical trials of ICB in glioblastoma have been disappointing (Lim et al., 2022).

The immune cycle in glioblastoma is a multi-step process that can be activated by combination therapies to overcome multifactorial immunosuppression (Bausart et al., 2022). For example, when in combination, chemotherapy improves cancer recognition by bolstering tumour antigenicity and immunogenic cell death, while ICB reduces immunosuppression by restoring T-cell activity (Leonetti et al., 2019). Hence, there has been an increased interest in
combining TMZ and ICB (Yan et al., 2018). The CheckMate 548 trial (Bristol-Myers and Ono Pharmaceutical Co, 2020) explored the efficacy of nivolumab with SOC. However, these trials did not substantially improve patient survival, highlighting the need to identify factors contributing to poor outcomes.

Many solid tumours exhibit significant inter/intratumor heterogeneity (Galon et al., 2006; Heindl et al., 2015), particularly in spatial distribution of cell types. The heterogeneous tumour microenvironment plays a significant role in determining cancer phenotypes by exerting selective pressure through a myriad of mechanisms (Yuan, 2016; Masud et al., 2022). With the advent of highly-multiplexed imaging technologies, including imaging mass cytometry (IMC), generating detailed information on the spatial configuration of the tumour became a reality (Chang et al., 2017; Elaldi et al., 2021). IMC is a labelling strategy that detects cellular biomarkers through magnetic labelling, providing simultaneous details on cellular phenotypes and spatial loci of cells.

Drug development involves lengthy clinical trials and substantial costs (Simoens and Huys, 2021). High failure rates, especially in oncology (Mohs and Greig, 2017), necessitate innovative approaches to guide drug development. Mathematical modelling is used in the development pipeline to offer insights into preclinical failures (Knight-Schrijver et al., 2016; Cassidy and Craig, 2019; Surendran et al., 2022). Previously, mathematical models have been employed to understand the effectiveness of mono/combination chemo/immunotherapies (Barazzuol et al., 2010; Lai and Friedman, 2017; Ayala-Hernandez et al., 2021), as well as to infer the growth and treatment response of glioblastoma (Harpold et al., 2007; Bottcher et al., 2018; Massey et al., 2019). However, most of these are continuum models based on ordinary and partial differential equations and do not incorporate the role of spatial heterogeneity and localized cell-to-cell interactions within the tumour microenvironment.

Agent-based models (ABMs) are a computational modelling framework that consider spatial configurations of distinct cell types in the tumour (Ghaffarizadeh et al., 2018; Surendran et al., 2018; Cess and Finley, 2020). They describe realistic interactions between
cells in tumours while accounting for the inherent stochasticity in cellular processes. Hence, ABMs are suitable for mapping the spatial organization of cancer, stromal, and immune cell components of glioblastomas to reflect the IMC output of patient resection data.

Building upon the previous success of ABMs in modelling glioblastoma treatments (Jenner et al., 2022), here we investigate the role of tumour spatial heterogeneity on combination chemo/immunotherapies. We construct an ABM of glioblastoma incorporating chemotherapy and immunotherapy and leverage IMC data of patient tumour resections to distinguish the spatial heterogeneity in glioblastoma tumours. By combining our data and model, we predict the influence of immune cells, particularly CD8+ T cells, as well as the impact of spatial heterogeneity on combination treatment success. Importantly, we validate our model's predictions against IMC data from brain metastases from patients with various types of primary tumours, which generally respond better to ICB compared to glioblastoma. Together, our work highlights the role of quantitative systems pharmacology frameworks for understanding the physiological basis behind drug efficacy and suggests new avenues to pursue experimentally and clinically to improve the use of immune checkpoint inhibitors to treat glioblastoma.

MATERIALS AND METHODS

Imaging mass cytometry acquisition and analyses

IMC on glioblastoma patient samples was performed as previously described (Karimi et al., 2023). Briefly, tissue microarrays containing formalin-fixed paraffin-embedded tissues were subject to deparaffinization and heat-mediated antigen retrieval according to manufacturer instructions (Ventana Discovery Ultra auto-stainer, Roche Diagnostics). Slides were rinsed with 1X PBS and blocked for 45 min at RT (Dako Serum-free Protein Block Solution). Metal-conjugated antibodies were combined into a cocktail at the appropriate dilution (Dako Antibody Diluent) and applied to slides at 4°C overnight. Slides were washed with 0.2% Triton X and 1X PBS, and a secondary metal-conjugated anti-biotin was applied for 1 hr at RT. Slides were washed with 0.2% Triton X and 1X PBS, and counterstained with Cell ID
Intercalator-Ir as per manufacturer instructions (Fluidigm/Standard Biotools). Slides were rinsed with distilled water for 5 min and air dried prior to image acquisition (Hyperion Imaging System). IMC data used in this study are from (Karimi et al., 2023), which details cell segmentation and lineage assignment. Briefly, cell segmentation was performed using a fully automated machine learning-based computer vision algorithm (Karimi et al., 2022). Lineage assignment was performed using a supervised approach based on canonical lineage markers.

**Agent-based model of glioblastoma**

We developed an ABM of glioblastoma that incorporates imaging mass cytometry visualizations of tumour resections from untreated glioblastoma patients to initialize the tumour. To the best of our knowledge, this is the first time that patient glioblastoma tumour imaging mass cytometry data have been combined with an ABM. Since glioblastomas show significant intra- and inter-tumour heterogeneity, with diverse cell populations contained in different spatial niches (Comba et al., 2021), the use of our modelling framework allows for realistically simulating inter-patient variability in factors such as constituent cell types, their distributions and proportions, and spatial heterogeneity. The model developed here extends our recent work modelling oncolytic viral therapy in glioblastoma (Jenner et al., 2022) to include the administration of chemotherapy and immunotherapy. These extensions are detailed here, with a brief overview of the existing model.

Our agent-based model was created using the PhysiCell software framework (Ghaffarizadeh et al., 2018), an open-source platform that enables biologically accurate modelling of cellular processes, including cell cycling, cell death and cell-to-cell interactions, among others. A partial differential equation-based biotransport system BioFVM (Ghaffarizadeh et al., 2016) is intertwined with PhysiCell to replicate diffusing substrates and cell signals within the tumour microenvironment. This biotransport system coupled with the agent-based model allows agents (cells) to dynamically update their phenotypes based on microenvironmental conditions. A detailed description of the PhysiCell platform is available in...
(Ghaffarizadeh et al., 2018) and (Jenner et al., 2022). In this work, we considered glioblastoma, macrophage, stromal, CD4+ and CD8+ T cells, their cell-to-cell interactions, and the effects of chemotherapy (temozolomide—TMZ) and immunotherapy (anti-PD1/PDL1 nivolumab). A schematic overview of the model is provided in Figure 1.

To mimic biologically realistic logistic growth in the absence of TMZ, glioblastoma proliferation was modelled to depend on the local cell density, as in (Jenner et al., 2022). For this, we assumed that cells which experience a net mechanical pressure from their neighbouring cells above a certain threshold \( P_{\text{max}} \) do not proliferate, whereas cells that experience overall mechanical pressure below this threshold proliferate have a proliferation rate \( \beta \). In this way, our growth model emulated a carrying capacity density for tumour growth where glioblastoma cell proliferation slows to a limiting value as more and more cells attain the threshold pressure and are unable to proliferate. As temozolomide is an alkylating agent that works by arresting the cell cycle to reduce cell division, eventually leading to apoptosis (Hotchkiss and Sampson, 2021), we assumed that its presence leads to reduced proliferation and increased death of glioblastoma cells.

To model the interactions between immune cells within the tumour, we included both innate (macrophages) and adaptive (CD4+ and CD8+ T cells) immune cell subtypes. Macrophages are known to eliminate dead cells through phagocytosis and can present antigens to T cells, thereby activating the latter cells (Hirayama et al., 2017). We therefore modelled macrophages as becoming activated through the phagocytosis of apoptosed glioblastoma cells (either through the effects of chemotherapy or through cytotoxic T cells, see below). Once activated, macrophages were modelled to activate any CD4+ or CD8+ T cells in their neighbourhood, defined to be twice the sum of diameters of macrophage and the corresponding T cell. CD4+ T cells can also become activated through direct interactions with a tumour cell in their neighbourhood. Once primed, CD4+ T cells secrete a chemokine which recruits CD8+ T cells through chemotaxis. These cytotoxic CD8+ T cells then target glioblastoma cells, causing their death through apoptosis (Jenner et al., 2022). We consider stroma as a composite of non-cancer and non-immune content of the tumour that holds
tumour tissue together. Hence, for simplicity, we model them simply as taking up space and acting as a structural component of the tumour, with no movement or proliferation.

Cell movement in PhysiCell is characterised by a net locomotive force ($\mathbf{F}_{loc}$) acting on a cell which contributes a velocity $\mathbf{v}_{loc}$ to that cell (Ghaffarizadeh et al., 2018). The cell velocity is then defined as

$$\mathbf{v}_{loc} = \psi \frac{(1 - b) \phi + b \mathbf{d}_{bias}}{\| (1 - b) \phi + b \mathbf{d}_{bias} \|}$$

where $\psi$ is the cell's movement speed, $\mathbf{d}_{bias}$ is the movement bias direction, and $b$ is the magnitude of the movement bias. Here, $\mathbf{v}_{loc}$ is thought to be a combination of random and biased motion, where the bias magnitude varies from $b = 0$ (corresponding to purely Brownian motion) to $b = 1$, which corresponds to deterministic motion along the direction of $\mathbf{d}_{bias}$. We set $b = 0$ for glioblastoma, macrophage, and CD4+ T cells because these cell types are modelled to undergo solely Brownian motion (i.e., no bias). Glioblastoma cells are known to move in a "stop and go" pattern, where the cell either moves or rests for a period of time (Gallaher et al., 2020). We follow the procedure described by (Gallaher et al., 2020) and Jenner et al., 2022, where cells are randomly assigned a migration status (stop or go) and the persistence times (time duration over which a cell either stays stationary or continues to move) are sampled from the distribution of persistence times (Gallaher et al., 2020). Cells that are assigned a "go" status move with Brownian motion. Once the cell exceeds its persistence time, a new migration status is randomly assigned. As mentioned above, the stromal cells are assumed to be stationary, implying that $\psi = 0$. The CD8+ T cells are biased to move up the chemokine gradient. Therefore, we fix $b = 0.85$ and $\mathbf{d}_{bias}$ to be the gradient vector of the chemokine at that cell location, i.e., $\mathbf{d}_{bias} = \nabla \rho_{chemokine}(\mathbf{r})$, where $\mathbf{r}$ is the location of the cell. With this choice, CD8+ T cells are designed to move up the chemokine gradient with some random motion (since the choice of $b < 1$).

Glioblastomas are known to express negative immunomodulatory surface ligands (e.g., PD-L1) that lead to reduced anti-tumour immunity and to T cell anergy (Himes et al., 2021). These PD-L1 receptors expressed on glioblastoma cells bind to checkpoint protein (e.g., PD-
1) present on CD8+ T cells to evade cytotoxic T cell targeting. Thus, we assumed that CD8+ T cell-induced apoptosis of glioblastoma cells can only occur in the presence of anti-PD-1 drug nivolumab which binds to PD-1 receptors and reactivates the immune response (see Treating glioblastoma using immune checkpoint blockade, below).

**Pharmacokinetic/pharmacodynamic models of temozolomide**

To model TMZ concentrations at the tumour site over time, we integrated the population pharmacokinetic (PopPK) model developed by Ostermann et al., 2004 to predict the concentration of TMZ in the cerebrospinal fluid (CSF). Ostermann et al. developed a three-compartment model with first-order absorption from the gastrointestinal tract to model plasma and CSF concentrations (Figure 2A) according to:

\[
\frac{dA_1}{dt} = -K_a A_1, \\
\frac{dA_2}{dt} = K_a A_1 - \frac{CL}{V_D} A_2 - K_{23} A_2 + K_{32} A_3, \\
\frac{dA_3}{dt} = K_{23} A_2 - K_{32} A_3, \\
C_{\text{plasma}} = \frac{A_2}{V_D}, \\
C_{\text{CSF}} = \frac{A_3}{V_P},
\]

where \(A_1, A_2\) and \(A_3\) is the amount of TMZ in the absorption, plasma and CSF compartments, respectively, \(K_a\) is the absorption rate constant; \(K_{23}\) is the rate constant from plasma to CSF; \(K_{32}\) the rate constant from CSF to plasma; CL, oral clearance; \(V_D\), volume of distribution in the central compartment. \(V_P\) is the volume of the distribution in the CSF and \(C_{\text{plasma}}\) and \(C_{\text{CSF}}\) are the TMZ concentrations in the plasma and CSF.

Within the agent-based model we developed, variations in local TMZ concentrations are tracked by the BioFVM biotransport system (Ghaffarizadeh et al., 2016). Hence, the TMZ concentration at the tumour site \(TMZ(t, x)\) is given by the equation:
\[
\frac{\partial TMZ}{\partial t} = D_{TMZ} \nabla^2 TMZ - \lambda_{TMZ} TMZ - \sum_{\text{cells } k} \delta(x - x_k) W_k U_k TMZ,
\]

where \( D_{TMZ} \) and \( \lambda_{TMZ} \) are the diffusion coefficient and the decay rate of TMZ, respectively. Here \( \delta(x) \) is the Dirac delta function, \( x_k \in \mathbb{R}^2 \) is the \( k \)-th cell’s position, \( W_k \) is its volume and \( U_k \) is its uptake rates. Equation 7 is solved using first order implicit operating splitting on a discretized grid of voxels with length 20\( \mu m \). We took the periphery of the tumour (denoted \( dB \)) to be the set of points \((x, y) \in \mathbb{R}^2 \) such that \( R^2 \leq x^2 + y^2 \leq (R + 20)^2 \), where \( R \) is the tumour radius. We imposed the boundary condition \( TMZ(t, dB) = C_{CSF}(t) \) such that at \( t = 0 \), \( TMZ(0, dB) = C_{CSF}(0) \) and 0 elsewhere.

The TMZ diffusion coefficient within the tumour can be estimated by using Stokes-Einstein equation (Valencia and González, 2011):

\[
D_{TMZ} = \frac{kT}{n \pi \eta r},
\]

where \( k \) is the Boltzmann constant, \( T \) is the absolute temperature, \( \eta \) is the solvent viscosity and \( r \) is the solute hard sphere molecular ratio. The parameter \( n \) is an integer whose value (6, 4 or 2) is dependent on the volume relationship between the solvent and solute and we set \( n = 6 \). The molecular mass of TMZ is 194.151 \text{ g/mol} (Ananta et al., 2016), which is equivalent to 0.194151 \text{kDa}. Converting this to a hydrodynamic radius gives \( r = 0.521 \text{ nm} \). The value of the Boltzmann constant is \( k = 1.38064852 \times 10^{-23} \text{ m}^2\text{kg s}^{-2}\text{K}^{-1} \). The absolute temperature of the cerebellum in the human brain is 37.3°C (Kiyatkin, 2010), or \( T = 310.45 \text{ K} \). The dynamic viscosity of the tumour was estimated using that of the extracellular matrix of glioblastomas (\( \eta = 5 \text{Pa s} \)), estimated by Santaguiliana et al. Thus, the diffusion coefficient of TMZ was taken to be \( D_{TMZ} = 5.2324 \mu m^2/\text{min} \) (Equation 8). As TMZ has a half-life of 1.8 hours - 2 hours (Svec et al., 2018), we set the decay rate to be \( \lambda_{TMZ} = 0.0064/\text{min} \). The uptake rate of TMZ by glioblastoma tumour cells \( (U) \) was estimated using the TMZ brain tumour influx and efflux rates determined by Ballesta et al. (Ballesta et al., 2014).
Assuming that the volume of the brain is 360ml and using their estimate for the volume fraction of the interstitial compartment gives an uptake rate of $U = 5.8639 \times 10^{-4}$/min.

TMZ induces cell death in proliferating cells by arresting the cell cycle. As such, to model the effect of TMZ and the induction of apoptosis in cells, we created a simple deterministic system describing the apoptotic effects of this interaction:

$$\frac{dGBM}{dt} = \beta \left(1 - \frac{TMZ}{TMZ + IC_{50}}\right) GBM,$$

$$\frac{dD}{dt} = \beta \frac{TMZ}{TMZ + IC_{50}} GBM,$$

$$\frac{dTMZ}{dt} = -\lambda_{TMZ} TMZ,$$

where $GBM$ is the number of glioblastoma cells, $TMZ$ is the temozolomide concentration, $D$ is the number of dead cells, and $IC_{50}$ is the half effect of TMZ on the death of glioblastoma cells. To adapt this system of equations for modelling stochastic cell division and death in the ABM, we leveraged PhysiCell’s inbuilt "Live" and "Apoptosis" models (Ghaffarizadeh et al., 2018). The "Live" cell division model allows each cell to stochastically divide according to its division rate $\varphi$ such that the probability of cell division in a given time interval, $[t, t + \Delta t]$ is given by (Ghaffarizadeh et al., 2018)

$$\mathbb{P}(\text{division}) = 1 - e^{-\varphi \Delta t} \approx \varphi \Delta t$$

We set $\varphi = \beta \left(1 - \frac{TMZ}{TMZ + IC_{50}}\right)$. Similarly, in the PhysiCell "Apoptosis" cell death model, the probability for a cell to die in the time interval $[t, t + \Delta t]$ is $\mathbb{P}(\text{death}) = \psi \Delta t$, where $\psi$ represents the death rate. Hence, we set the death rate of glioblastoma cells as $\psi = \beta \frac{TMZ}{TMZ + IC_{50}}$. As mentioned previously, tumour cells in the absence of TMZ proliferate at the exponential rate $\beta$. (Saha et al., 2020) computed dose-responses of the glioblastoma stem cells by measuring the viability of these cells 4 days after an initial dose of TMZ. Fitting the standard Emax effects curve, i.e.,

$$E(TMZ) = E_0 \left(1 - \frac{IC_{50}}{IC_{50} + TMZ^h}\right),$$
to their measurements gave an estimate of $IC_{50} = 0.0162 \mu g/\mu L$ using the molecular mass of TMZ (Supplementary Figure 1).

Finally, we considered the dosing schedule where $175 mg/m^2/day$ of TMZ was administered for 5 days, consistent with standard-of-care for glioblastoma (Ostermann et al., 2004; Stupp et al., 2005). Considering an average adult male has a body surface area of $1.9m^2$, this schedule corresponds to $332.5 mg$ of TMZ per day for 5 days (Figure 2B).

**Treating glioblastoma using immune checkpoint blockade**

The approved flat dosage regimen for the anti-PD-1 drug nivolumab is $240 mg$ every two weeks. A study by Lee et al. (Lee et al., 2018) established that the flat dosage results in similar exposure to regimens dosing $3 mg/kg$ once every two weeks. To model the concentration of anti-PD-1 drug at the tumour site, we leveraged the method developed by Storey et al., 2020 who used pharmacokinetic data from the phase 1 study (Brahmer et al., 2010) to derive a linear relationship between the dose of anti-PD-1 drug ($D_o$, in mg/kg) to plasma concentrations $C_{max}(D_o) (\mu g/mL)$ (Supplementary Figure 2). This relationship is given by,

$$C_{max}(D_o) = 20D_o + 9.2.$$  

Converting the units of $C_{max}(D_o)$ to $\mu M$ using the molecular mass of nivolumab as $143599.39$ g/mol (Chaudhari, 2017), we get

$$C_{max}(D_o) = 0.139D_o + 0.064.$$  

The change in concentration of the anti-PD-1 immunotherapy drug at the periphery of the tumour $dB$, denoted by $APD$, is represented by the equation:

$$\frac{d APD}{dt} = A(t),$$  

where $A(t)$ represents the source of the anti-PD-1 drug as a function of time. As mentioned above, the standard administration schedule of nivolumab is a single intravenous dose of $3mg/kg$, administered for one hour every two weeks. Computing $C_{max}$ for this dose gives
$C_{\text{max}}(3) = 0.481 \mu M$. For simplicity, we assumed this value as the baseline estimate for $A(t)$ during the one hour of anti-PD-1 drug administration (Storey et al., 2020). This implies that for each time $t_d$ (in the unit of minutes) at which anti-PD-1 is administered,

$$A(t) = \begin{cases} 0.481, & t_d < t < t_d + 60 \\ 0, & \text{otherwise.} \end{cases}$$

Variations in the local concentration of the anti-PD-1 drug through diffusion and decay are tracked by the BioFVM partial differential equation,

$$\frac{\partial ICB}{\partial t} = D_{\text{ICB}} \nabla^2 ICB - \lambda_{\text{ICB}} ICB.$$  

Similar to Equation 7, we used the boundary condition $ICB(t, dB) = APD(t)$ and initial condition $ICB(0, dB) = APD(0)$ and $ICB(0, \sim dB) = 0$ to solve Equation 18.

Using the Stokes-Einstein equation given in Equation 8 and taking the molecular mass of nivolumab as $143599.39 \text{ g/mol}$ (Chaudhari, 2017), we computed the diffusion coefficient of the immune checkpoint inhibitor to be $D_{\text{ICB}} = 0.6094 \mu m^2/\text{min}$. As nivolumab has a half-life of 15 days (Brahmer et al., 2010), we estimated the decay rate to be $\lambda_{\text{ICB}} = 3.21 \times 10^{-5}/\text{min}$.

We assumed that CD8+ T cells induce apoptosis in glioblastoma cells only when the number of ICB-bound PD-1 receptors on CD8+ T cells is above the threshold $N_T$. For each CD8+ T cell, we tracked the number of ICB-bound PD-1 receptors ($R_B$) and the number of the unbound PD-1 receptor ($R_U$). The immune checkpoint inhibitor and PD-1 receptor binding kinetics were modelled by the system of equations:

$$\frac{d}{dt} R_B = k_{\text{on}} N_{\text{ICB}} R_U - k_{\text{off}} R_B,$$

$$\frac{d}{dt} R_U = -k_{\text{on}} N_{\text{ICB}} R_U + k_{\text{off}} R_B,$$

where $k_{\text{on}}$ and $k_{\text{off}}$ are the binding and unbinding rates and $N_{\text{ICB}}$ is the amount of anti-PD-1 drug present at the location of CD8+ T cell. We converted the concentration of ICB in the
unit of $\mu M$ to molecules/$\mu m^3$ by multiplying it by the Avogadro number ($6.022 \times 10^{23}$). We set the initial number of unbound PD-1 receptors on CD8+ T cells as 3096, which is found to be the average number of PD-1 receptors expressed on CD8+ T cell (Cheng et al., 2013).

Quantification of spatial structure using pair correlation function

Since we were interested in exploring how the spatial heterogeneity of glioblastoma tumours affect combination chemotherapy and ICB therapies, we sought to define a metric to compare and quantify the spatial configuration of the patient samples measured by IMC. For this, we leveraged the pair correlation function (PCF) ($C_{ij}(r)$) expressed as a function of separation distance between cells ($r$) as in previous work in ecology (Agnew et al., 2014; Surendran et al., 2019; Surendran et al., 2020b; Surendran et al., 2020a). A PCF is defined as the ratio of density of pairs of cells with the square of the density of cells such that, in a population where cells are randomly distributed (complete absence of spatial structure), $C_{ij}(r) = 1$. Here, the subscripts $i$ and $j$ represent the cell types of the two respective cells that form the pairs. To calculate the PCF for any population of cells, a reference cell at a location $x_i$ is randomly chosen and the distances $r = |x_j - x_i|$ from all other cells to the reference cell are computed. This procedure is repeated for all the cells in the sample (i.e., all cells act once as the reference cell). Once all possible pair distances are calculated, the PCF is constructed by enumerating the distances between pairs of agents that fall into the interval $[r - \delta r/2, r + \delta r/2]$ ensuring a bin width of $\delta r$. Normalizing the bin count by a factor of $N_iN_j(2\pi r\delta r)/L^2$ ensures that $C_{ij}(r) = 1$ in the absence of spatial structure, where $N_i$ and $N_j$ are the number of cells of type $i$ and $j$, respectively, and $L$ is the length of the computational domain.

In a clustered spatial configuration, where pairs of cells are more likely to be found in close proximity, the PCF will have values $C_{ij}(r) > 1$ for short distances of $r$. Conversely, when cells are less likely to be found close together resulting in a segregated spatial
pattern, $C_{ij}(r) < 1$ for short distances of $r$. Thus, we measured the PCFs in each of IMC images to quantify the extent of spatial structure in live cell populations which adopt uniform, clustered, or segregated spatial structure to varying degrees. Note that we only considered clustering of cancer cells, given that CD8+ T cells were sparse in our patient samples.

However, plotting and comparing PCFs as a function of distance for a large cohort of patients can be computationally challenging. To compare various possible combinations of spatial structure among multiple tumours, we also defined a simplified measure of spatial structure that expresses the type and extent of spatial structure as a scalar quantity. Hence, we defined a summary statistic based on the area under the PCF curve, i.e.,

$$F_{ij} = \int_0^H (C_{ij}(r) - 1) \, dr,$$

as a convenient way to simply express the nature and extent of the spatial structure as a single number (Surendran et al., 2019). Thus, if cells are more (less) likely to be in proximity to others, then $F_{ij} > 0$ ($F_{ij} < 0$). If there is no correlation between cell locations, then $F_{ij} = 0$. Supplementary Figure 3 shows the distribution of $F_{CC}$ (i.e., the metric measuring the correlation between glioblastoma cells) for the patient tumour samples in the glioblastoma cohort. We found that $F_{ij}$ is insensitive to maximum distance between the cells, $H$, when we choose $H$ to be sufficiently large since the PCFs approach unity for large separation distances. In our simulations, we set $H = 500\mu m$.

**RESULTS**

**Increasing CD8+ T cell counts can improve immune checkpoint blockade efficacy**

We first investigated how mono- and combination therapy impact tumour growth. For this, we compared the increase in the number of glioblastoma cells under various treatment conditions: 1) no treatment (baseline), 2) TMZ monotherapy, 3) ICB monotherapy, and 4) combination treatment with TMZ and ICB. To simulate each of the four cases, the respective therapies not being administered were set to zero. In Jenner et al., two model initializations were established based on random cell distributions within the tumour (according to cell
counts established in ex vivo tumour slices) or “patchy” distributions (for more details, see the methods in (Jenner et al., 2022)). Before integrating patient IMC data to understand individual heterogeneity, we first studied the effects of treatment using these model initializations as a reference case to compare effectiveness of each of the different treatments. A snapshot of the initial configuration of all constituent glioblastoma, stromal, macrophage, CD4+, and CD8+ T cells for the random distribution of cells within the tumour is shown in Figure 3. To create this, we placed cells randomly on the computational domain by sampling an angle ($\theta \in U(0, 2\pi)$) and a radius ($d = \sqrt{U(0, 1)R}$), where $R$ is the radius of the tumour.

Beginning from this random initialization, we sought to understand how each of the four treatment scenarios described above impact on overall tumour dynamics (Figure 4A-D). While our model predicted an overall reduction in cancer cell growth under TMZ monotherapy (Figure 4B) compared to the case with no treatment (Figure 4A), this did not result in total removal of glioblastoma cells and there is still an overall positive growth in the presence of TMZ. This finding is consistent with clinical observations that patients receiving TMZ do not experience significant tumour reductions, particularly over short treatment periods. The ICB therapy was predicted to have a negligible effect on overall tumour growth (Figure 4C), most likely due to the low number of adaptive immune cells initialised in these simulations. While the notion that the central nervous system and brain are completely immune-privileged is increasingly being challenged, the overall presence of CD8+ T cells in the brain is low compared to other tissues (Urban et al., 2020). Our results suggest that the failure of ICB monotherapy in glioblastoma can be in part attributed to the low number of CD8+ T cells present in the tumour (see Figure 3). Lastly, in the case of combination therapy, although we found reduced glioblastoma growth, it was largely driven by the effects of TMZ and not the presence of ICB (Figure 4D), again due to the lack of cytotoxic T cells in the tumour field.
To better understand how CD8+ T cell recruitment to the tumour site affects treatment outcomes, we next varied the initial number of CD8+ T cells in the tumour domain by 5, 10, and 20 fold the initial amount (22 CD8+ T cells, see (Jenner et al., 2022)). We again simulated the administration of TMZ and ICB for each of these cases and looked at the change in glioblastoma cell counts in each of these scenarios. Note that initial parameters in ABM simulations, such as the number of glioblastoma cells and the tumour radius, can influence the individual tumour growth trajectories due to the pressure-dependent proliferation. However, our focus here is on looking at how varying initial CD8+ T cell count for a specific tumour sample impacts its growth dynamics. Our results showed a reduction in glioblastoma cell growth as the number of CD8+ T cells in the tumour increased (Figure 5). This result suggests that patients with higher numbers of cytotoxic T cells within their tumour would benefit more greatly from treatment with ICB, and further, that this biomarker may act as a differentiator between response and non-response.

**Reference source not found.**

**Spatial configuration of the tumour impacts on therapeutic success**

Glioblastomas are significantly spatially heterogeneous, as shown in the histopathological analyses in Jenner et al. (Jenner et al., 2022) which revealed critical differences in the size and distribution of cancer cell-enriched regions within their ex vivo tumour spheroids. Jenner et al. showed that this spatial heterogeneity determines the efficacy of oncolytic virotherapy beyond the impact of CD8+ T cell numbers. Therefore, to similarly elucidate how glioblastoma cell clustering and spatial heterogeneity impact on the therapeutic success of combination TMZ and ICB therapy, we next used our agent-based glioblastoma model to simulate two tumours with different (uniform and clustered) spatial configurations under combination TMZ and nivolumab. To generate the clustered configuration, we used the method described in Jenner et al. (Jenner et al., 2022) where they considered 50% of the tumour was comprised of cancer cell-enriched regions. We then randomly assigned five points as the centre of the clusters and sampled the size (radius) of
each of the clusters from a distribution informed by the approximate radius of clusters in the patient samples described in Jenner et al., 2022. Snapshots of the initial tumour samples with the uniform and clustered configuration are shown in Figure 6A and Figure 6B, respectively.

For the uniform and clustered tumour samples, we simulated the agent-based glioblastoma model under treatment by the combination regimen and quantified the change in glioblastoma cell counts. We found that the clustered tumour results in worse treatment outcomes (Figure 6D) compared to a more homogeneous cell distribution (Figure 6C). As seen in Figure 6E, the uniformly randomized tumour showed drastically decreased tumour growth over the short treatment period, suggesting that glioblastoma cells within clusters may get shielded from the drug action and thereby evade therapy within heterogenous tumours. This result may also explain the failure of mono-ICB in glioblastomas. Additional simulation results of tumour growths in absence of treatment in both clustered and uniform tumours (Supplementary Figure 4) further verify this observation, since both types of tumours have similar growth dynamics when no treatment is applied.

Interpatient spatial heterogeneity and treatment outcome

Given our previous results, we returned to our IMC data to predict how interpatient variability in tumour spatial configuration influences treatment outcomes. We used the pair correlation function expressed as a scalar metric of spatial structure ($F_{CC}$, see Equation 21) to rank patient samples according to the extent of spatial clustering. We then compared and quantified the extent of spatial structure and observed significant variability in the spatial organization these samples (Figure 7).

We then initialized our agent-based model according to four representative patient samples (Figure 7A-D) and simulated the administration of combination chemo-immunotherapy, focusing on the change in glioblastoma cell counts. Note that the number of cells of each type is not identical among these samples since they correspond to distinct
patients. Hence for comparison, we computed fold increases to glioblastoma cell numbers to accommodate differences in initial glioblastoma cell counts across samples. Consistent with the findings from our simulations based on the synthetic patient tumour sample (see Section: Spatial configuration of the tumour impacts on therapeutic success), we found that treatment outcomes worsened with increased clustering (Figure 8). These findings suggest that the variability in the spatial configuration of the tumours is also a significant factor in determining treatment success, and as in oncolytic virotherapy, efforts should be made to account for spatial heterogeneity for clinical treatment decision making.

**Predicted attributes of better responses remain consistent in brain metastases**

In the results described thus far, we identified that the extent of T cell recruitment and infiltration, and the spatial architecture of the tumour have a significant impact on responses to combined TMZ and ICB. To validate our findings, we used data from IMC analyses (see Methods) of brain metastases from primary lung cancers, breast cancers, and melanomas (Karimi et al., 2023) to establish whether their architectures and degrees of T cell infiltration differed from our glioblastoma samples, and whether these potential differences lead to better predicted responses. Brain metastases typically respond better than primary brain tumours to ICBs (Aquilanti and Brastianos, 2020; Lim et al., 2022). Hence, we investigated whether brain metastases had attributes similar to the predicted features of better responders, such as the presence of more T cells (Figure 5).

Leveraging the IMC spatial snapshots from our glioblastoma and brain metastases samples, we first computed CD8+ T cell counts and the cross-correlation between cancer cells and CD8+ T cells expressed as a scalar metric, i.e., \( F_{CT} \) (Figure 9A-B). Note that \( F_{CT} \) can be computed by following the method described in section quantification of spatial structure using pair correlation function, and by specifically considering the distances between cancer cells and CD8+ T cells. Computing the correlation between CD8+ T cells (\( C_{TT} \)) may provide insight into the possible occurrence of CD8+ T cell clusters and their potential impact on treatment efficacy. However, the number of CD8+ T cells in all the
samples we considered are relatively low, and under these conditions, accurately computing $C_{TT}$, is not possible. A two sample T-test performed using `ttest2` function in MATLAB with $\alpha$-significance level of 0.05 revealed a significant difference in the mean number of CD8+ T cells between these two cohorts, with brain metastases exhibiting overall higher CD8+ T cell counts (p-value of 0.000475). No statistically significant difference in the mean value of $F_{CT}$ was found between these two cohorts (p-value of 0.8403).

To explore potential differences in treatment responses between glioblastoma and brain metastases, we again chose a representative subset of patients and simulated the administration of combination TMZ and ICB therapy. We selected three brain metastases (Figure 9C-E) and two glioblastoma patients (Figure 9F-G) based on the density of CD8+ T cells and the relationship between CD8+ T cell and cancer cell localization calculated as the product of a sample’s CD8+ T cell count and $F_{CT}$ (with $F_{CT}$ calculated from the cross-correlation function, $C_{CT}(r)$, as described above—see Figure 9H-L). Put otherwise, this metric distinguishes patients with higher CD8+ T cell counts positioned closer to cancer cells. In all the cases considered here, we choose samples that maximized this metric.

Our results showed the fold increase in cancer cell counts after combination treatment to be consistent with our previous predictions, namely that tumours with higher recruitment/infiltration of CD8+ T cells respond better to combination chemotherapy and ICB, and clinical observations that brain metastases have better responses to immune checkpoint inhibitors as compared to primary glioblastomas (Figure 9M), thus validating our model’s predictions. These results further underline the role of tumour architecture and CD8+ T cell infiltration as a biomarker of response and non-response.

**DISCUSSION**

Glioblastoma continues to be one of the most lethal brain tumours, despite intensive standard-of-care treatment. New treatment modalities, like ICB, have been extensively tested both experimentally and in clinical trials with the goal of improving treatment
outcomes for this difficult-to-treat cancer. Regrettably, ICB has thus far failed to show efficacy with respect to survival in clinical trials. Consequently, a better understanding of the tumour microenvironment and spatial effects determining responses to ICB, particularly in combination with approved treatment modalities like temozolomide, is paramount. In response, we developed a computational agent-based model that incorporates chemotherapy and PD1/PD-L1 immune checkpoint inhibition to treat glioblastoma to predict the key factors affecting the effectiveness of combination therapeutic approaches. Notably, our model describes key immunological (e.g., macrophages, CD4+ and CD8+ T cells), phenotypic (e.g., stromal cells), and spatial interactions to provide a rational modelling framework for preclinical study.

Consistent with our previous study using an agent-based models of glioblastoma (Jenner et al., 2022), we first explored the role of CD8+ T cell numbers and localization on short-term therapeutic outcomes after monotherapies (TMZ or ICB alone) and in combination. Our model predicted that the efficacy of the immune response is a function of the amount of recruited CD8+ T cells. Since glioblastoma tumours are highly immunosuppressive and the ICB works not by directly targeting the cancer but rather enabling the patient's CD8+ T cells to attack and remove cancerous cells, a sufficient number of CD8+ T cells must be present in the tumour for ICB to be effective. Indeed, these results reiterate the fact that both CD4+ and CD8+ T cell recruitment and specificity play a major role in ICB therapy. A potential avenue for further research here is to test combinations of ICB with immunostimulatory agents or oncolytic viruses that kill tumour cells and causes the release of danger signals to elicit an effective and sufficiently strong antitumoral immune response.

Facilitated by imaging mass cytometry visualizations of patient tumour resections, we quantified the spatial configuration of the tumour microenvironment to assess the role of spatial heterogeneity on treatment outcomes. To better understand the effect of the tumour microenvironment, we considered random (i.e., largely spatially homogeneous) and “patchy” (i.e., spatially heterogeneous) tumours and used our ABM to predict how spatial
heterogeneity determines therapeutic efficacy. Varying microenvironmental conditions such as immune infiltration, nutrients, and drug diffusion exert selective pressure and shape cancer development. Treatment resistance due to selection is a major barrier to effective cancer treatments. Considering our results from a cancer evolution perspective, spatial heterogeneity can amplify these effects. For example, we found that the glioblastoma cells within clusters may get shielded from the drug action and thus evade therapy within heterogenous tumours. Thus, together, our results suggest that the varied spatial structure of glioblastomas contributes greatly to the challenges of treating these tumours. This is due to therapies not being equally effective throughout heterogeneous tumours (Figures 7 and 8), highlighting the need for customizing the treatments to target specific tumour microenvironments. However, taking account of tumour heterogeneity when designing treatment is practically difficult to achieve and should be an area of focus going forward, particularly in preclinical models for which we can obtain better quantification. Crucially, we validated our model’s predictions using IMC data from brain metastases, which have shown greater responsiveness to immune checkpoint inhibition (Aquilanti and Brastianos, 2020). Comparing representative samples from patients with glioblastoma to patients with brain metastases, we found that the number of recruited and infiltrating CD8+ T cells was determinant of the response to treatment, corroborating our model’s predictions of why ICB has not shown efficacy for the treatment of glioblastoma and underlining the role of CD8+ T cells for immune checkpoint inhibition success.

There are limitations to our approach. Most notably, we considered only the short-term (i.e., up to 7 days) effects of therapy on glioblastoma due to the computational complexity of long-term simulations of our model (which models several tens of thousands of cells/agents). In future work, we will integrate both homogenous (ordinary differential equations) models of combination glioblastoma treatment with TMZ and ICBs and our ABM to provide more comprehensive and long-term predictions of therapeutic scheduling while still accounting for the spatial effects of treatment. Further, our model simplifies the immunological response within the tumour (e.g., a thresholding effects for the ICB efficacy,
simplified accounting of immune cell subsets acting within the tumour). Future work should explore the impact of increasing the complexity of immunological interactions on model predictions.

Quantitative systems pharmacology and computational modelling are increasingly recognized for their contributions to preclinical drug development and for improving our understanding of the mechanisms underlying drug responses. Given the severity of glioblastoma, better insight into the drivers of response to new treatment modalities, like ICB, have the potential to provide significant benefits to patients. The modelling framework developed here is general enough to be adapted and extended to include other treatments, such as oncolytic viral therapy, in addition to the ones considered in this study. Thus, our study provides a rationale for mechanistic modelling in the preclinical space, and motivates the continued integration of experimental, clinical, and predictive models.

AUTHORSHIP CONTRIBUTIONS
Participated in research design: Surendran, Jenner, Quail, Walsh, and Craig.

Conducted experiments: Karimi, Quail, Walsh, and Craig.

Contributed new reagents or analytic tools: Surendran, Jenner, and Craig.

Performed data analysis: Fiset, Surendran, Jenner, and Craig.

Wrote or contributed to the writing of the manuscript: Surendran, Jenner, Karimi, Fiset, Quail, Walsh, and Craig.

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DATA AVAILABILITY STATEMENT
The computational code to implement our agent-based model is available on GitHub at
https://github.com/Anudeep-Surendran/GBM_TMZ_ICB
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**Footnotes**

**a) Funding information**

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b) **Financial disclosure statement**

No author has an actual or perceived conflict of interest with the contents of this article.

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**FIGURE LEGENDS**

**Figure 1. Glioblastoma TMZ and immune checkpoint inhibitor agent-based model.**

Schematic summarizing the interactions between different cell types as well as drugs. The agent-based model describes and tracks glioblastoma tumour cells, stromal cells, macrophages, and CD4+ and CD8+ T cells (see inset legend). The chemotherapy drug temozolomide (yellow) reduces proliferation and enhances the death of glioblastoma cells (purple). Macrophages (green) become activated through the phagocytosis of apoptosed...
glioblastoma cells (black). CD4+ T cells (orange) become activated through direct interactions with a glioblastoma cell. Once activated, CD4+ T cells secrete chemokines that attract CD8+ T cells (blue) through chemotaxis. CD8+ T cells are activated either by CD4+ T cells or macrophages. CD8+ T cell-induces apoptosis of glioblastoma cells in the presence of the immune checkpoint inhibitor drug nivolumab which binds to PD-1 receptors and reactivates the immune response.

Figure 2. Summary of the three-compartment pharmacokinetic TMZ model developed by Ostermann et al. A) The amount of TMZ in the GIT, blood, and brain (CSF) is modelled with Equations 2-6. Details on the population pharmacokinetic model can be found in Ostermann et al. 2004. B) Model simulations predicting the amount of TMZ in the GIT and plasma (top), and the concentration in the CSF (bottom).

Figure 3. Snapshot of random initial tumour configuration. Purple: glioblastoma cells. Pink: stromal cells. Green: macrophages. Red and blue: CD4+ and CD8+ T cells, respectively.

Figure 4. Treatment outcomes under different treatment modalities. ABM predictions of therapy effects on tumour growth under A) no treatment (baseline growth), B) TMZ monotherapy, C) ICB monotherapy, and D) Combination TMZ and ICB. Blue solid lines: mean predicted growth (6 replicates). Orange shaded region: variance of 6 model realizations. The insets in A-D show the snapshots of the final tumour configuration for each of the four treatment scenarios.

Figure 5. Enhancing CD8+ T cell numbers improves combination treatment outcomes. Glioblastoma cell numbers as a function of time after treatment with TMZ and ICB Red:
baseline number of CD8+ T cells. Blue: 5-fold increase. Green: 10-fold increase. Purple: 20-fold increase.

**Figure 6. Impact of spatial configuration of the tumour on treatment success.** A) Uniform random and B) clustered tumour initial configurations. Combination TMZ and ICB treatment outcomes on C) uniform and D) clustered tumours. E) Predicted effects of combination TMZ and ICB treatment on uniform (red) and clustered (blue) tumours.

**Figure 7. Patient samples display significant variability in spatial heterogeneity.** A-D) Representative samples of the initial tumour configurations of four patients according to IMC visualization results. Samples were visualized in our agent-based model based on cell coordinates measured by imaging mass cytometry (see Methods). E-H) Corresponding pair-correlation functions (red) for the tumours in A-D, respectively. From left to right: higher pair-correlation ($C_{cc}(r)$) values indicate higher clustering, whereas a $C_{cc}(r)$ value close to 1 denote tumours with uniform (homogeneous) configurations. The dotted black lines in E-H shows $C_{cc}(r) = 1$. The $F_{cc}$ values corresponding to the tumour samples in A-D are 130.36, 82.48, 79.81 and 6.18, respectively.

**Figure 8. Interpatient spatial heterogeneity affects combination treatment outcomes.** Fold changes in glioblastoma cell counts as a function of time predicted from simulations of combination TMZ + ICB blockade after initializing the agent-based glioblastoma model to representative patient spatial configurations (**Figure 7A-D**). The $F_{cc}$ values corresponding to the tumour samples in A-D are 130.36, 82.48, 79.81 and 6.18, respectively.
Figure 9. Comparison of combination treatment outcomes in primary glioblastomas and brain metastases. A-B) Violin plots of the distribution of CD8+ T cell counts and cross-correlation metric ($F_{cr}$), respectively. A two-sided T-test showed a statistical difference in the mean number of CD8+ T cells counts in brain metastases versus glioblastomas. No significant difference was found in the cross-correlation metric. Representative IMC visualization of the initial cell configuration in C-E) brain metastases (BM) and F-G) glioblastomas (GBM). H-L) Corresponding cross correlation functions for the tumors in C-G, respectively. M) Fold changes in cancer cell counts as a function of time predicted through simulations of the agent-based model initialized to the spatial configurations in C-G.
A

Dose

Gastrointestinal tract

Absorption

Plasma

CSF

Brain

Tumour

Elimination

B

Time (hours)

Plasma TMZ (mg)

GIT TMZ (mg)

CSF TMZ (mg/L)

Time (hours)
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Supplementary Information

Agent-based modelling reveals the role of the tumour microenvironment on the short-term success of combination temozolomide/immune checkpoint blockade to treat glioblastoma

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Supplementary Figure 1. Standard Emax effects curve fitting associated with section Pharmacokinetic/pharmacodynamic models of temozolomide. The standard Emax effects curve, $E(TMZ) = E_0 \left(1 - \frac{I_{max} TMZ}{IC_{50} + TMZ}\right)$ was fit to the dose response data of glioblastoma stem cells treated with TMZ from Saha et al., 2020. Fitting provided an estimate of $IC_{50} = 83.4 \mu M$ or $IC_{50} = 0.0162 \mu g/\mu L$. Blue dots show the dose response measurement along with error bar and red curve show the fit obtained using lsqcurvefit function in MATLAB.
Supplementary Figure 2. Figure showing the linear relationship between the dose of anti-PD-1 drug ($D_o$, in mg/kg) and plasma concentration $C_{\text{max}}(D_o)$ (μg/mL), related to section Treating glioblastoma using immune checkpoint blockade. Linear fit is obtained using polyfit function in MATLAB and the pharmacokinetic data from Brahmer et al., 2010.

Linear fit: $C_{\text{max}}(D_o) = 20 \, D_o + 9.2$
Supplementary Figure 3. Distributions of $F_{cc}$ (the metric measuring the correlation between glioblastoma cells) for patient tumour samples in the glioblastoma cohort. A) Patient samples arranged in the order of increasing clustering (increasing $F_{cc}$ values) and corresponding $F_{cc}$ values. B) Histogram of the metric $F_{cc}$. 
Supplementary Figure 4. Increases in glioblastoma cell counts in the absence of treatment for both uniform (red) and clustered (blue) tumours shown in Figure 6 in the Main Text.