



Escuela Internacional de Doctorado UFV

Programa de Doctorado en Biotecnología,
Medicina y Ciencias Biosanitarias

Tesis Doctoral

A Precision Oncology Framework for Adult Diffuse Gliomas Using Patient-Derived Organoids and Translational Drug Screening

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Madrid, 2026

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**A Precision Oncology Framework for Adult
Diffuse Gliomas Using Patient-Derived
Organoids and Translational Drug Screening**

Memoria que presenta

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Por la presente, la **Dra. Noemí García Romero** y el **Dr. Ángel Ayuso Sacido**, en calidad de **directores**, certifican que:

D. Álvaro Monago Sánchez, Graduado en Biotecnología por la Universidad Francisco de Vitoria, ha realizado bajo nuestra dirección su Tesis Doctoral, titulada:

“A Precision Oncology Framework for Adult Diffuse Gliomas Using Patient-Derived Organoids and Translational Drug Screening”, realizada en el Brain Tumor Laboratory, en los laboratorios de investigación de la Universidad Francisco de Vitoria.

Y para que conste, firmamos la presente autorización para la defensa de esta Tesis en Madrid a 28 de mayo de 2026.



Dra. Noemí García Romero



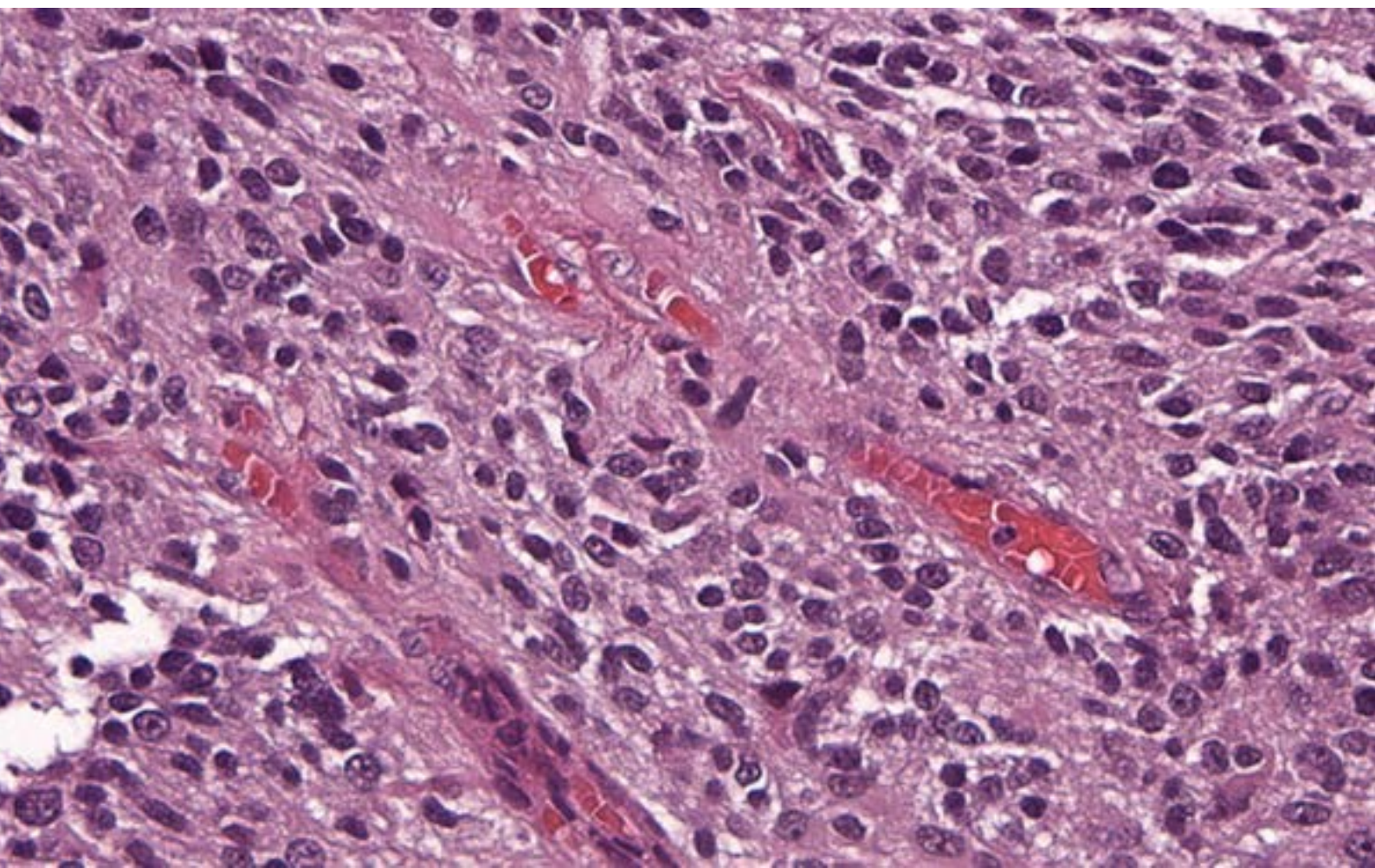
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La realización de esta tesis doctoral ha sido posible gracias a una ayuda de **Formación del Profesorado Universitario (FPU)**, otorgada por el **Ministerio de Ciencia, Innovación y Universidades de España**, con referencia **FPU21/02281**.

The completion of this doctoral thesis was made possible thanks to a **Formación del Profesorado Universitario (FPU)** grant, awarded by the **Spanish Ministry of Science, Innovation and Universities** under reference **FPU21/02281**.

AGRADECIMIENTOS



**“Si vienes, por ejemplo, a las cuatro de la tarde; desde las tres yo
empezaría a ser feliz.”**

- Antoine de Saint-Exupéry.

Hay un refrán muy famoso que dice algo así como “es de bien nacido ser agradecido”, y, en mi caso, tengo mucho que agradecer a mucha gente. Aunque mi nombre sea el que aparece en la portada de este documento, la realidad es que nada de esto sería posible sin todos los que me rodeáis o habéis formado parte de este viaje. A pesar de esto, con la locura que es una tesis, muchas veces se me ha olvidado agradeceros vuestro apoyo y todos lo que me habéis aportado en este camino. Así que, voy a aprovechar estas páginas para hacer esto mismo. Ya sabéis que soy un escritor nefasto, así que no puedo expresar con palabras todo lo que siento por todos vosotros (y, menos mal, porque si no sería más largo que la tesis en sí).

Quiero empezar por el **BTL**, el que ha sido mi segundo hogar estos años, un equipo con el que iría al final del mundo sin pensarlo dos veces, que no duda en quedarse hasta la hora que haga falta para ayudar a un compañero, y que se atreve desde a cortar tumores hasta a meterse con ordenadores cuánticos. Hemos pasado muchas cosas juntos estos años, bodas, nacimientos, becas, papers, pero también otras no tan bonitas, y, aun así, seguimos llegando cada mañana con ilusión (menos el lunes, por lo que sea). No creo que haya un equipo mejor en el mundo.

En concreto, a **Ángel**, muchas gracias en primer lugar por responder a aquel primer correo que envié (quizás al último al que me respondiste (es broma)) y confiar en mí para un proyecto tan ambicioso de los GBOs (ahora GOs), y continuar apostando por mí cuando llevábamos casi un año con unos 0 organoides que valiesen para algo, y mil problemas más. Creo que eres muy buen líder, nos tratas a todos con respeto y cariño, incluso cuando hacemos como que trabajamos ... Como científico, poco tengo que añadir que no se haya dicho ya, siempre me sorprenderá esa capacidad de hacer preguntas que a ninguno se nos habían ocurrido de una figura y de ahí sacar hasta un nuevo proyecto de tesis, un contacto con una empresa, etc. Ojalá, si algún día acabo dirigiendo a un equipo, pueda aplicar algo de que he aprendido de ti. A pesar de que ahora empieza una época de cambio para ti y el equipo, creo que vas a poder estar mucho más al pie del cañón, que me parece que es lo que más disfrutas, y, estoy seguro de que va a ser un nuevo comienzo fantástico.

A **Noemí**, no sé muy bien qué decirte aquí que no hayamos hablado los dos en persona. Gracias por dirigirme tan bien estos años, por los ratos en el fumadero (no os preocupéis que no fumo jajaja), por quedarte hasta horas infinitas cuando algo salía mal, por animarme cuando no salía absolutamente nada, por presionarme para que siempre dé el 100% y me supere. Sinceramente creo que hay poca gente como tú y que he tenido mucha suerte de encontrarte, a pesar de que sea un tipo desorganizado, caótico y a

AGRADECIMIENTOS

veces un poco soñador, creo que hacemos un equipo increíble. Gracias por haberme ayudado a creer más en mí mismo, por haberme forzado a hacer eso del “networking” y porque no me rindiera. Alabo tu capacidad de llevar 30 cosas a la vez y que todas salgan adelante, como me has enseñado a “responder mails”, así como todo el caos de estudiantes y proyectos. Nunca me olvidaré de estos ratos juntos y de todos los consejos que me has dado, algunos incluso los he apuntado y todo. Me gustaría creer que algún día pueda aportar en un equipo todo esto que tú nos aportas. Ahora también empiezas una nueva etapa en esto de la ciencia, pero no tengo dudas de que te va a ir bien, ya sabes, el que se atreve vence (o algo así dicen por ahí).

A **Pepa**, que más que una lab manager es una experta en todo lo que necesites y faro de sabiduría. Qué habría hecho sin ti... en cuanto a experimentos hacer la mitad de las cosas mal seguro, y en cuanto a lo personal, gracias por aquellas tardes en la café o desayunos cuando nos fallaban las fuerzas, cuando llegaban 3 pacientes en una semana (o día) o cuando no salía ninguna inmuno. Gracias por guiarnos a todos con infinita paciencia y repetirnos mil veces donde estaba todo o qué había que añadir a no sé qué, que a ver si alguna vez leíamos el protocolo. Gracias por llamarme para rescatar a Claudio (tremenda aventura) o por las risas manteniendo un equipo random con un “bueno desatornilla ahí del citómetro, si total ya está roto”. Creo que eres una científica brillante y una persona de mucho valor y cariño (aunque haya mañanas que no sonrías a nadie). Sé que te va a ir muy bien en lo que hagas, porque hay poca gente más perseverante y cabezota (en serio no he visto a nadie hacer la misma inmuno 20 veces).

A **Ana (Hernández)**, mi compañera de tesis desde el principio, cómo se te echa de menos en el laboratorio; eres una de las personas con mejor fondo de las que conozco, entregada a los demás y cariñosa; aparte de una estrella mundial del baile (que jamás me enseñaste ni un paso, ni que se me diese mal eso del baile). Uno de los 3 miembros originales del triángulo del mal aquí abajo cuando teníamos espacio en las mesas incluso. Gracias por todos los ratos e incluso tu ayuda cortando tumores hasta la 1 de la mañana alguna vez (mientras hacíamos un curso de no se sabe muy bien qué) a pesar de ser nuestra bioinformática de referencia. Gracias por invitarme a tu boda y por tus consejos y filosofía de vida también. Sé que no estás pasando tu mejor momento ahora mismo, pero también sé que podrás superar todo esto y más, tómate tu tiempo y vuelve con más fuerza, que esa tesis está ya a puntito a puntito (al final te gané, aunque alguna vez dijimos que no era una competición, pero ya sabes que yo no soy casi nada competitivo).

A **Laura**, la primera estudiante que después de pasar por nuestras manos y ver esta locura decidió empezar a hacer una tesis con nosotros. Creo que eres una persona maravillosa, alegre, bondadosa, trabajadora y soñadora, no pierdas eso nunca, incluso cuando la noche sea muy oscura y no se adivine el amanecer. Gracias por haberme aguantado ya unos cuantos años, por las risas, las bromas y todas nuestras cosas, por los congresos, por la ilusión de cada día. Estás en el camino de convertirte en una gran investigadora. Estoy muy orgulloso de que se te haya pegado algo de mí y hayas decidido ser igual de caótica que yo, que no creas en los cuadernos ni en apuntar las cosas, aunque de vez en cuando debas escuchar a tu directora. No te preocupes que esté donde esté vendré a presenciar tu tesis. Ya sabes si estás agobiada pues no te agobies y para adelante.

A **Sandra**, la más reciente incorporación al grupo. Qué alegría que estés con nosotros por fin, estoy seguro de que van a venir muchas cosas buenas aquí, papers, becas, etc. Creo que eres muy muy buena persona, trabajadora, sacrificada y servicial a todos tus compañeros. No sólo eso, sino que es una cantante excepcional, aunque a veces te de vergüenza. Gracias por contagiarnos a todos tus risas y ganas siempre, incluso después de llevar horas y horas de prácticas con alumnos tremendos, de frustraciones y de malos momentos. Ojalá todos pudiésemos ser un poquito más como tú cuando las cosas no salen bien. Yo opino que la gente buena atrae la suerte, así que estoy seguro de que tú la vas a tener siempre en la vida. Además, seguro que tu pedazo de paper está ya a la vuelta de la esquina.

A **Tomás**, uno de los tipos más fenómenos que conozco, médico, científico, escritor, influencer, etc. Me habría encantado poder coincidir más contigo y hacer aquello del metabolismo óptico. Gracias por enseñarme a estar un poco loco en el laboratorio, y hacer como que no escuchaba a la jefa cuando me convenía, la verdad es que me fue bastante bien cuando se me pegó un poquito de ti. Gracias también por haberme acogido para unas burgers y un paseo cuando nos reencontramos en Boston no hace mucho. Siento bastante haberte pegado el COVID (ya te vale no haberte tomado un solo paracetamol) y prometo acabar de leerme tu libro, aunque siento que carezco del léxico necesario para hacerlo cada vez que lo abro.

A **Rodrigo**, nuestro estadístico bioinformático o lo que haga falta de confianza, gracias por toda tu ayuda estos años, por intentar explicarnos a todos estos conceptos inexplicables y por tener paciencia en todos tus labmeetings.

A **Irina**, por su paciencia infinita con la ddPCR, por decir “buenos días jueves” cuando era martes y por haberme invitado al día más feliz de tu vida.

AGRADECIMIENTOS

A todos los estudiantes que han pasado por mi mano, especialmente a **Claudia**, **Carlos**, **Raquel (de la Flor)**, **Lucía (Parejo)** y **Nerea**; gracias por haberme ayudado tanto, animado, enseñado cosas de estas que dicen los jóvenes, por haberme enseñado a gestionar gente, a corregir mil veces póster de congresos y haberos quedado hasta que hiciese falta con una sonrisa siempre. Sois todas unas cracks y vais a conseguir lo que os propongáis, espero me invitéis a vuestras diversas defensas/tesis aunque hayáis tenido que aguantar mis chapas todos los días.

Por supuesto no me podía olvidar de la más reciente ampliación del **BTL**, toda nuestra sección matemática, en cuyos labmeetings entiendo palabras sueltas como “GBM”, “cáncer” o “fármacos”; es una suerte poder contar con todos vosotros, y ya por fin esta colaboración tan curiosa esté dando sus frutos. Supongo que nos queda un buen rato entrelazados cuánticamente. En concreto, a **Cynthya**, la que inició toda esta aventura y no tuvo miedo en meterse en la biología, así como de convencer a los estudiantes de venirse con nosotros; a **Jorge**, experto cuántico indiscutible, qué haríamos sin ti en todos estos proyectos; que conste en acta que, aunque Noe no entienda ninguna de tus bromas frikis, a mí me parecen una maravilla. También quería agradecer a **Natalia** y **Yedra**, por su trabajo con nosotros y lanzarse a la aventura del GBM e incluso ayudar en papers.

No puedo seguir sino con todos los demás compañeros de fuera del BTL, los que están ahí hasta horas interminables, los de las risas, los de comer a la 1 y el crotolamo ...

Empezamos por **Cristina**, la primera persona que me saludó (y me contó su vida entera) cuando entré hace ya unos años por la puerta. Gracias por todos estos años, por las risas por las bobadas y tus consejos; a pesar de tu carencia absoluta por cualquier criterio estético (a tus cubiertos tornasol me remito). Me da mucha pena que te hayas ido después de tantos años juntos, pero estoy seguro de que conseguirás lo que quieres, como siempre con mucho esfuerzo, pero con aún más cabezonería (no tanto como la mía) gritando algo así como “me ***** el *****”, tú ya me entiendes.

No podía faltar **Santi**, un auténtico macho alfa ibérico, pero que, en el fondo, tiene un corazón enorme. El tipo que instauró la tradición legendaria del Miyuki cuando había una buena noticia y con el que me ponía amarillo en ese mismo sitio. Qué habría hecho sin colgar memes por ahí contigo, sin hablar de fútbol de manera totalmente objetiva, sin nuestras bromas como la del piponazo y similares. Eres un tío grande (aunque los viernes no trabajes), y tu tesis va a ser una pasada. Más te vale no abandonar a tu futura mujer el día del parto si juega el Madrid la final de la Champions.

Seguimos con **Lucía (Benito)**, la única que sabe sumar del laboratorio (pero aparentemente eso no se enseña en la carrera de matemáticas). Gracias por jugar al ajedrez alguna noche a las 9 mientras esperaba un tumor, tu compañía y amistad inquebrantable. Gracias por reírte siempre de mis chistes malos (no por cumplir, sino porque aparentemente te hacen gracia). Confía en ti misma y sigue adelante cuando te caigas, porque creo que eres tremendamente capaz. Tranquila, también iré a tu tesis, de la cual seguro que no entiendo nada de nada, así que no creo que pueda preguntar. Ya sabes, si estás triste no estés triste, lloradita y pa adelante.

A **María (Ponce)**, una de las personas más buenas que tenemos aquí abajo en el sótano, gracias por apoyarnos a todos siempre, por estar ahí cada vez que alguien te necesita y poner un poco de cabeza y calma en este grupo de locos.

A **Lucía (Montero)**, una de mis personas favoritas de la UFV, gracias por tu alegría, por tu pasión constante, entusiasmo energía, etc. Por bailar algunas tardes, por las risas y las bromas. Qué contentos estamos todos cuando tú estás feliz.

A **Sara (Temprano)**, la todoterreno de los podoplaninos, gracias por enseñarme a seguir siempre adelante, a luchar para conseguir lo que me propongo, porque al final las cosas buenas acaban llegando siempre. Por aguantarnos al Santi y a mí (aunque te hacemos gracia) y querernos a todos mucho.

A **Ana (Lobo)** y **Marta**, y sí, las meto juntas en un pack porque son inseparables, gracias también por vuestras risas, por vuestros ánimos y por haber finalmente cedido a comer a la 1 todos los días. ¡Ya os queda menos de lo vuestro, seguid empujando todos los días!

Por supuesto a los más nuevos, a **David**, el otro macho alfa (deconstruido) del labo, amable, gracioso y siempre cercano, me alegro mucho de que al final te hayas decidido por esta aventura de la tesis a pesar de saber en la que te estabas metiendo. También a **Raquel**, que tuvo el valor de convertirse en Pepa durante unos meses y aún así mantuvo la cordura; y a **María (Ortiz)**, por su alegría en las comidas.

También a las chicas de **Cocina**, especialmente a **Sonia** y a **Estrella**, por todas esas tardes por los pasillos del sótano de la UFV, nuestras bromas y nuestras risas, por dejarme prestado algún reactivo o algo de extranjs y por vuestro cariño diario.

No me podría olvidar tampoco de mis **alumnos**, que por algún motivo desconocido deciden escucharme a las 8 de la mañana (a veces), y me animan a esforzarme y mejorar también todos los días para poder enseñarles, mientras a la vez me enseñan

AGRADECIMIENTOS

palabras y expresiones nuevas que ellos aseguran son en español; así como ruidos que ellos califican como “música” y “arte”.

Quiero también agradecer a la **UFV**, que ha sido mi casa durante muchos años ya, acogiéndome siempre con cariño y amor. A **Maite** y **Cruz** por su gestión, cercanía y alegría; a **Olga**, por haberme dado la oportunidad de participar en docencia y haber aguantado todos mis emails; a **Estela, Bárbara, Dani** y **Ester** por ser mis referentes en docencia, haberme enseñado cómo se hace esto de dar clase y haber compartido sus asignaturas conmigo.

Obviamente, no podría evitar mencionar a los de **esgrima**, otra gran familia y un pedazo de apoyo las tardes que necesitaba simplemente relajarme y liberar tensión. Quiero empezar por **Javi** y **Rodri**, aquel equipo que se forjó en el **Europeo**, que luego sería **CELC**, un equipo que apuntaba a poco, y que, al más puro estilo de la épica tiró de compañerismo y valor para ganar medallas en campeonatos de España. A **Álex**, que me ha tratado de entrenar (aunque siga sin hacerle caso en cómo colocar la punta) prácticamente desde el principio, y que me acogió en su club cuando lo necesité. A todos los demás, desde entrenadores como **Carlos** y **Fabián**, que se parten la espalda para que este club crezca cada día; a los chavales de competición, **Pablo(s), Calleja, Guille(s), Marqueta, Torres, Silvia, y los mongos varios ...** que le ponen ganas cada tarde y se atreven con cualquier campeón del mundo que pase por allí; a mi veterano favorito, **Damas**, por haberme enseñado que las lecciones de la esgrima a veces también valen para la vida; y a los de ocio, en especial a **Néstor** y **Jose**, que después de un día duro siempre están ahí para tirarte 5 asaltos con sus revanchas si es necesario. Sois todos enormes.

Cómo iba a seguir si no con los del cole. Gracias **Íñigo** por ser ese mejor amigo que está ahí todo el rato, por nuestras noches en tu casa jugando o viendo lo que sea. Por tu comprensión y apoyo cuando cancelaba alguna cena después de que me llegase un paciente sorpresa. Gracias por haberme enseñado 3000 juegos de mesa esto años, a pesar de que apenas entiendo las reglas de ninguno, pero oye, al final me gustó MTG, quién me lo iba a decir... quizás algún día consiga convencerte de jugar a DCS...

También a **Pelon** y a **Ixi**, ojalá nos viéramos más los tres, pero siempre es un lujo cuando nos juntamos y hablamos de los viejos tiempos y de los nuevos, gracias por estar ahí también después de tantos años.

No podían faltar aquí los chavales de **Ce mamó**, especialmente **Luis**, uno de los tipos más leales y comprometidos que he conocido, que apoya a sus amigos hasta el final de

las consecuencias y con el que te enfrentarías a cualquier cosa sin pensarlo dos veces, gracias por esas tardes de verano en las terrazas de Algete, por el fútbol, por los bocatas y los paseos interminables.

Para acabar, no podría ser de otra forma que, agradeciendo a mi **familia**, mi soporte y apoyo durante toda la vida, pase lo que pase.

Voy a empezar por mis **abuelos**, luchadores, trabajadores, que amaban a los suyos más que a nada. A **Agustina, Félix, Juan y Pilar**, gracias por todo el cariño a vuestros nietos, entre los que tengo la fortuna de encontrarme, y por todas esas enseñanzas que no te pueden dar en ninguna universidad.

A mis tíos, **Pilar, Jesús, Javier, Elena, Ana y Félix**, y a mis primos **Adrián, Sara, Sonia, Natalia y David** por todas las comidas, cenas, risas, cariño y amor que me habéis dado siempre.

A mis **padres**, mis primeros maestros que siempre me ayudaban a recitar la lección o a hacer los deberes, gracias a vosotros es que he podido llegar hasta aquí. Gracias por vuestra paciencia, vuestro amor incondicional e incluso aguantarme cuando era inaguantable. A **Papá**, por transmitirme esa chispa inextinguible de curiosidad infinita, por buscar cómo funciona cada cacharrito y encontrar un hobby nuevo cada semana, pero también por enseñarme algo de paciencia, de calma y de reflexión, a pensar y cuestionarlo todo. A **Mamá**, por enseñarme a amar a los demás y entregarse totalmente a los que quieres, por abandonarlo todo por mí, pero también por enseñarme sobre el valor y la lucha constante. Sois los mejores padres del mundo, y os quiero muchísimo, aunque no siempre os lo demuestre. Ojalá si alguna vez soy padre sea la mitad de bueno que vosotros.

A mi compañera en esta vida, **Paula**, a la que quiero con locura. No hay nada que me duela más que estar separados ni que me guste más que abrazarte siempre que tenemos ocasión. Eres la persona con el mejor corazón que conozco, y ojalá se me haya pegado algo de tu bondad (e incluso capacidad de planificar y organizar todas las cosas). Gracias por estar a mi lado todos estos años, por quererme pase lo que pase como lo haces siempre, por los paseos, los besos y las caricias. También por aguantarme todas las noches cuando nos llamamos y decía que iba a dejar la tesis, que no podía más que no quería más jornadas interminables, que nos habían rechazado el paper otra vez. Gracias por haberme animado y apoyado todas estas veces, dándome tu amor infinito todos los días. Al final, como muchas otras veces tenías razón y aquí estoy, aunque a veces no me lo crea. Estoy muy orgulloso de ti, eres un ejemplo para todos.

AGRADECIMIENTOS

Hay muchas más personas que también me han ayudado o han formado parte de mi vida a lo largo de estos años y seguro estoy olvidando, gracias a todos por formar parte de esto. Creo sinceramente que el amor y la música son las dos cosas que separan al ser humano del resto de criaturas de este planeta, así que gracias a todos por el amor que me habéis dado todos estos años, porque, como decía al principio, esta tesis es también de todos vosotros.

Contents

Resumen	1
Abstract	2
Abbreviations	3
1. Introduction	5
1.1 Introduction to cancer and CNS tumors	6
1.2 IDH-mutant gliomas	9
1.3 Glioblastoma, IDH-wildtype	17
1.4 Glioma modeling techniques	29
2. Hypothesis	38
3. Objectives	40
4. Materials & Methods	42
5. Results	63
5.1 Glioma organoids (GOs) establishment performance	64
5.2 GO characterization and model validation	65
5.3 Molecular characterization of GOs	85
5.4 Evaluation of immune system landscape in GOs	95
5.5 Guided drug-response profiling of GOs using clinically relevant glioma therapies	102
5.6 Unbiased screening and validation of non-glioma compounds in GOs	109
5.7 Validation of GO results in glioma cell lines and GSCs	119
5.8 <i>In vivo</i> validation of GO drug screening	123
6. Discussion	126
7. Conclusions	139
8. References	141
9. Annexes	179

Resumen

Los gliomas constituyen el tumor cerebral primario más frecuente en adultos. Este grupo de tumores incluye desde los gliomas IDH-mutantes, generalmente asociados a grados histológicos más bajos y a una evolución clínica más favorable, hasta el glioblastoma (GBM), el subtipo más frecuente y agresivo. Sin embargo, ambos presentan una marcada heterogeneidad inter- e intratumoral, así como una elevada capacidad de infiltración, progresión y recurrencia, lo que limita la eficacia terapéutica. En el GBM, el tratamiento estándar, consistente en resección quirúrgica máxima seguida de radioterapia y quimioterapia concomitante con temozolomida (TMZ), ofrece una mediana de supervivencia de aproximadamente 15 meses, y estrategias recientes como la inmunoterapia no han logrado mejoras clínicamente relevantes. En este contexto, existe la necesidad de desarrollar plataformas preclínicas que reproduzcan fielmente las características específicas de cada tumor y permitan identificar nuevas alternativas terapéuticas. Los organoides derivados de paciente representan una herramienta prometedora para el cribado terapéutico, al preservar rasgos clave del tumor original y facilitar potencialmente la selección de tratamientos.

El objetivo de esta tesis fue establecer y validar organoides de glioma derivados de paciente (GOs) como plataforma translacional y emplear un cribado transcriptómico para priorizar candidatos terapéuticos.

En este trabajo se establecieron GOs a partir de 61 pacientes con GBM (GOwts) y gliomas IDH-mutantes (GOMuts). Tras optimizar las condiciones de cultivo, se analizaron sus características histológicas y patrones moleculares, confirmando su conservación respecto a los tumores parentales y validando así el uso de los GOs como avatares tumorales. Una vez demostrada su fidelidad en ambos grupos, el estudio se centró en el GBM, por ser el subtipo más frecuente y agresivo. En este caso, se realizó un cribado *in silico* basado en datos transcriptómicos mediante la plataforma DiSCoVER para identificar fármacos candidatos, cuyos resultados se validaron *in vitro* en GOs y en otros modelos celulares clásicos de glioma, así como *in vivo* en modelos de xenotrasplante derivados de GOs y de líneas celulares de glioma.

En conjunto, estos resultados respaldan el uso de los GOs como una plataforma preclínica fiel y útil para el descubrimiento de nuevas terapias en gliomas. Además, la integración de modelos 3D con estrategias bioinformáticas se perfila como una aproximación prometedora para abordar la resistencia terapéutica. Como resultado de este estudio, alectinib y ruxolitinib se proponen como candidatos para futuros estudios preclínicos y, potencialmente, clínicos en gliomas.

Abstract

Gliomas are the most common primary brain tumors in adults. This group of tumors includes a spectrum ranging from IDH-mutant gliomas, generally associated with lower histological grades and a more favorable clinical course, to glioblastoma (GBM), the most common and aggressive subtype. However, both are characterized by marked inter- and intratumoral heterogeneity, as well as a high capacity for infiltration, progression, and recurrence, which limits therapeutic efficacy. In GBM, the standard of care treatment, consisting of maximal surgical resection followed by radiotherapy and concomitant chemotherapy with temozolomide (TMZ), provides a median survival of approximately 15 months, and recent strategies such as immunotherapy have not achieved clinically meaningful improvements. In this context, there is a need for preclinical platforms that faithfully reproduce the individual characteristics of each tumor and enable the identification of new therapeutic alternatives. Patient-derived organoids represent a promising tool for therapeutic screening, as they preserve key features of the original tumor and may facilitate treatment selection.

The aim of this thesis was to establish and validate patient-derived glioma organoids (GOs) as a translational platform and to apply a transcriptomic screening approach to prioritize therapeutic candidates.

In this work, GOs were established from 61 patients with GBM (GOwts) and IDH-mutant gliomas (GOMuts). After optimizing culture conditions, their histological features and molecular patterns were analyzed, confirming their preservation relative to the parental tumors and validating the use of GOs as tumor avatars. Once model fidelity had been demonstrated in both groups, the study focused on GBM, as it is the most common subtype with the worst prognosis. In this context, an *in silico* screen based on transcriptomic data was performed using the DiSCoVER platform to identify candidate drugs, and the results were validated *in vitro* in GOs and other classical glioma models, as well as *in vivo* in xenograft models derived from GOs and glioma cell lines.

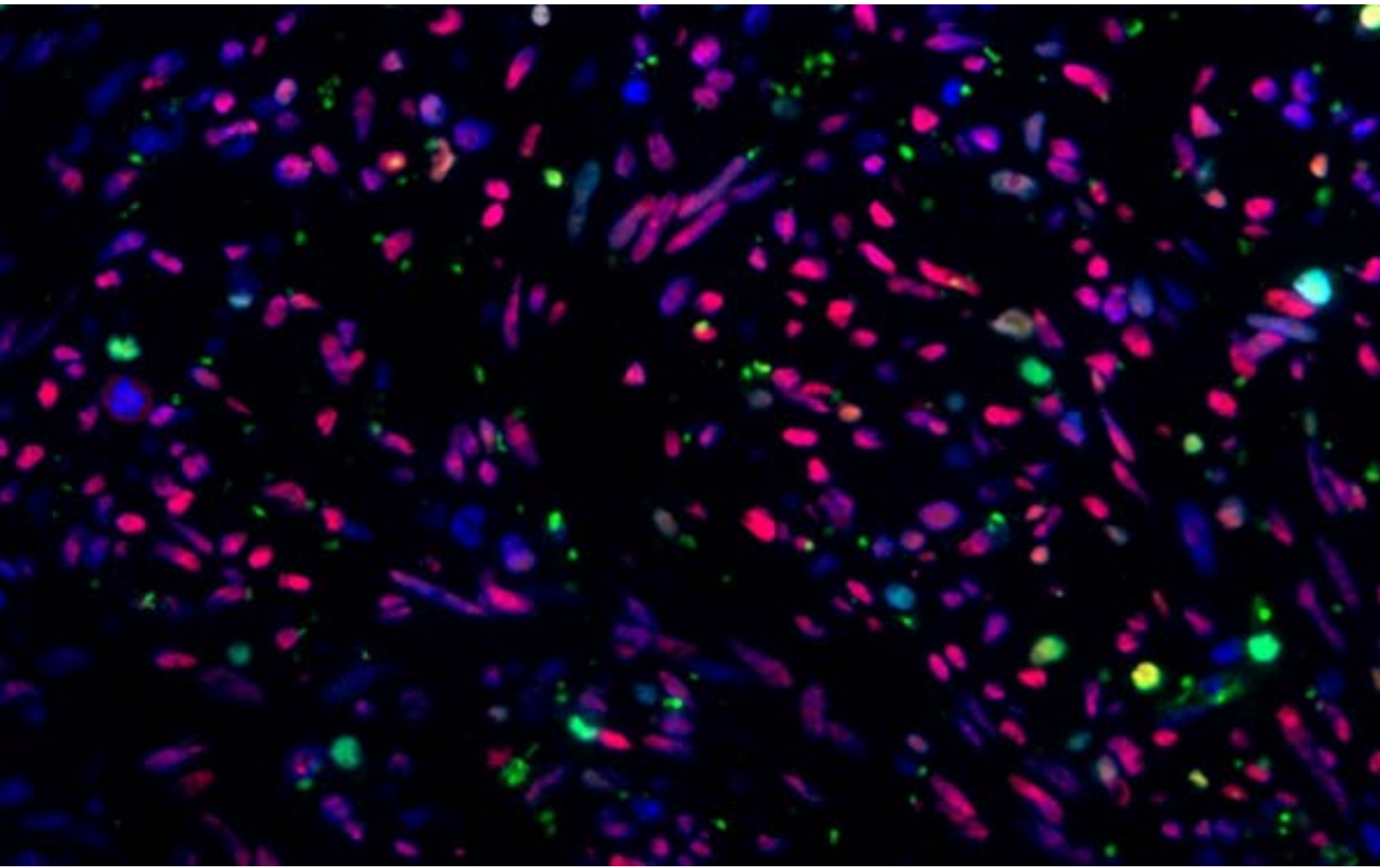
Overall, these results support the use of GOs as a faithful and useful preclinical platform for the discovery of new therapies in gliomas. In addition, the integration of 3D models with bioinformatic strategies emerges as a promising approach to address therapeutic resistance. As a direct outcome of this study, alectinib and ruxolitinib are proposed as candidates for future preclinical and potentially clinical studies in gliomas.

Abbreviations

- ALK: Anaplastic lymphoma kinase.
- BBB: Blood-brain barrier.
- BrdU: Bromodeoxyuridine.
- CAR-T: Chimeric antigen receptor T cells.
- cDNA: Complementary DNA.
- CNS: Central nervous system.
- CNV: Copy number variation.
- D-2HG: D-2-hydroxyglutarate.
- ddPCR: Droplet digital PCR.
- ECM: Extracellular matrix.
- EMT: Epithelial to mesenchymal transition.
- ER: Endoplasmic reticulum.
- FC: Flow cytometry.
- GBM: IDH-wildtype glioblastoma.
- GOmuts: IDH-mutant-derived glioma organoids.
- GOs: Patient-derived glioma organoids.
- GOWts: GBM-derived glioma organoids.
- GSCs: Glioma stem-like cells.
- GSEA: Gene set enrichment analysis.
- H&E: Hematoxylin and eosin.
- hEGF: Human epidermal growth factor.
- hFGF: Human fibroblast growth factor.
- IBDV: Infectious bursal disease virus.
- IC: Immune checkpoints.
- ICIs: Immune checkpoint inhibitors.
- IDH: Isocitrate dehydrogenase.
- IF: Immunofluorescence.
- IHC: Immunohistochemistry.
- IQR: Interquartile range.
- mOS: Median overall survival.
- MRI: Magnetic resonance imaging.

- MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
- MVP: Microvascular proliferation.
- NDV: Newcastle disease virus.
- O/N: Overnight.
- OOC: Organ-on-chip.
- PAS: Periodic acid-Schiff.
- PDE: Patient-derived explant.
- PDX: Patient-derived xenograft.
- PFA: Paraformaldehyde.
- PFS: Progression-free survival.
- PI: Propidium iodide.
- qPCR: Real-time quantitative PCR.
- RNA-seq: RNA sequencing.
- RT: Radiation therapy.
- RT^o: Room temperature.
- Sanger-seq: Sanger DNA sequencing.
- scRNA-seq: Single cell RNA sequencing.
- SD: Standard deviation.
- SNP: Single nucleotide polymorphism.
- SOC: Standard of care therapy.
- TEM: Transmission electron microscopy.
- TME: Tumor microenvironment.
- TMZ: Temozolomide.
- TTFs: Tumor treating fields.
- WHO: World Health Organization.

1. INTRODUCTION



“Somos la manera que tiene el Universo de mirarse a sí mismo”

- Carl Sagan.

1.1 Introduction to cancer and CNS tumors

1.1.1 General cancer overview and statistics

Cancer is a major cause of morbidity and mortality worldwide (1). Globally, more than 20 million new cancer cases are diagnosed each year with approximately 9.7 million deaths attributed annually, corresponding to an estimated lifetime risk of roughly one in five individuals developing cancer (2). In Spain alone, close to 300,000 new cases are diagnosed per year, and cancer remains among the leading causes of death, with overall 5-year survival across all cancer subtypes of approximately 50% (3,4).

Cancer comprises a heterogeneous group of diseases that can arise in many tissues but share core biological features. Historically, cancer was described as the consequence of cells escaping normal growth-control mechanisms and proliferating in an unregulated manner; in malignant disease, additional traits such as invasion and dissemination underpin morbidity and mortality (5,6). However, contemporary studies instead emphasize that tumor development and progression reflect diverse genetic, metabolic, and immunological processes, and that the tumor microenvironment (TME) is an active determinant of tumor behavior and treatment response (7,8).

Cancer burden differs substantially by tumor type and by sex. Overall, both cancer incidence and cancer-related mortality are slightly higher in men than in women. Among men, the most frequently diagnosed cancers are lung, prostate, and colorectal cancer, whereas among women breast, lung, and colorectal cancer are the most common. Mortality trends are comparable, though not identical: in men, lung, liver, and colorectal cancer are the leading causes of cancer death, while in women breast, lung, and colorectal cancer account for the highest mortality burden (2,9).

While not ranking amongst the most prevalent tumors in population-based registries, malignant primary brain and other central nervous system (CNS) tumors consistently rank among the cancers with the lowest survival, with overall 5-year relative survival on the order of 30%, depending on histology and age. Within this group, high-grade diffuse gliomas represent the poorest-prognosis entities, with 5-year survival in some subgroups approaching only 1-2% of cases (10,11).

1.1.2 Overview of CNS tumors

Malignant primary CNS tumors are relatively uncommon cancers, encompassing about 1.6% of all newly diagnosed cases, which translates to about 6.4 cases per 100,000 inhabitants globally (12). They arise within the intracranial and intraspinal compartments,

INTRODUCTION

affecting structures such as the cerebral hemispheres, brainstem, cerebellum, and spinal cord. Among primary CNS malignancies, gliomas, tumors with astrocytic, oligodendroglial, or ependymal differentiation, represent the predominant group and account for the large majority of malignant primary CNS tumors in population registries, corresponding to about 80% of cases (13,14).

Historically, diffuse gliomas were classified and graded by the World Health Organization (WHO) primarily using histopathological criteria, including cytologic atypia, mitotic activity, microvascular proliferation, and necrosis, often accompanied by pseudopalisading. This approach assigned tumors to grades I–IV, with higher grades generally associated with more aggressive clinical behavior and poorer prognosis (15).

However, histopathology alone can have limited reproducibility and prognostic resolution, and tumors with similar histologic grade may follow divergent clinical behaviors. Therefore, and thanks to the rise of omic technologies, the latest classification of CNS tumors by the WHO, published in 2021, has reclassified gliomas in accordance with both histological and molecular parameters, with an increasing emphasis on the latter, as a way to better subclassify gliomas into more homogenous entities to increase diagnostic precision, and, hopefully, therapeutic stratification. Moreover, in this classification, CNS WHO grades are expressed with Arabic numerals rather than the Roman numerals used in previous editions, to avoid confusion with histological grading (13).

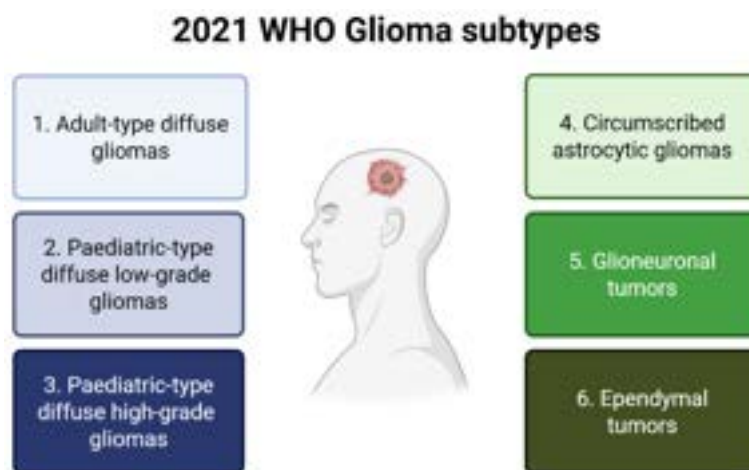


Figure 1: WHO glioma subtypes. Schematic representation of the 6 main subtypes of gliomas as defined in the 2021 WHO classification of CNS tumors (16). Each box represents a different tumoral entity.

According to the WHO, gliomas can be divided into 6 groups (Fig. 1):

1. Adult-type diffuse gliomas.
2. Paediatric-type diffuse low-grade gliomas.
3. Paediatric-type diffuse high-grade gliomas.
4. Circumscribed astrocytic gliomas.
5. Glioneuronal tumors.
6. Ependymal tumors.

Groups 1-3 represent the grand majority of cases, with this latest classification placing special emphasis on the distinct characteristics of adult and paediatric diffuse gliomas. Group 1 constitutes the bulk of neoplasias treated in neuro-oncology practice, as these tumors more frequently arise in adults. Within group 1, we only find tumors with astrocytic and oligodendroglial origin. Moreover, this new classification improves on the previous grouping by presence of mutations in the isocitrate dehydrogenase (IDH) gene (16–18) (Fig. 2).

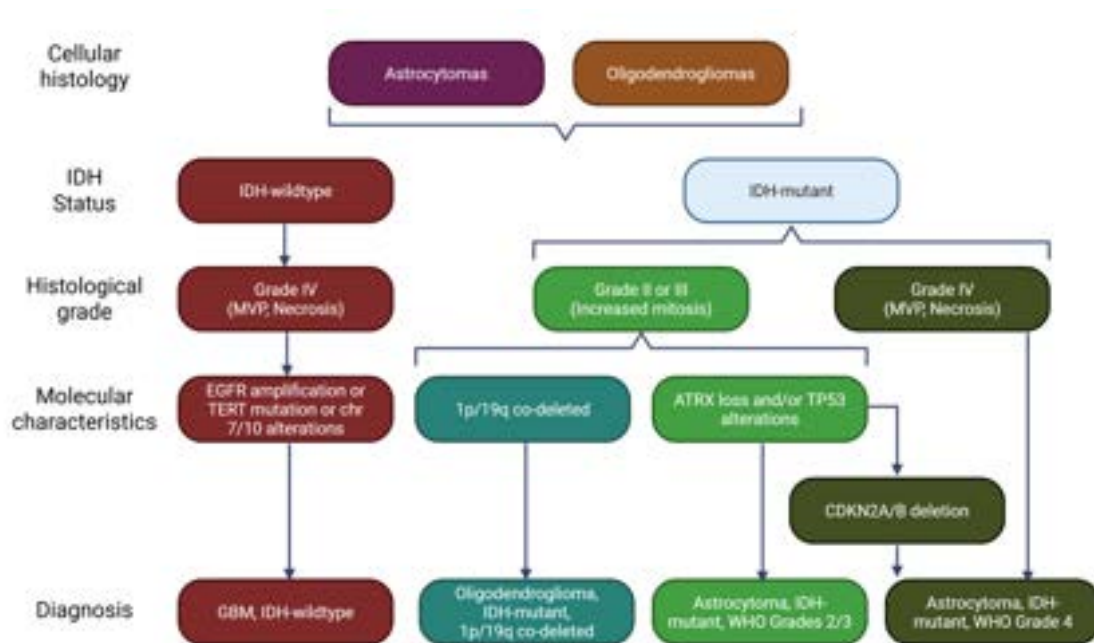


Figure 2: Overview of WHO group 1 adult gliomas classification. Flow chart of diagnostic workflow. Group 1 tumors include astrocytomas and oligodendrogliomas, which are broadly subdivided into groups according to their IDH mutation status, and then again into smaller subdivisions based on molecular characteristics. Each tumoral entity is color coded. Adapted from WHO CNS5 2021.

1.2 IDH-mutant gliomas

1.2.1 IDH genes and common mutations in gliomas

The *IDH* gene family consists of 5 distinct genes: *IDH1*, *IDH2*, *IDH3A*, *IDH3B* and *IDH3G*, which encode for the IDH1, IDH2 and IDH3 enzymes, respectively. IDH1 and IDH2 are the predominant isoforms, and are involved in cellular metabolism, catalyzing the conversion of isocitrate to α -ketoglutarate, a key metabolic node in the Krebs cycle, while generating NADPH. Mutations in these enzymes often confer gain of function, allowing for subsequent conversion of α -ketoglutarate into D-2-hydroxiglutarate (D-2HG) and consumption of NADPH. The accumulation of D-2HG not only affects normal cellular metabolism, but also has deeper effects at the epigenetic level, by inhibiting α -ketoglutarate dependent TET DNA demethylases and JmJc histone demethylases, leading to widespread epigenetic reprogramming and characteristic hypermethylation patterns (Fig. 3) (19–21).

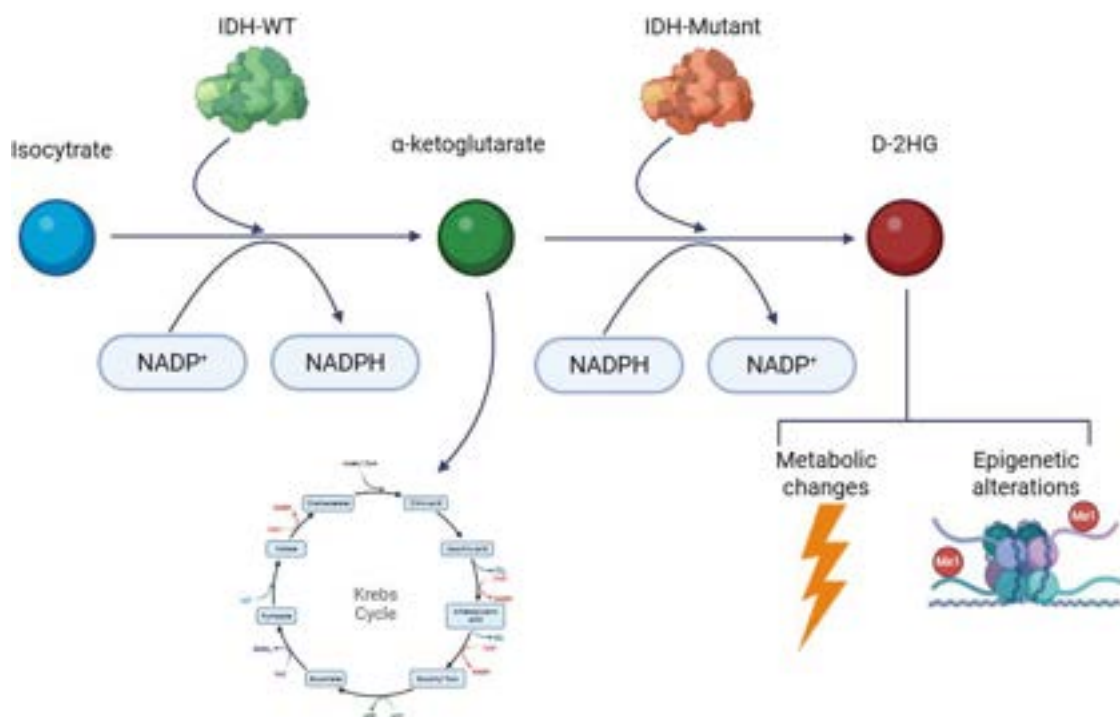


Figure 3: IDH reactions. Schematic representation of reactions catalyzed by IDH1 and IDH2 enzymes under physiological conditions, with mutant enzymes producing D-2HG, which leads to metabolic and epigenetic alterations.

In gliomas, the *IDH1* gene is most commonly mutated, accounting for about 97% of cases. This gene is located on chromosome 2 (2q33) and most often displays heterozygous mutations in position 395, via a nucleotide change from G to A (G395A)

which affects codon 132 of the resulting protein, changing a conserved arginine to a histidine (R132H). This codon is located in the active center of the enzyme, which is directly responsible for its catalytic activity. Other much less common mutations in IDH1 can also appear in the same 132 codon such as R132G (C394G), R132S (C394A), R132C (C294T) and R132L (C395T). IDH2 can also present mutations to its catalytic center in codon 172, but they only account for 3% of patients. Both *IDH1* and *IDH2* mutations occur in a single allele and are mutually exclusive (22–24).

These IDH mutations were identified in glioma patients through genome-wide sequencing approaches, and were rapidly recognized as clinically relevant, since they marked a subset of tumors with distinct behavior and improved clinical outcomes with a median survival of 32 months for some tumors. This increase in survival, coupled with their distinct methylation and metabolic pattern has led the WHO to reclassify all IDH-mutant gliomas as a separate category in 2021, which can present WHO histology grades II-IV (13,16).

1.2.2 IDH-mutant glioma diagnosis and classification

Since the integration of molecular markers into routine neuropathological classification, IDH mutation testing has become a core component of glioma diagnosis. A widely adopted approach recommended by major clinical guidance begins with immunohistochemistry (IHC) for IDH1 R132H on tumor tissue, as this variant accounts for the majority of IDH-mutant gliomas. If IDH1 R132H IHC is negative, sequencing of IDH1 codon 132 and IDH2 codon 172 is recommended in all WHO grade 2 and 3 diffuse astrocytic and oligodendroglial gliomas, and in grade 4 patients younger than 55 years, to avoid missing non-canonical IDH1 variants and IDH2 mutations and to enable integrated diagnosis (25,26). In addition to direct genotyping, mutant IDH activity can be inferred by measuring the oncometabolite D-2HG; rapid tissue-based detection by mass spectrometry has been demonstrated in pre-/intraoperative research workflows, but this approach is not currently positioned as a primary diagnostic recommendation in standard clinical guidelines (27,28).

After IDH profiling, tumors are classified into astrocytomas and oligodendrogliomas by combining IHC for nuclear *ATRX* loss and FISH or sequencing for detecting 1p/19q codeletions. Nuclear *ATRX* retention and 1p/19q codeletion classifies as oligodendroglioma, IDH-mutant and 1p/19q codeleted, which can fall into histological grades II or III based on mitotic activity. Currently WHO grade 4 IDH-mutant oligodendrogliomas are not recognized. In contrast, absence of 1p/19q codeletion supports astrocytoma, IDH-mutant, for which CNS WHO grading spans 2–4 using

INTRODUCTION

combined histologic and molecular criteria. On the other hand, loss of nuclear *ATRX*, with no codeletions, points to astrocytoma, which are then classified into histological grades from II to IV according to a pathologist. When *CDKN2A/B* homozygous deletions are present, IDH-mutant astrocytomas are automatically classified as WHO grade 4 astrocytomas, even if they don't meet classical grade IV histological criteria (25,26,29) (Fig. 4).

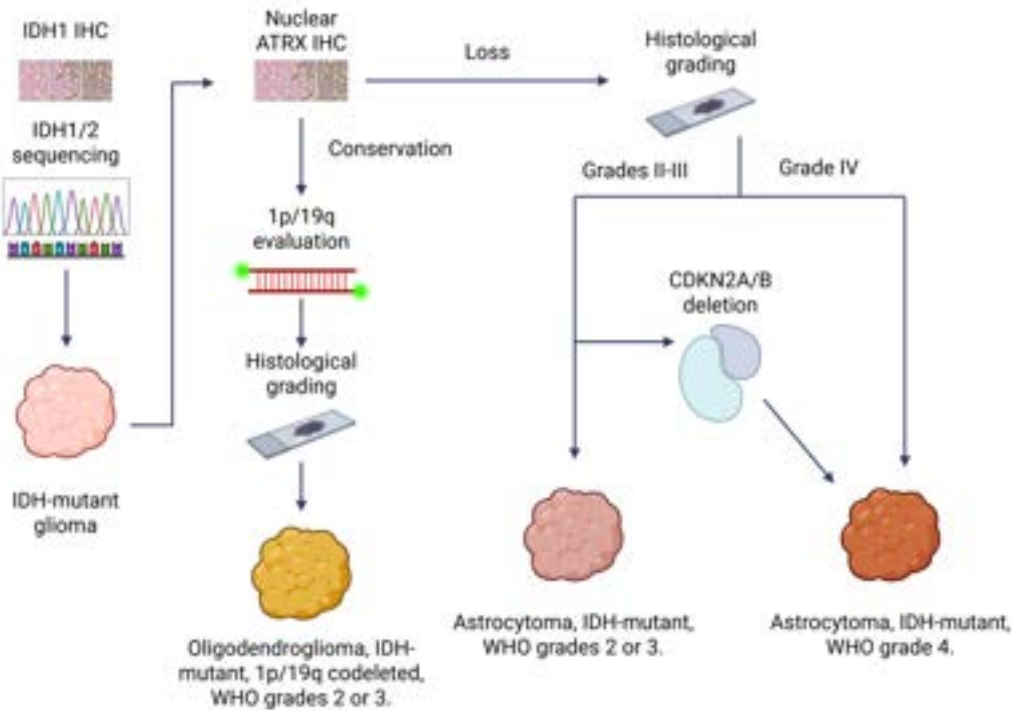


Figure 4: Diagnostic workflow for IDH-mutant gliomas. Schematic representation of diagnostic classification following IDH mutation confirmation. Gliomas are then subdivided according to ATRX status, 1p/19q codeletion, CDKN2A/B status and classical histological grading in an integrated pipeline.

1.2.3 IDH-mutant astrocytomas

Astrocytoma, IDH-mutant is an infiltrative diffuse glioma that occurs across CNS WHO grades 2–4, according to mitotic activity, cellular differentiation criteria and CDKN2A/B status. These tumors are frequently supratentorial and often involve the frontal lobe, although they can arise throughout the CNS, including the brainstem and spinal cord (30).

Population-based evidence suggests that these malignancies have an incidence of around 0.46 patients per 100,000 persons, corresponding to about 7% of primary CNS malignancies and roughly 10% of gliomas. Most patients are in their thirties or forties,

with a median onset age of 38 years old for WHO grade 2 and 3, while WHO grade 4 tumors appear in older patients (11).

Etiology is still largely unknown, with IDH-mutant astrocytomas appearing sporadically. Certain loci are associated with an increased risk, such as *FAM20C* or *D2HGDH* (31), and it is the most commonly associated tumor with the rare genetic syndrome Li-Fraumeni (32).

Clinically, symptoms often develop gradually, with seizures representing a frequently presenting manifestation of IDH-mutant astrocytomas. Additional deficits may include speech or language alterations, subtle motor impairment, and behavioral or executive dysfunction, particularly when lesions involve the frontal and prefrontal regions (33,34). Symptoms may precede diagnosis by months or even years. This slow clinical course likely reflects the infiltrative growth pattern and, in many cases, a low proliferative rate, which may allow partial functional compensation over time (35,36).

When symptoms prompt evaluation, diagnosis is initiated with neuroimaging. On magnetic resonance imaging (MRI), WHO grade 2-3 IDH-mutant astrocytoma appears as poorly demarcated areas of abnormal signal. These areas, often seen as bright regions on T2/FLAIR sequences, show little or no contrast uptake, with variable swelling or pressure on nearby brain tissue. In more aggressive disease, enhancement becomes more common and imaging may show areas of necrosis and increased edema, although grade assignments are ultimately based on integrated histologic and molecular criteria (37) (Fig. 5).

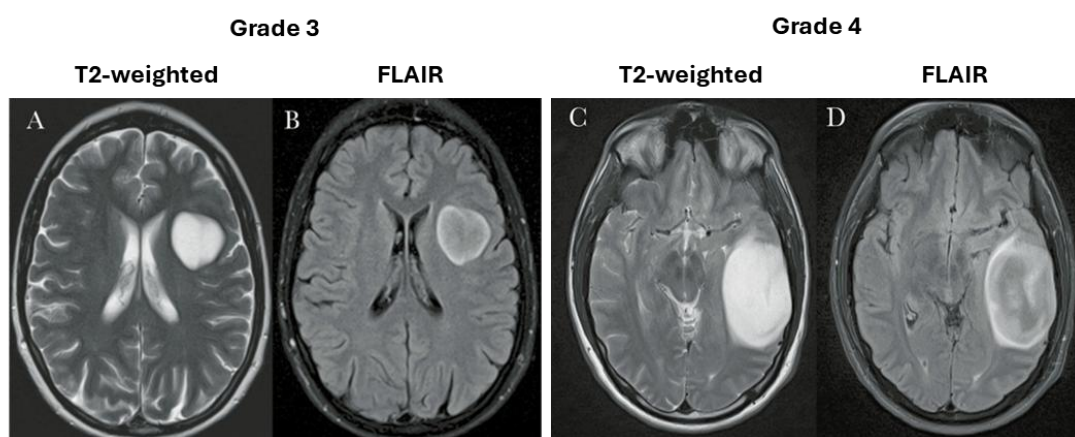


Figure 5: MRI presentation of IDH-mutant astrocytomas. Representative T2-weighted (A and C) and FLAIR (B and D) MRI images of WHO grade 2 (left) and WHO grade 4 (right) IDH-mutant astrocytomas. Note the more irregular composition and central necrosis in the WHO grade 4 tumor. Adapted from Jain et al. 2020 (38).

Besides the IDH mutations, IDH-mutant astrocytomas often present concomitant *TP53* mutations and *ATRX* loss of expression, which supports their astrocytic lineage by, for example, repressing the *SOX2* gene (39). Moreover, hypermethylation is a common phenotype as a side effect of D-2HG demethylase inhibition, with its consequent influence on further alterations and disease progression (40). The last major alteration, the homozygous deletion of *CDKN2A/B*, is a key marker of adverse disease (41).

Although malignant cells constitute the bulk component of tumors, extensive evidence shows that non-malignant cellular populations together with acellular components such as the extracellular matrix (ECM) actively contribute to tumor initiation and progression, shaping invasion and promoting therapy resistance. These cellular and acellular elements are collectively referred to as the tumor microenvironment (TME) (8,42). The microenvironmental features of IDH-mutant astrocytomas can vary with tumor progression and local tissue states (e.g., hypoxia-related regions in more aggressive lesions) (43). These tumors generally show a more immunosuppressive microenvironment, with lower tumor-infiltrating lymphocyte density across multiple cohorts (44,45). Accumulation of D-2HG has been implicated as one contributor to this phenotype by directly impairing antitumor immune function and promoting immune escape mechanisms (46,47).

IDH-mutant astrocytoma standard-of-care (SOC) therapy is stratified by tumor grade (Fig. 6). All patients receive maximal safe surgical resection with radiation therapy (RT); dosage is dependent on grade, with WHO grade 4 patients sharing RT regimen with GBM patients, with the exception of young (<40 years old) asymptomatic patients who require constant MRI follow-up following resection. Chemotherapy is usually composed of temozolomide (TMZ) or nitrosoureas with certain WHO grade 4 patients benefiting from bevacizumab (25,26,48,49).

Survival and disease progression are quite variable and linked directly to tumoral grades. WHO grade 2 patients can survive up to 20 years, but malignant progression into higher grades is common within 4-5 years (50,51). WHO grade 3 patients have a mOS of 8 years, with around half progressing to WHO grade 4 within 4 years (52,53). Finally, WHO grade 4 patients display the worst prognosis, with 2 to 5 years of survival, depending on molecular background (54,55).

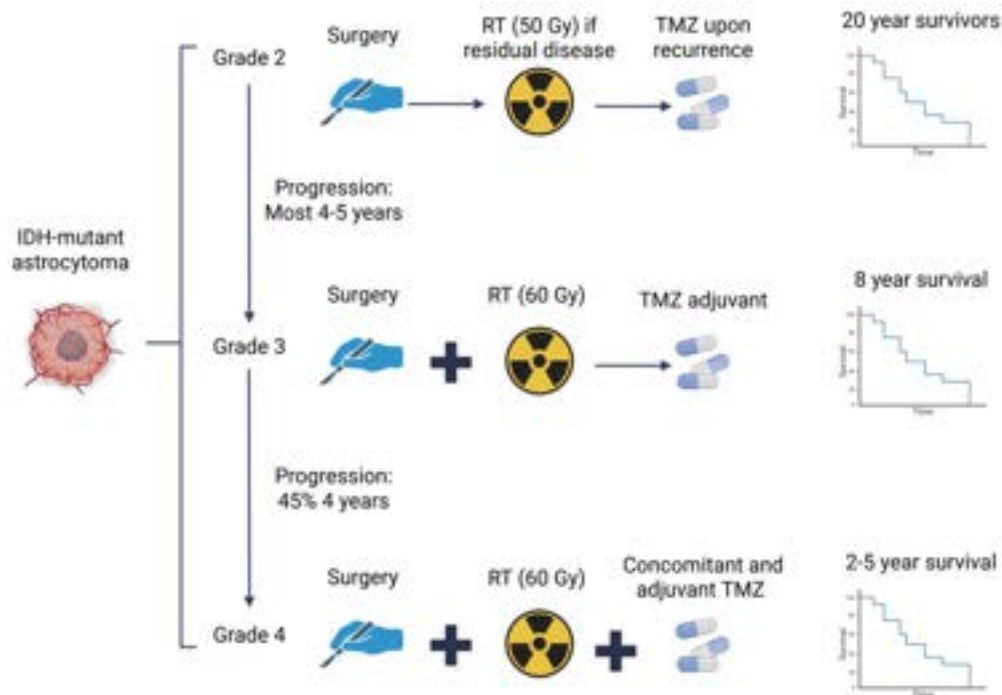


Figure 6: SOC therapy for IDH-mutant astrocytomas. Representative diagram of the 3 potential treatment regimens for IDH-mutant astrocytomas, stratified by WHO tumoral grade with their estimated progression and median survival times.

1.2.4 IDH-mutant oligodendrogliomas

Oligodendroglioma, IDH-mutant and 1p/19q-codeleted is the last IDH-mutant adult-type diffuse glioma entity recognized by the WHO. This tumor is assigned WHO grades 2 or 3, and there is currently no grade 4 category for oligodendroglioma. These tumors are most frequently supratentorial and commonly involve the frontal lobe, although they can occur in other regions; leptomeningeal spread has been described, particularly in the recurrent setting, but is not a defining feature (16,56,57).

Population studies place these tumors with an incidence of around 0.29 per 100,000 individuals, corresponding to around 4% of primary CNS malignancies, or 5% of all gliomas, thus making them one of the least common primary gliomas. Median onset age is dependent on grade, with WHO grade 2 at 43 years old, and 48 years old for WHO grade 3 tumors (11,58).

As with other gliomas, etiology remains largely unknown, and most cases are sporadic, with rare familial cases reported (59). Certain single nucleotide polymorphisms (SNPs) have been associated with the disease in genes *BICRA*, *ERCC2*, *GSTT1* and *D2HGDH* (60–62). To date, no hereditary cancer syndrome has been consistently linked to the

INTRODUCTION

development of IDH-mutant oligodendrogliomas. Other etiological factors like viral infections or immunodeficiency show conflicting evidence, with no clear association with the disease (63).

Clinical presentation is often indolent, with seizures the first common symptom, accompanied by headaches and cognitive changes. These symptoms are heterogeneous and based on anatomical location (64,65). MRI imaging shows oligodendrogliomas as cortical or subcortical invasive lesions that commonly present calcifications. In general, WHO grade 2 tumors show little contrast enhancement, while WHO grade 3 tumors enhance more clearly and also display aggressiveness features like edema (56,66).

Besides the classifying IDH and 1p/19q alterations, oligodendrogliomas share a common molecular background of shared genomic alterations. Most adult tumors present activating alterations in the *TERT* gene, promoting telomerase activity. *CIC* and *FUBP1* are commonly inactivated (67,68). The NOTCH1 and PI3K pathways also present multiple alterations in a subset of these tumors (69).

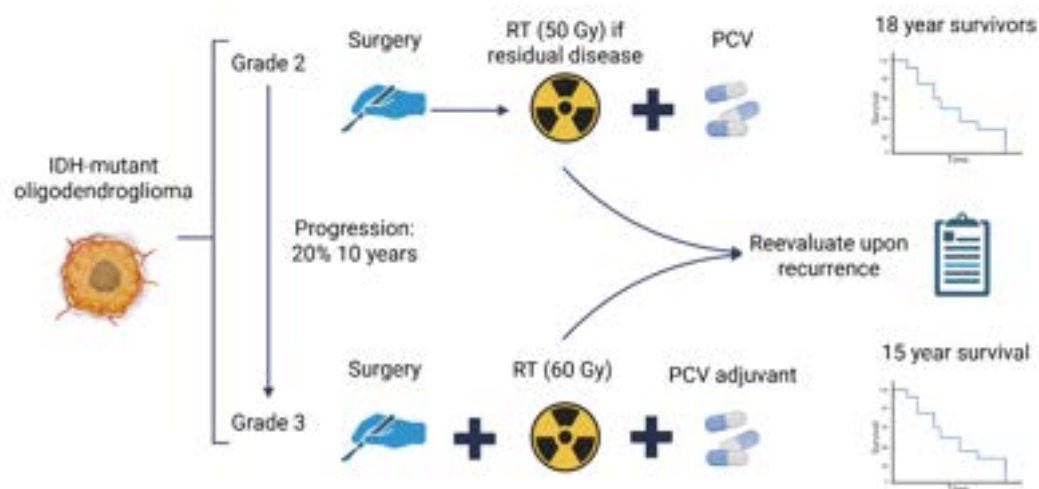


Figure 7: SOC therapy for IDH-mutant oligodendrogliomas. Representative diagram of the 2 potential treatment regimens for IDH-mutant 1p/19q codeleted oligodendrogliomas, stratified by WHO tumoral grade with their estimated progression and median survival times.

SOC therapy and survival for IDH-mutant oligodendrogliomas are also stratified by assigned grade (Fig. 7). Surgery is again the first recommended approach for all cases. For WHO grade 2 patients with progressing disease and all WHO grade 3 cases, RT is provided followed by chemotherapy in the PCV regimen, containing procarbazine (P), lomustine (C) and vincristine (V) (25,26). Interestingly, TMZ is used in some practices, but long-term randomized survival studies show no clinical improvements over PCV (70).

WHO Grade 2 patients have a mOS of 18 years, with 20% progressing within 10 years, while WHO grade 3 patients present a mOS of around 15 years with common recurrence events (71,72).

1.2.5 IDH1 vaccine

Even though prognosis for IDH-mutant gliomas is substantially better than for GBM, these tumors remain incurable, and many patients ultimately experience progression and recurrence with associated neurological morbidity. This has motivated research into novel therapeutic strategies. One of the most advanced approaches is mutation-specific vaccination, particularly the peptide vaccine targeting *IDH1* R132H (IDH1-vac) (73).

IDH1-vac is designed to generate an immune response against the shared R132H neoantigen by promoting antigen presentation on MHC class II molecules and inducing CD4 positive T-helper responses. In translational analyses, vaccine-induced immune responses have been detected systemically and within the TME. In some patients, treatment has also been associated with pseudoprogression, an inflammatory radiological pattern that can reflect increased immune cell infiltration and activation within the lesion (74).

In the first human trial (NOA-16), a phase I study in patients with newly diagnosed IDH1-mutant gliomas, vaccination was feasible, demonstrated an acceptable safety profile, and elicited immune responses in a substantial proportion of patients. Subsequent follow-up suggested durable disease control in subsets, although these observations remain exploratory given the non-randomized design (75). Despite these promising results, several factors currently limit its incorporation into SOC regimes. First, randomized studies are required to determine the incremental benefit over modern multimodal therapy and to define optimal combinations and timing (76,77). Second, efficacy may be attenuated by treatment-related immunosuppression, including corticosteroid use and the effects of chemoradiation on immune competence (78). Finally, immune-related imaging changes such as pseudoprogression can complicate response assessment and clinical decision-making (79,80).

1.3 Glioblastoma, IDH-wildtype

1.3.1 Definition and molecular landscape

Glioblastoma, IDH-wildtype (GBM), formerly known as GBM in the 2016 WHO classification, corresponds to diffuse astrocytic tumors located within brain structures. This tumor is the most frequent glioma, and accounts for over 50% of primary brain malignancies identified in adults (81).

GBM comprises a biologically heterogeneous set of tumors with substantial diversity at the genomic, epigenetic, and transcriptional levels. Large-scale molecular profiling has identified recurrently altered core pathways but also marked interpatient variability in driver events and regulatory programs. Moreover, GBM exhibits pronounced intratumoral heterogeneity (82,83).

At the genomic level, GBM frequently displays a characteristic copy-number variation (CNV) signature with whole chromosome 7 gain and whole chromosome 10 loss alterations that often occur simultaneously and affect multiple oncogenic and tumor-suppressor loci. These alterations can be identified in around 50% of GBM patients. In this context, *EGFR* (on chromosome 7p11.2) is one of the most commonly amplified oncogenes in GBM, present in over 60% of cases and represents a major driver event in a substantial subset of tumors. Beyond CNV gains, *EGFR* is also frequently altered by structural rearrangements and sequence variants (84–86).

These alterations in *EGFR* commonly promote the activation of multiple signaling pathways, most notably, the PI3K-AKT-mTOR and MAPK pathways, while also signaling through the JAK-STAT3 pathway in parallel. EGFR pathway activation may be further reinforced by additional alterations, including the *EGFRvIII* variant (87,88).

In parallel, one common alteration as a direct consequence of chromosome 10 loss is the deletion of *PTEN* (10q23.31), which further promotes the direct deregulation of the PI3K pathway (87,89). Other common CNVs occur in the loci of *CDKN2A/B* located on chromosome 9 and usually presenting deletions, leading to accelerated cell cycle progression (90,91).

Besides CNVs, mutations in multiple genes are present, most notably *TERT* promoter alterations, present in 80% of GBMs, which leads to reactivation of telomerase, further supporting tumoral growth (92–94). The TP53 pathway also suffers from multiple alterations, further facilitating tumoral progression (95) (Fig. 8).

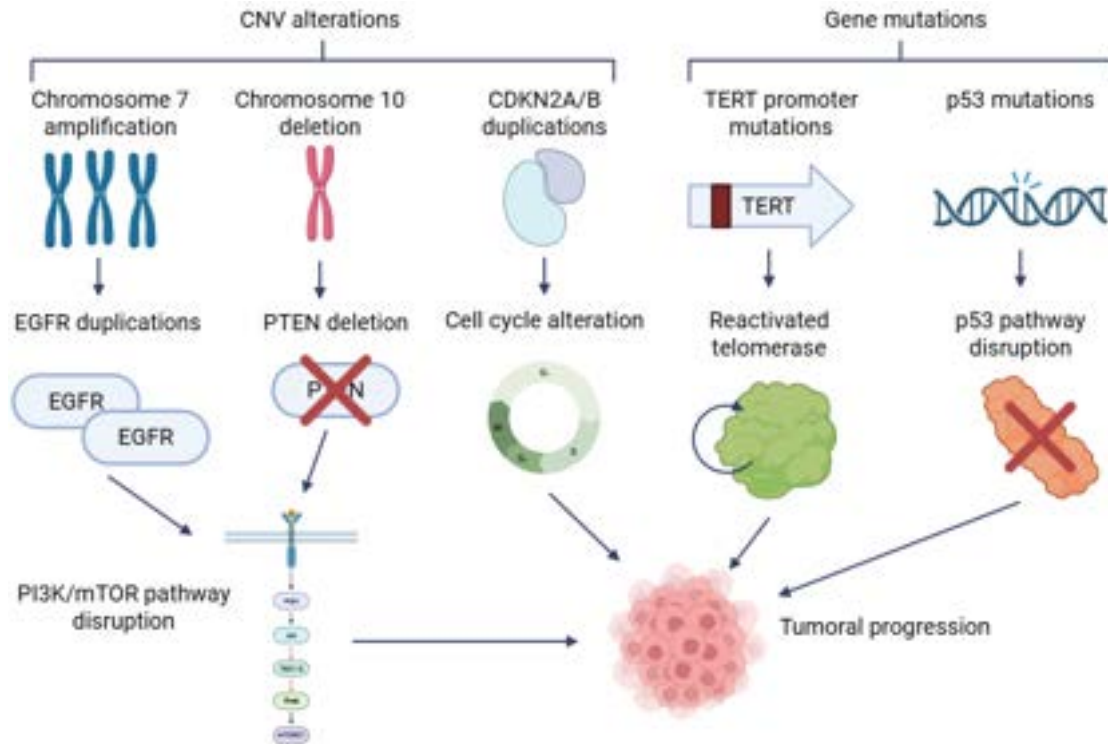


Figure 8: Genetic alterations common to GBM. Schematic diagram of common CNV and gene mutations that occur within GBM and lead to disruptions in key antitumoral pathways, supporting tumoral progression.

The advent of accessible bulk transcriptomic sequencing allowed to examine these heterogeneous tumors in order to identify common expression patterns, which allowed for the classification of GBMs into 4 distinct molecular subtypes, later reduced to 3 when the neural subtype was determined as contamination from healthy brain tissue samples (96). The remaining 3 subtypes: proneural, classical and mesenchymal recapitulate distinct biological programs and gene expression patterns, which have allowed for a deeper understanding of the disease. Proneural GBMs are associated with the presence of multiple *PDGFRA* alterations, together with the reactivation of various developmental programs within tumoral cells. Classical GBMs are enriched in *EGFR* signaling programs as well as various proliferation pathways. Lastly the mesenchymal subtype is characterized by an increased inflammatory response, together with tissular remodeling signatures and myeloid presence (97,98). Patients with proneural signatures present the best prognosis, with the worst for mesenchymal subtypes; however, GBMs tend to evolve into the mesenchymal subtype as the disease progresses (Fig. 9) (99,100).

While these bulk analyses can explain some of the transcriptomic complexities of GBM they are still insufficient, as more contemporary studies using single cell RNA-seq (scRNA-seq) and more fine area dissections have pointed out the coexistence of multiple

INTRODUCTION

subtypes within the same tumor, often in an area dependent manner (101,102). These findings have led to a paradigm shift, where GBM tumoral cells have been classified into 4 distinct transcriptional states that mirror neuronal lineages and stress programs: neural progenitor-like (NPC-like), oligodendrocyte progenitor-like (OPC-like), astrocyte-like (AC-like) and mesenchymal-like (MES-like). NPC and OPC-like cells show increase in progenitor and cycling cellular programs and often are associated with tumors with higher proliferation and growth-factor alterations. AC-like cells show astrocytic differentiation programs and a strong activation of *EGFR*, while MES-like cells are enriched in injury and inflammation signaling, upregulating *NF1*. Importantly, these states are inherently plastic, suggesting that GBM cells can easily transition between them under environmental factors (103).

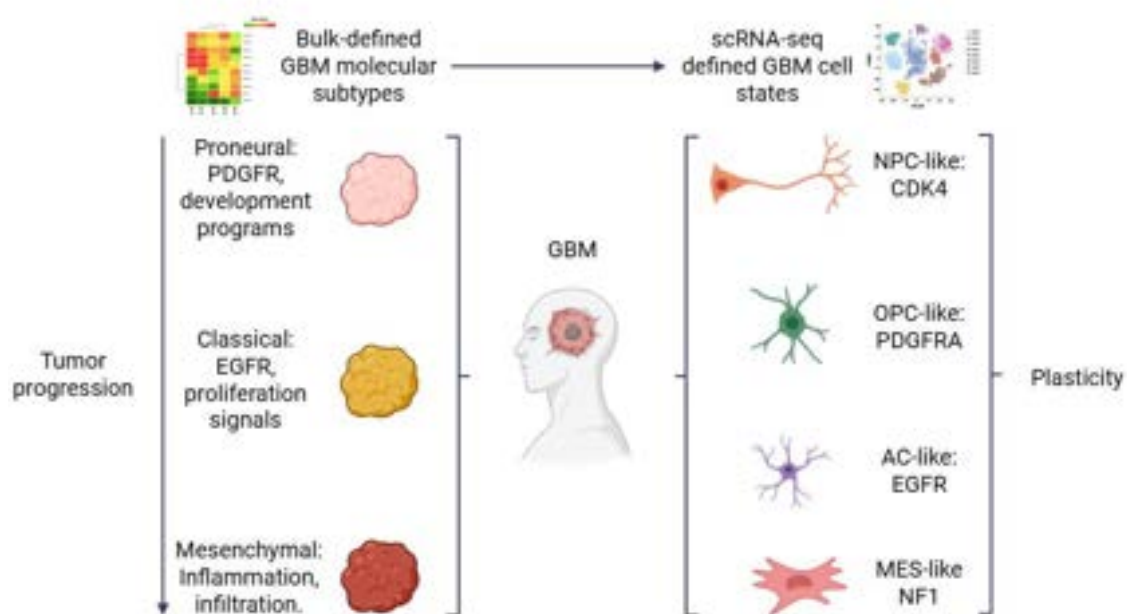


Figure 9: Molecular classification of GBM subtypes. Schematic diagram of GBM subtypes derived from bulk RNA-seq data (left) and the paradigm shift towards tumoral cell states with scRNA-seq, which introduces a high degree of tumoral cell plasticity.

1.3.2 WHO molecular classification

According to the WHO, the main classifying feature of GBM is the absence of *IDH* mutations, distinguishing it from IDH-mutant diffuse gliomas. All GBMs are assigned CNS WHO grade 4, in contrast to the previous WHO classification frameworks, where histological grade IV IDH-mutant astrocytomas were also classified as GBM IDH-mutant. Moreover, this updated classification recategorizes histological grade II and III astrocytomas (previously classified as lower-grade gliomas) as GBM as long as they

present certain molecular alterations, since more contemporary research demonstrated that they behave biologically as “classical” GBM. IDH-wildtype tumors outside of this classification have been reassigned to other tumoral groups (104,105).

From a histopathological point of view, GBM is characterized by 2 key features: microvascular proliferation (MVP) and/or necrosis. MVP refers to prominent, abnormal vascular remodeling with smaller caliber vessel proliferation, reflecting increased angiogenic activity. Necrosis is often extensive and, in GBM, can display the characteristic pseudopalisading pattern, in which densely packed tumor cells surround necrotic foci (Fig. 10). Additional features such as neuroinflammation (106) and prominent myeloid infiltration (107) are frequently observed, but they are not, by themselves, defining criteria for tumor classification (16).

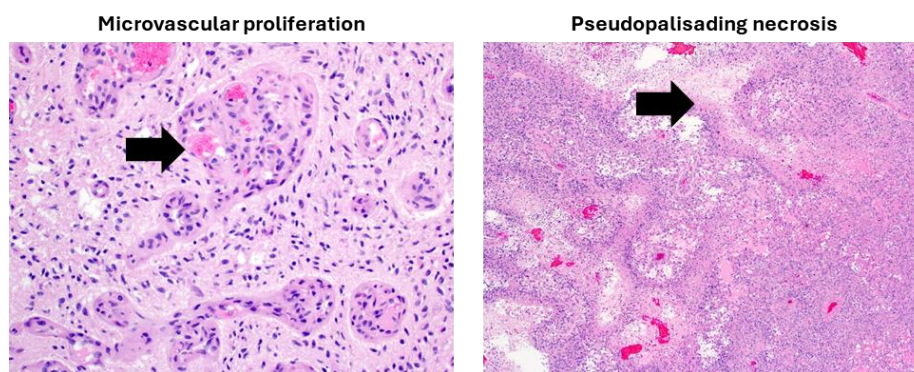


Figure 10: Defining histopathological characteristics of GBM. Representative H&E images of MVP (left) and pseudopalisading necrosis (right) in GBM, indicated by black arrows. Modified from PathologyOutlines.com, as provided by P.J. Cimino, M.D., Ph.D.

Histopathological classification is then complemented by molecular profiling to obtain an integrated diagnosis. For GBM classification at least one of the previously mentioned alterations must appear: chromosome 7 duplications and/or chromosome 10 deletions, amplifications in the *EGFR* locus, and *TERT* promoter mutations. Moreover, histological grade II and III malignancies are also classified as GBM when these characteristics are present, even when classic grade IV histological features are not apparent on the examined tissue. Other recurrent molecular alterations like alterations in *CDKN2A/B*, the PI3K pathway or *TP53* are not criteria for classification (13,16).

From this broad GBM denomination, the WHO defines 3 subtypes: giant cell GBM, gliosarcoma and epithelioid GBM. These entities are separated by histological morphologies, tend to appear at different ages but with little implications in clinical outcomes or management (108–110).

1.3.3 Epidemiology, clinical progression and diagnosis

GBM has an incidence of 3-6 cases per 100,000 people, which varies slightly from country to country, due to difficulties in diagnosis and age-gaps in less developed regions. GBM can manifest in patients of any age, but it is more frequent in older adults, with a median diagnosis age of 65 years. In children, this tumor type accounts for only 3% of CNS malignancies (11,111,112). Male to female ratios are slightly tilted towards males with a proportion of 1.38:1 (113).

The etiology of GBM is currently unknown, with most cases being sporadic and occurring in isolated individuals within a family (114,115). Certain mutations in genes like *TERT*, *EGFR*, *CCDC26*, *CDKN2B*, *PHLOB1*, *TP53*, and *RTEL1* have also been associated with increased risk (116). Due to the recent increases in diagnosis, environmental factors have been suggested to play a key role in GBM generation, but, so far, only exposure to ionizing radiation has been validated as a risk factor (117,118).

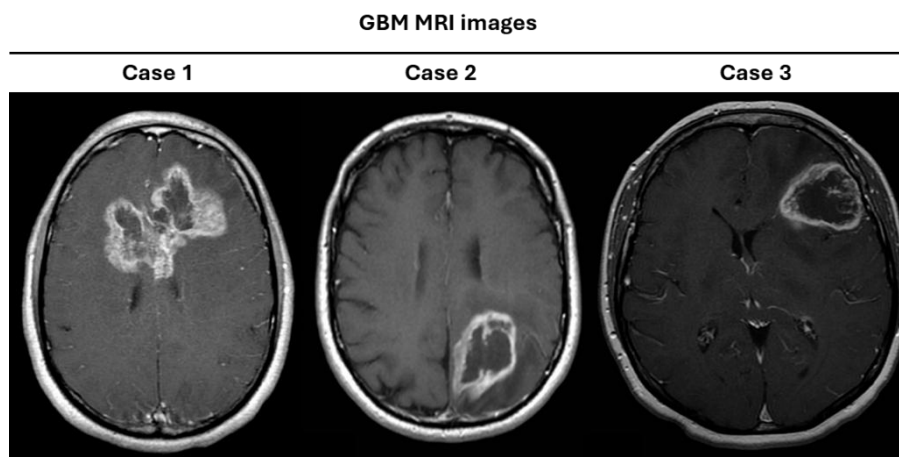


Figure 11: Magnetic resonance imaging of GBM. Representative FLAIR MRI images of 3 independent GBM patients. Note the evident necrotic core with multiple edematous areas, as well as the interpatient heterogeneity. Adapted from Radiopaedia.org.

GBM most frequently arises within the cerebral hemispheres, often centered at the gray and white matter interface or subcortical white matter, and can involve any lobe, with frontal and temporal locations commonly reported (30). Directly influenced by its diffuse nature, GBM is highly infiltrative and commonly invades adjacent brain parenchyma and may extend to other structures, most notably the corpus callosum (119). Clinical presentation is heterogeneous and largely determined by tumor location but typically includes a combination of intracranial pressure symptoms (headache, nausea) and focal neurological or neurocognitive deficits (weakness, language disturbance, seizures,

personality or cognitive change). Symptoms may become clinically apparent within approximately 3 months of tumor onset (120).

Diagnosis typically begins after patients report neurological or headache symptoms, which prompt neuroimaging. Contrast-enhanced MRI is the preferred technique for GBM and other high-grade gliomas. GBM appears to be irregularly shaped lesions with a dark non-enhancing necrotic core and frequent edema on the outside (Fig. 11). MRI is also used to guide surgical interventions and assess response to treatment. Definitive diagnosis requires tumoral tissue, so biopsies are obtained from preoperative biopsy and surgical resection tissue. A combined histological and molecular protocol is performed on these samples in accordance with the latest WHO criteria (25,26,121).

1.3.4 Standard-of-care therapy and resistance

SOC therapy for newly diagnosed GBM consists of a multimodal approach which combines maximal surgical resection followed by RT with concomitant and adjuvant chemotherapy in the form of the DNA alkylating agent TMZ. This treatment is known as the Stupp regime, and has been used in clinical practice for the last couple of decades (122).

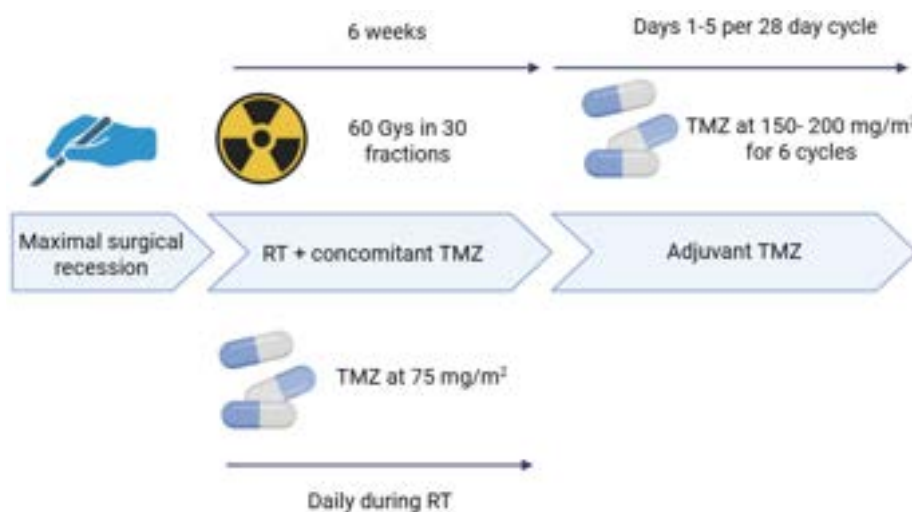


Figure 12: SOC therapy in GBM. Schematic diagram of the standard multimodal Stupp regime received by GBM patients combining surgery, RT and chemotherapy.

RT is provided in the form of external beam radiation for 30 fractions during 6 weeks, with a total radiation of 60 Gy. During RT, concomitant TMZ is administered at a dosage of 75 mg/m² daily and as an adjuvant after RT at 150-200 mg/m² for 5 days every 28 days for 6 cycles (Fig. 12). These parameters are shared by clinical guidelines in Europe and the USA (25,26).

Despite this multimodal approach, GBM prognosis remains dismal, with a median overall survival (mOS) of 15 months and a 5-year relative survival of 5–6% (11,123). Prognostic factors include younger age, better functional status and lower comorbidity burden, as well as methylation of the *MGMT* promoter, which is associated with greater benefit from TMZ (124,125). It is therefore clear that intrinsic and acquired resistance mechanisms limit the long-term efficacy of current standard therapy in GBM.

This resistance to therapy is mediated by distinct and often overlapping mechanisms:

- **Anatomical surgical constraints:** A major barrier to effective surgical removal is tumor location relative to eloquent brain regions as well as the diffusely infiltrative growth pattern which may be located in areas not detectable by MRI. These factors contribute to residual disease and faster recurrence of patients (126,127).

- **Blood-brain barrier (BBB) and limited drug penetration:** Systemic chemotherapy faces unique challenges when penetrating the brain due to the BBB. Its heterogeneous nature in GBM disrupts uniform drug delivery into the tumoral mass which creates drug gradients that allow for poorly penetrated compartments to resist treatment. On top of that, tumor-associated ECM composition and biophysical properties can further impede diffusion and promote these protective niches (128–130). In order to improve drug delivery, 2 strategies have been tested. Catheter-enhanced delivery is a delivery approach in which intracranial catheters infuse therapeutic agents directly into tumor under positive pressure to increase tissue penetrance. However, its impact is limited by the need for invasive placement, leakage into adjacent spaces and strong dependence on catheter positioning and infusion parameters (131). In parallel, nanoparticle-based carriers aim to improve pharmacokinetics and tumor delivery through engineered formulations, yet their efficacy is limited by heterogeneous BBB permeability, rapid clearance/sequestration, restricted intratumoral penetration, manufacturing concerns and frequent failure of preclinical delivery gains in human GBM (132).

- **DNA damage tolerance and repair:** RT and TMZ exert cytotoxicity through DNA damage, yet GBM frequently limits lethality via DNA repair and checkpoint responses. *MGMT*-mediated reversal of O6-methylguanine lesions is a central mechanism of TMZ resistance and underlies the predictive value of *MGMT* promoter methylation. Alterations affecting mismatch repair, base excision repair, and double-strand break repair pathways are also common and can further modulate sensitivity, including treatment-induced selection for resistant clones (124,133).

- **Cellular plasticity:** As explained above, GBM tumoral cells are not a single static entity, but a heterogeneous and highly plastic entity organized around different specialized niches. Under therapy, this plasticity enables rapid adoption of stress-tolerant programs and promotes selection of resistant populations that repopulate the tumor (103,134).

- **Glioma stem-like cells (GSCs):** GSCs are a key tumoral subpopulation with self-renewal and tumor-propagating capacity, and they are often favored under treatment because they can adopt stress-adapted states and repopulate the lesion after clinical challenge. GSCs display multiple features that support resistance, including enhanced DNA damage signaling and repair and relative slow-cycling behavior. As a result, radio- and chemotherapy can act as selective pressures that increase the relative abundance of these cells (135).

- **Immunosuppressive microenvironment:** The GBM microenvironment is typically myeloid-enriched and immunosuppressive, with limited effective T-cell infiltration and functional impairment of anti-tumor immunity. Although immune checkpoint pathways can be present, the overall immune composition and systemic immunosuppression contribute to poor responses to immunotherapies in most unselected patients (136,137).

1.3.5 Tumor microenvironment in GBM

In GBM, the TME is particularly relevant because the CNS provides a distinct and tightly regulated tissue context including brain-resident cell types, BBB constraints, and a generally immunosuppressive environment, while GBM itself exhibits pronounced intratumoral heterogeneity that is partly shaped by microenvironmental cues (138). Moreover, GBM presents distinct tissular niches. These niches can be defined by their spatial location as well as nutrient availability and cellular infiltration, constituting the leading edge of the tumor representing the limit of malignancy, the infiltrating tumor with malignant cells infiltrating brain tissues, cellular tumor presenting the bulk of tumoral cells with minimal healthy tissue, vasculature proliferation areas where angiogenesis is most active and peri-necrotic areas surrounding necrosis with increased levels of hypoxia (102).

Starting with the cellular compartment, the immune system microenvironment in GBM is typically dominated by myeloid lineage cells, which are composed of resident microglia complemented by infiltrating macrophages. Analysis of these populations suggests that they exist in heterogeneous activation states, which are dependent on tumoral niche and tumoral development stage, via elements like necrosis, hypoxia and tumoral cell signals.

INTRODUCTION

GBM-associated myeloid cells support tumoral processes like angiogenesis, tissular remodeling or even the suppression of cytotoxic immune cells (139–141).

On the other hand, lymphoid populations are scarcer and with impaired functionality. CD4 and CD8 T cells can be detected in patients, but mostly reflect phenotypes consistent with exhaustion and disfunction, driven by tumoral signaling as well as myeloid cells (142–144). Regulatory T cells are often present, further disrupting the action of effector T cells and contributing to immunosuppressive effects of myeloid cells (145). B lymphocytes and NK cells are generally the least prominent lymphoid population (139).

Coupled to immune alterations, the vascular microenvironment in GBM is greatly altered, with dysregulated angiogenesis that often yields poor and heterogeneous perfusion, promoting regional hypoxia. Importantly, BBB dysfunction is not uniform: leaky regions can coexist with infiltrative areas where barrier properties remain relatively preserved. Pericytes, key components of the neurovascular unit, contribute to BBB regulation and show tumor-associated changes in GBM, indicating active participation in vascular dysfunction rather than a purely structural role. Consequently, the perivascular niche acts both as a structural element governing nutrient distribution and cellular infiltration as well as a signaling hub to support the malignancy (146–148).

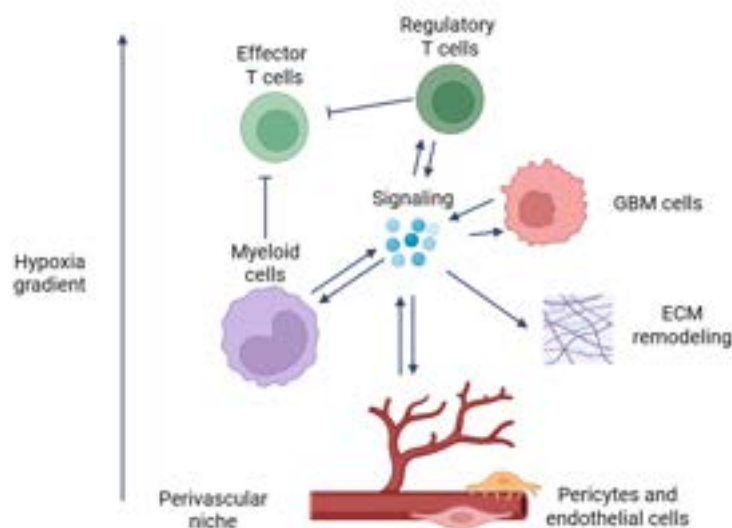


Figure 13: TME in GBM. Representative illustration of cellular and non-cellular components of the TME present in GBM together with their crosstalk through signaling molecules.

Besides the cellular component of the TME, non-cellular factors like ECM and signaling molecules are key to GBM progression. Remodeling of the ECM benefits tumoral progression by promoting cellular migration and invasion as well as controlling the influx

of infiltrating immune cells and substances into the densely cellularized tumoral core. In parallel, diverse gradients of cytokines, chemokines, and growth factors produced by tumor and stromal cells coordinate angiogenesis, immune modulation, and niche maintenance (149,150).

Collectively, these TME components engage in dynamic crosstalk and respond to local environmental cues, shaping tumor cell states, adaptation, and persistence (Fig. 13).

1.3.6 Novel therapeutic approaches

Given the abysmal efficacy of SOC therapy and considering the many mechanisms of resistance of GBM, many alternative therapies have been proposed in the last decades, with limited effects so far.

In light of the particular microenvironment of GBM, immune system-based approaches have also been extensively tested in GBM, with very limited success so far. One of them is the use of chimeric antigen receptor T-cells (CAR-Ts) to target tumor-associated antigens such as *EGFRvIII*. While CAR-T therapies have been very successful in hematologic malignancies, clinical translation in GBM has so far yielded limited durable benefit, with evidence of antigen loss and adaptive resistance (151,152). Newer multi-target CAR designs like trivalent CAR-Ts show encouraging preclinical activity but remain to be validated in larger clinical cohorts (153,154). In parallel, immune checkpoint inhibitors (ICIs) targeting the lymphoid compartment have shown limited efficacy in phase III GBM trials (155,156). More recent preclinical work has therefore shifted toward targeting myeloid ICIs, which may better match the myeloid-dominant immune landscape of GBM (157).

Another promising immune approach is the utilization of oncolytic viruses. These viruses work by selectively infecting tumoral cells, inducing lysis by their direct replication and converting the “cold” immune microenvironment into a more inflammatory state via immunogenic cell death (158,159). In the context of GBM Newcastle disease virus (NDV) is the most clinically studied platform, which has shown minimal toxicity and even complete response for some patients in phase I and II trials (160). However, larger phase III cohorts remain to be studied. More recently, infectious bursal disease virus (IBDV) has been proposed as a novel oncolytic candidate, with preclinical and *ex vivo* human GBM data suggesting both antitumor activity and partial remodeling of the immunosuppressive microenvironment, but clinical studies have not yet been reported (161).

One of the few innovations to demonstrate an OS benefit in a phase III trial in newly diagnosed GBM is the implementation of tumor treating fields (TTFs). TTFs are a non-invasive antimitotic therapy that applies low-intensity alternating electric fields (typically 200 kHz for GBM) through transducer arrays placed on the shaved scalp. In combination with TMZ, randomized clinical trials have reported a modest increase in progression-free survival (PFS) together with an increase in median overall survival from 15 to 20.9 months (Fig. 14) (162). Despite this success, therapeutic adoption has not been uniform. The FDA has approved its use under the name Optune for newly diagnosed patients with supratentorial GBM in an adjuvant setting with TMZ or as monotherapy in recurrent patients, as reflected by the NCCN guidelines (25). In practice, however, adoption across multiple centers remains variable in the USA (163), owing to factors such as the high initial cost of the therapy (164). In Europe, in contrast, the EANO clinical guidelines acknowledge the increase in survival observed in the phase III clinical trial for select patients, but they do not present them as SOC, raising concerns on trials controversies, cost-effectiveness and limited availability. Furthermore, their use is discouraged beyond progression (26). Therefore, European adoption is country dependent. In the case of Spain, TTFs have been introduced since 2025 through a monitored implementation framework with strict constraints on patient selection (165).

In line with the molecular alterations described in GBM, novel targeted therapies against the PI3K and MAPK pathways, CDKN2 and metabolism have also been proposed. However, clinical impact has been modest because pathway inhibition is frequently undermined by molecular heterogeneity, parallel pathway redundancy and rapid adaptive rewiring, and suboptimal/heterogeneous drug exposure across the BBB. These failures point to the necessity of better patient stratification coupled with combinatory and flexible treatment regimens (166–168).

Lastly, another pharmacological strategy is drug repurposing for GBM, which leverages compounds with established pharmacokinetic and safety profiles, thereby reducing development costs and potentially shortening the time required for clinical translation (169,170). However, a major limitation is that repurposed agents are often evaluated in molecularly heterogeneous, poorly stratified patient populations, which can dilute clinical signals. In addition, translation is hindered by the historically limited use of high-fidelity functional screening platforms that better preserve intra-tumoral heterogeneity and can support biomarker-linked patient selection (171,172).

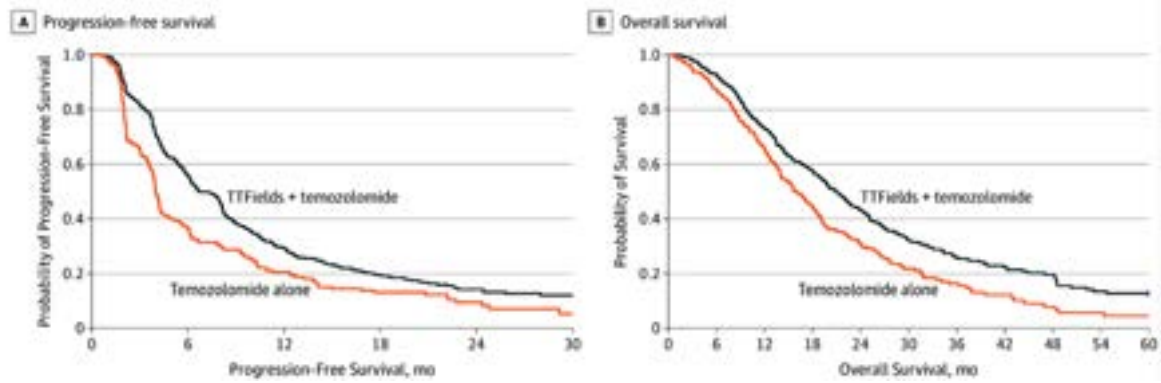


Figure 14: TTFs in combination with TMZ increase progression-free survival and overall survival in GBM. Kaplan-Meier survival curves of randomized clinical trial of GBM patients treated with SOC TMZ (orange) and TTFs + TMZ (black). Curves depict PFS (left) and overall survival (right). Adapted from Stupp et al. 2017 (162).

1.4 Glioma modeling techniques

1.4.1 Overview of glioma models

Gliomas are biologically heterogeneous and spatially complex tumors whose growth, invasion, and treatment response are strongly conditioned by the distinct CNS environment (103,138,173). In clinical practice, the study of gliomas is constrained by limited access to longitudinal tumor material, sampling bias across anatomical regions and substantial inter- and intratumoral variability that may not be captured by a single biopsy specimen (174). Moreover, therapeutic resistance arises through a combination of intrinsic tumor cell programs and adaptive processes shaped by microenvironmental cues and treatment-driven selection (138,175). For these reasons, experimental and computational models are essential complements to patient-derived data to further study tumoral mechanisms as well as novel potential therapeutic approaches (172).

One key concept when comparing glioma models is fidelity, which can be defined as the degree to which the model reproduces relevant features of the disease. Importantly, this fidelity is not an absolute property but rather depends on the relevant biological question asked to the model. For example, a model could be optimal for high-throughput screening of compounds but not maintain the complete cellular complement of the original tumor. Therefore, fidelity can be defined across several complementary axis: First, molecular fidelity, which refers to the preservation of key genomic and transcriptomic alterations, as well as their maintenance over time. Second, histological and spatial fidelity refers to the capacity of the model to recapitulate tumoral tissular architecture. Third, TME fidelity refers to the capacity of the model to conserve not only tumoral cells, but also non tumoral cellular and acellular elements. Finally, therapeutic response fidelity addresses the similarities between model response to treatment compared to the parental tumor (176–178).

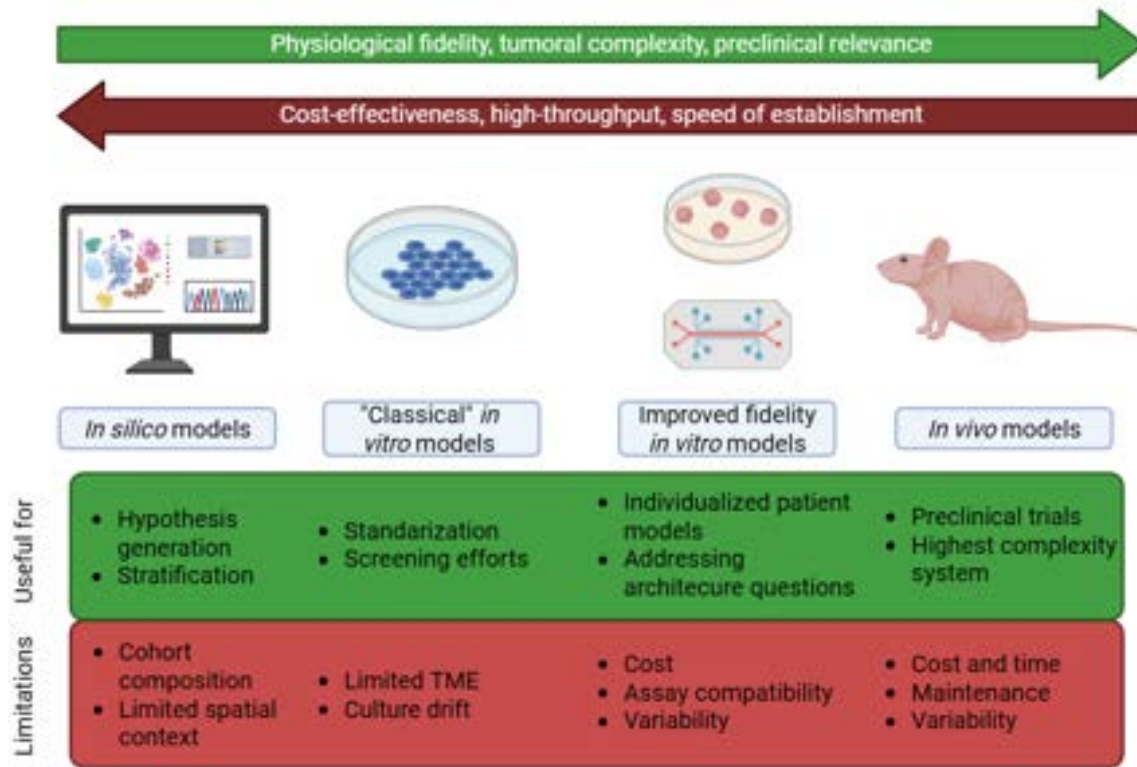


Figure 15: Experimental glioma models. Representative diagram of the main categories of models used in glioma research, with their usefulness and limitations.

Glioma models can be grouped into three broad categories. First, *in silico* or computational approaches which comprise analytical, mechanistic, and predictive frameworks using multi-omics and functional datasets. Second, *in vitro* models encompassing cell and tissue culture systems ranging from established cell lines to patient-derived three-dimensional platforms. Third, *in vivo* models enabling investigation of glioma biology within a living organism. Each category offers distinct strengths and limitations, and their use is typically matched to the stage of investigation, spanning discovery, mechanistic interrogation, and translational validation (Fig. 15) (172,177,178).

1.4.2 *In silico* approaches

In silico approaches comprise computational methods that leverage existing molecular and functional datasets to describe, infer, and predict key aspects of glioma biology. These methods typically integrate multi-omics profiles derived from patient cohorts (e.g., genomic alterations, DNA methylation, and transcriptomic programs) with functional information such as genetic dependency screens, perturbation datasets, or drug-response resources (82,83,179). Consequently, *in silico* analyses are most commonly applied at the discovery and prioritization stages. They enable identification of dysregulated pathways and regulatory programs and support the definition of disease-relevant cell states that may underlie malignancy, invasion, or therapy resistance.

Computational approaches can also facilitate patient stratification by associating molecular signatures with clinical outcomes (180–182).

A major advantage of computational analyses is their speed and cost-effectiveness relative to laboratory experimentation, while directly leveraging patient-derived datasets and thereby capturing clinically observed inter- and intratumoral molecular diversity. Moreover, the increasing availability of single-cell and spatially resolved omics datasets has expanded *in silico* analysis towards resolving cellular subpopulations and inferring spatially structured transcriptional programs and microenvironmental niches. Nevertheless, these approaches remain sensitive to cohort composition, technical confounders such as batch effects, and the assumptions embedded within the analytical framework (183,184). For these reasons, *in silico* findings require functional validation in experimental systems, where causal relationships and therapeutic responses can be assessed under controlled conditions.

1.4.3 Classical *in vitro* approaches

Among *in vitro* tissue culture models, classical approaches rely on long-established but reductionist cell culture systems that prioritize scalability, reproducibility, and experimental control. These platforms have been pivotal for mechanistic studies and high-throughput analyses, owing to their cost-effectiveness, ease of genetic manipulation, and compatibility with standardized assays of apoptosis, proliferation, migration, and drug response. Consequently, they are well suited for target validation, pathway dissection, and compound screening. However, these advantages often come at the expense of model fidelity. Classical cultures typically consist of relatively homogeneous, clonal tumor cell populations and therefore only partially recapitulate key cellular and acellular determinants of glioma biology *in situ*, including ECM features, vascular-associated cues, physiologically relevant gradients and native tissue microarchitecture. In addition, culture conditions impose strong selection pressures that favor rapidly proliferating cellular subsets, leading to underrepresentation of the inter- and intratumoral heterogeneity characteristic of glioma. Finally, prolonged *in vitro* propagation can promote genetic and transcriptional drift as cells adapt to artificial culture environments, directly impacting the molecular fidelity of the system (185–187).

Within this category, 2D adherent established cell lines represent the most widely used and experimentally tractable platform. These lines are typically maintained as monolayers under serum-containing conditions, enabling robust expansion, standardized manipulation, and reproducible phenotyping, and have therefore been central to early mechanistic studies and large-scale drug screening efforts. Nevertheless,

long-term passaging and adaptation to serum-rich environments can drive substantial divergence from the tumor of origin, including altered transcriptional programs, reduced cellular heterogeneity, and limited representation of invasion-relevant behaviors that depend on three-dimensional tissue context. Widely used examples include U87, U373 and LN229 cell lines (188–190).

A commonly used alternative is neurosphere-based culture, in which glioma cells are propagated in suspension under serum-free conditions supplemented with mitogens (commonly EGF and FGF2). Neurosphere culture has historically been employed to enrich for stem-like features and to maintain cells in a less differentiated state than serum-grown monolayers, and in some contexts may better preserve selected molecular programs and tumorigenic capacity *in vivo*. However, neurosphere formation is influenced by technical factors such as aggregation and culture conditions. Moreover, neurosphere cultures remain subject to clonal outgrowth and molecular drift and still lack the non-malignant cellular compartments and structured tissue architecture that shape glioma behavior in patients (191). Building on neurosphere methodologies, patient-derived GSCs constitute a widely adopted model class intended to retain clinically relevant tumor-initiating properties while remaining experimentally tractable. GSCs are derived directly from surgical specimens and maintained in serum-free, defined media, either as neurospheres or as adherent stem-like cultures on defined substrates. They are typically characterized by functional properties including self-renewal, multilineage differentiation potential, and tumorigenicity following orthotopic implantation, and often preserve key driver alterations and transcriptional programs present in the tumor of origin (192,193). For these reasons, GSCs are generally considered more biologically representative than long-term established cell lines for investigating glioma cell states, plasticity, and treatment resistance, while still remaining reductionist, presenting clonal drift (194) and being limited in their capacity to recapitulate TME interactions unless incorporated into more complex experimental systems (195).

1.4.4 Improved fidelity *in vitro* approaches

Improved fidelity *in vitro* models have emerged to address key limitations of classical glioma cultures, with the explicit aim of preserving clinically relevant molecular and cell-state features while reintroducing essential aspects of 3D organization (172). Conceptually, these approaches increase biological realism along complementary axes. 3D cultures can recreate oxygen and nutrient gradients while supporting more physiologically relevant cell-to-cell and cell-to-matrix interactions, thereby improving histological and spatial fidelity. In parallel, strategies that incorporate selected non-

malignant components (e.g., immune, glial, or vascular cells) enhance TME fidelity and enable interrogation of paracrine signaling and therapy-induced adaptive responses. Finally, microengineered platforms provide controlled delivery of cues such as flow, shear stress, and compartmentalization, supporting more faithful modeling of barriers and niche-like contexts. Despite these advances, improved fidelity systems typically involve increased cost, variability and reduced throughput compared with classical models (196,197).

A couple of differing systems have been proposed:

- **Co-culture systems:** In these systems, glioma cells are cultured together with one or more supporting cell types that are abundant in glioma tissue either in direct contact or separated using transwell configurations, thus allowing to distinguish cell–cell contact from paracrine signaling. Co-cultures enhance TME fidelity and enable interrogation of mechanisms that are poorly captured in tumor-only monocultures, including invasion programs, immunomodulatory crosstalk, and therapy-induced adaptive responses. However, co-culture models remain simplified and highly dependent on experimental design, as outcomes can be strongly influenced by the choice of cellular partners, their activation state and ratios, and whether the system is implemented in 2D or 3D.

- **Organoid systems:** Organoids are 3D self-organizing tissue cultures that arise from stem cells or primary tissue and partially recapitulate key aspects of the cellular composition and architecture of the tissue of origin. In the CNS field, cerebral organoids generated from pluripotent stem cells using the widely adopted Lancaster–Knoblich protocol (198) provide a scalable platform that develops region-like brain identities and cytoarchitecture, enabling disease modeling in a human-relevant context.

In glioma research, organoid-based modeling has converged on three main approaches (Fig. 16). First, genetically engineered brain organoid models introduce oncogenic drivers into otherwise normal cerebral organoids to model early tumorigenesis. For example, neoplastic cerebral organoids have been generated by introducing combinations of oncogenic alterations using CRISPR–Cas9, resulting in GBM-like tumoral growth within cerebral organoids (199). Second, tumor cell–based organoid systems generate 3D cultures from glioma-like cells that form tumor organoids capable of recapitulating features such as hypoxic gradients and heterogeneous stem-like compartments observed *in vivo* (200). Third, patient-derived GOs can be established directly from fresh surgical tissue, often using minimal dissociation to better preserve tumor architecture and cellular heterogeneity. These cultures can be generated in extracellular-matrix support systems such as Matrigel (201) or even as matrix-free

cultures under agitation (202,203). This approach has gained substantial traction for drug screening in many cancers, as patient-derived organoids provide very high levels of molecular and spatial fidelity while capturing aspects of patient-specific treatment response (204–206).

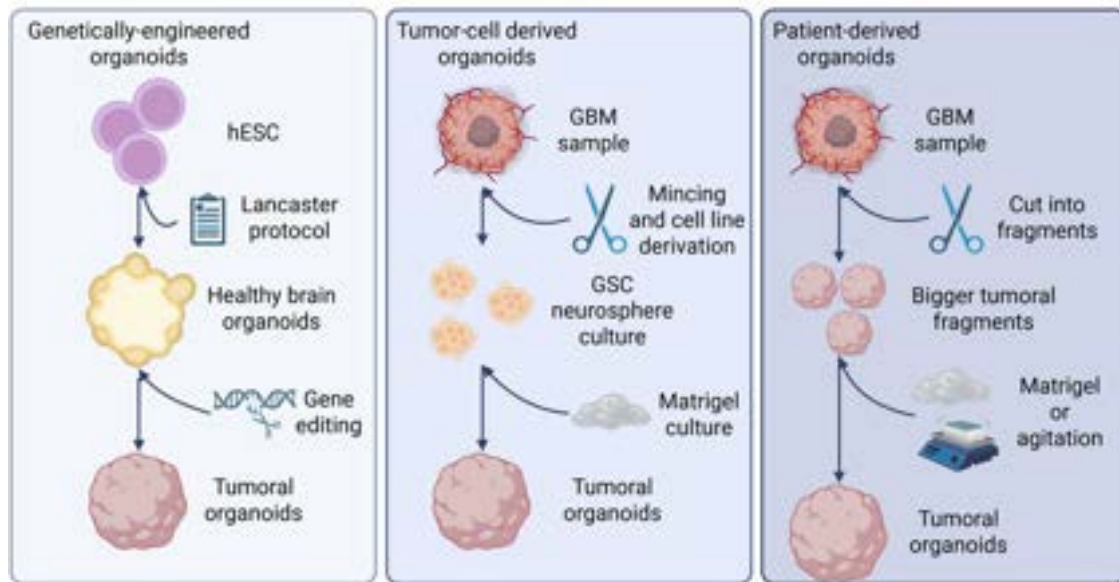


Figure 16: Overview of glioma organoid generation strategies. Representative diagram of the 3 main strategies used for GO generation consisting of genetically engineered organoids, tumoral-cell derived organoids and patient-derived organoids.

However, organoids also introduce practical trade-offs, including increased variability, lower throughput, and typically incomplete representation of vasculature and immune compartments unless explicitly engineered (207,208).

- **Patient-derived explants (PDEs):** These models are *ex vivo* cultures of minimally dissociated tumoral pieces, which provides a very high level of structural fidelity by preserving tumoral architecture and TME directly. In addition, these systems are also very quick to establish directly after surgery, making them ideal to interrogate therapeutic response or short-term phenotypes in a short clinically-relevant timeframe which also avoids selective *in vitro* pressures that affect other systems (209–211). Their main limitation is their restricted longevity, which doesn't surpass the barrier of two weeks in culture due to nutrient availability limitations. This downside compromises expansion and biobanking as well as long term experiments. Furthermore, standard cell culture assay techniques are difficult to adapt to these systems due to their larger sizes. Finally, model standardization is not uniform across laboratories, due in part to their novelty as a platform (212,213).

- **Assembloid systems:** These systems are a fusion of healthy brain organoids with other tumoral organoids or tumoral cells (214). Assembloids are particularly useful to interrogate the brain-tumoral cells interactions in glioma. A well-established example is the GLICO model, in which GSCs are co-cultured with human cerebral organoids to “retro-engineer” patient-specific GBM growth within organoid tissue. In this system, glioma cells migrate towards the organoid, invade and proliferate within the parenchyma, and can reproduce tumor behaviors that are difficult to capture in tumor-only cultures, including infiltrative growth and network-like structures associated with invasion (215). These systems share spatial/histological fidelity with conventional tumoral organoids, with comparable limitations like variability and incomplete vasculature and immune compartment recapitulation. In addition, model characteristics depend strongly on the tumor model used to seed the assembloid (216,217).

- **Organ-on-chip (OOC) systems:** OOC systems integrate cell culture with microfluidic devices that use microscale channels to control fluid flow, compartmentalization, and the spatiotemporal delivery of biochemical cues. In glioma research, these platforms are particularly useful for modeling context-dependent processes that are difficult to reproduce in static cultures, including reproducible gradients of oxygen, nutrients, and soluble factors, as well as controlled perfusion of immune or vascular-associated compartments (197). Microfluidic architecture can also be adapted to incorporate key elements of the BBB, making OOC platforms valuable for investigating drug transport and treatment responses under more physiologically relevant conditions than conventional static 3D systems (218). However, the fidelity of these models depends strongly on chip design and microfluidic parameters, and practical constraints include technical complexity and limited scalability for high-throughput applications (219).

1.4.5 *In vivo* approaches

In vivo models enable interrogation of glioma phenotypes that depend on organism-level context, including invasive growth within brain parenchyma, vascular remodeling, BBB constraints, pharmacokinetics/dynamics and immune system interactions. These advantages come with important practical limitations, including higher cost, longer experimental timelines, lower throughput, increased administrative burden, and ethical considerations. Accordingly, *in vivo* experiments are most commonly used for mechanistic confirmation and preclinical validation (220,221). The most frequently used organism for glioma modeling is the mouse (*Mus musculus*), owing to its relative ease of husbandry, extensive characterization, and the availability of numerous engineered

strains (222). *In vivo* glioma models can be stratified according to tumor induction strategy and host immune competence (Fig. 17).

- **Genetically engineered mouse models** generate glioma-like tumors *in situ* by introducing defined oncogenic alterations into CNS lineages. Because tumors arise within the native brain microenvironment and an intact murine immune system, they are informative for studying tumor initiation, lineage relationships, evolutionary trajectories, and therapy-induced selection under physiological constraints. However, variable latency and penetrance, model-dependent heterogeneity, and species-specific differences can limit reproducibility, scalability, and direct translational interpretation (223).

- **Syngeneic models** involve implantation of murine glioma cell lines into immunocompetent mice, typically in an orthotopic location. Their principal advantage is preservation of an intact immune system, making them useful for investigating tumor–immune interactions and for preclinical testing of immunomodulatory strategies. Limitations include the murine origin of tumor cells and potential divergence from human glioma genetics and cellular states, which can reduce human fidelity (224,225).

- **Xenograft models** implant human glioma cells into immunodeficient mice and can be established orthotopically (intracranial) or heterotopically (most commonly subcutaneous flank). Orthotopic xenografts better reproduce glioma-relevant invasion patterns and BBB-constrained therapeutic exposure than subcutaneous models. When derived from primary patient material, these models are referred to as patient-derived xenografts (PDXs) and can retain key driver alterations and aspects of interpatient heterogeneity. The principal limitation is the requirement for immunosuppression, which restricts evaluation of immune-dependent biology and therapy responses (226,227). To recover this immune response, **humanized mouse models** aim to partially restore human immune context by engrafting immunodeficient mice with human hematopoietic cells and then performing a tumoral xenograft. This approach allows us to study immunotherapies and mechanisms of immune evasion in the presence of human immune components. Nevertheless, humanized models are technically demanding and costly, exhibit substantial variability in immune reconstitution, and remain imperfect representations of the human CNS immune niche (228,229).

INTRODUCTION

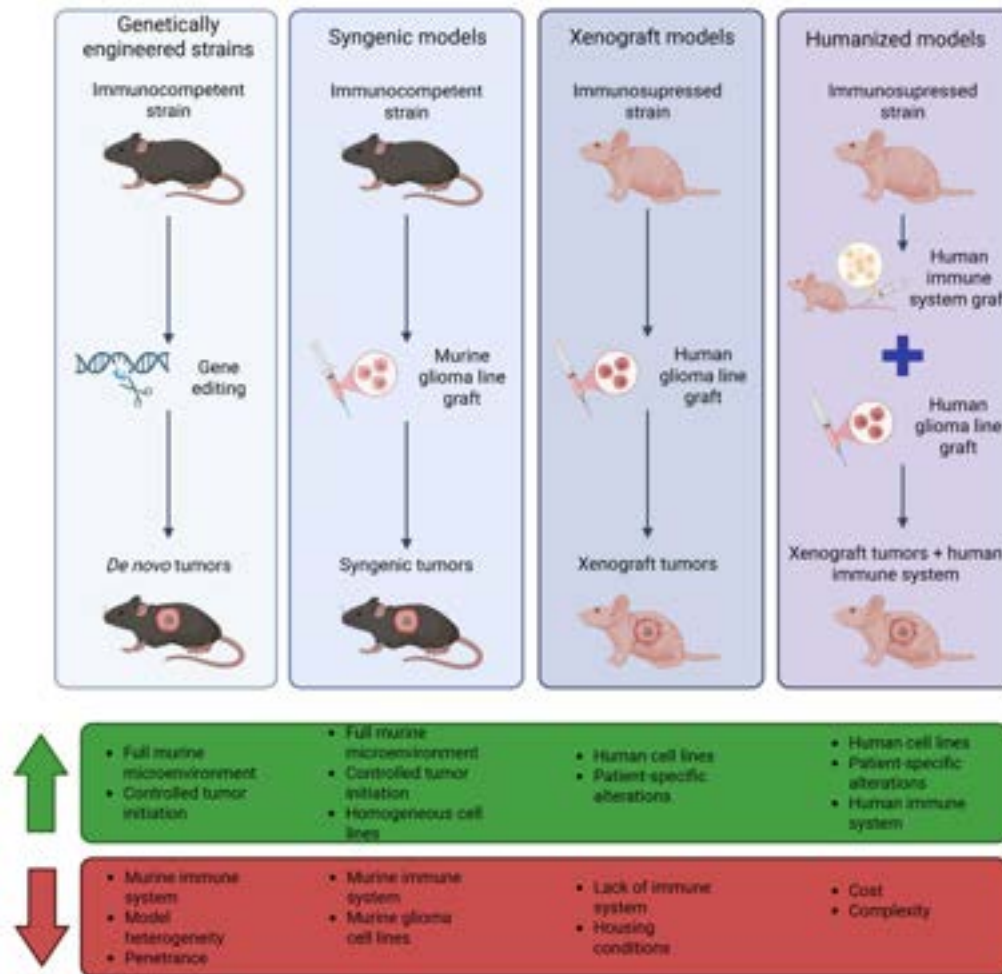
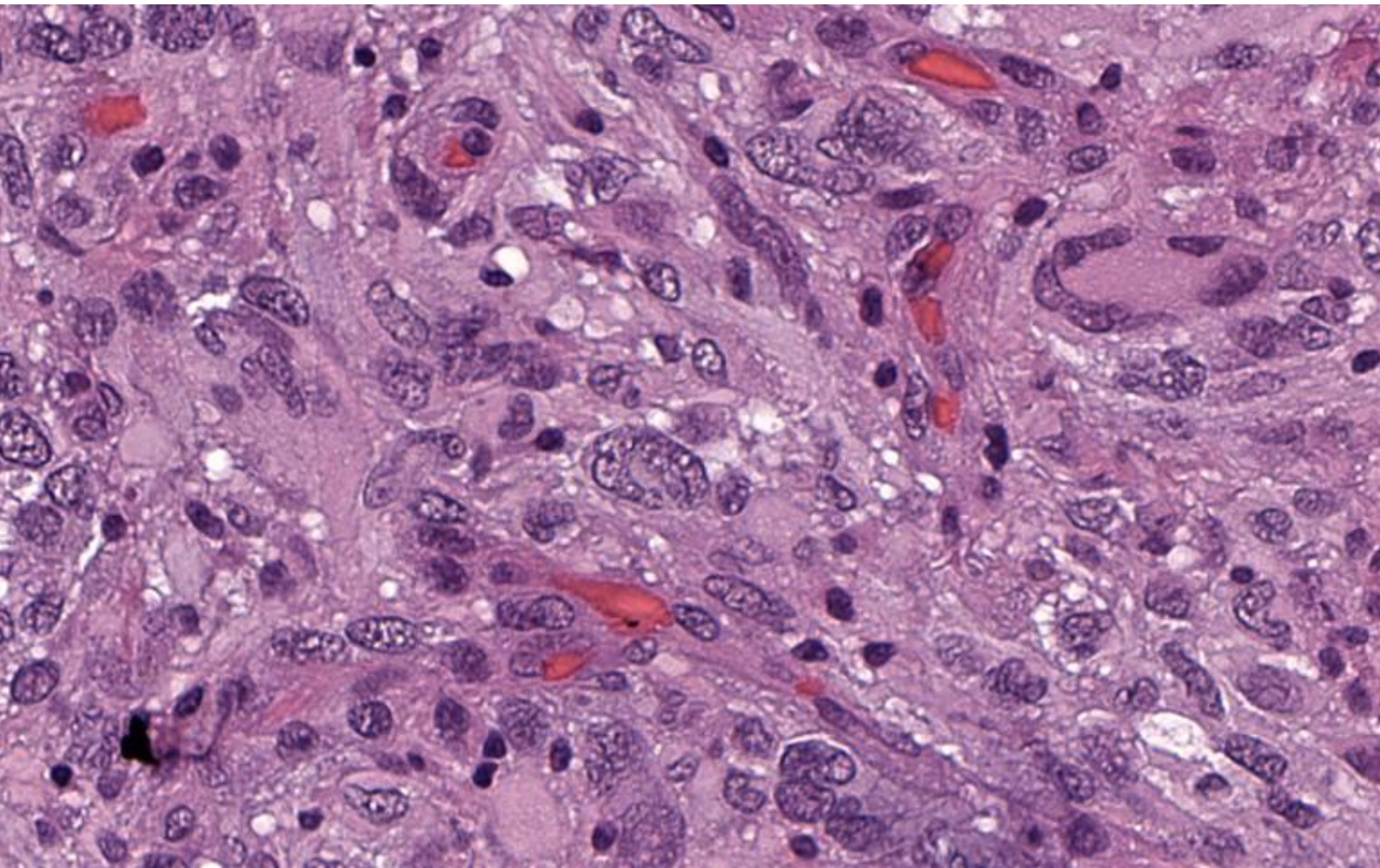


Figure 17: Overview of glioma murine models. Representative diagram of the 4 main strategies for in vivo glioma modeling from both immunocompetent and immunosuppressed strains. Advantages and limitations of each approach are also listed.

In short, adult diffuse gliomas, particularly GBM, remain among the most lethal malignancies, with current SOC therapy providing only limited and often transient benefit despite decades of clinical effort. A central obstacle to successful treatment is their marked cellular and molecular heterogeneity, coupled with profound therapy-driven adaptation that enables residual populations to survive, repopulate the lesion, and drive near-universal recurrence. In this context, experimental systems that better preserve patient-specific tumor features are required to improve biological understanding and to support more predictive preclinical testing. Patient-derived organoids offer a promising platform because they can recapitulate key aspects of inter- and intra-tumoral variability while enabling functional interrogation of treatment response in a controlled setting. As such, organoid-based approaches may help bridge the gap between molecular profiling and actionable vulnerabilities, facilitating the development and prioritization of innovative, patient-relevant therapeutic strategies for glioma.

2. HYPOTHESIS

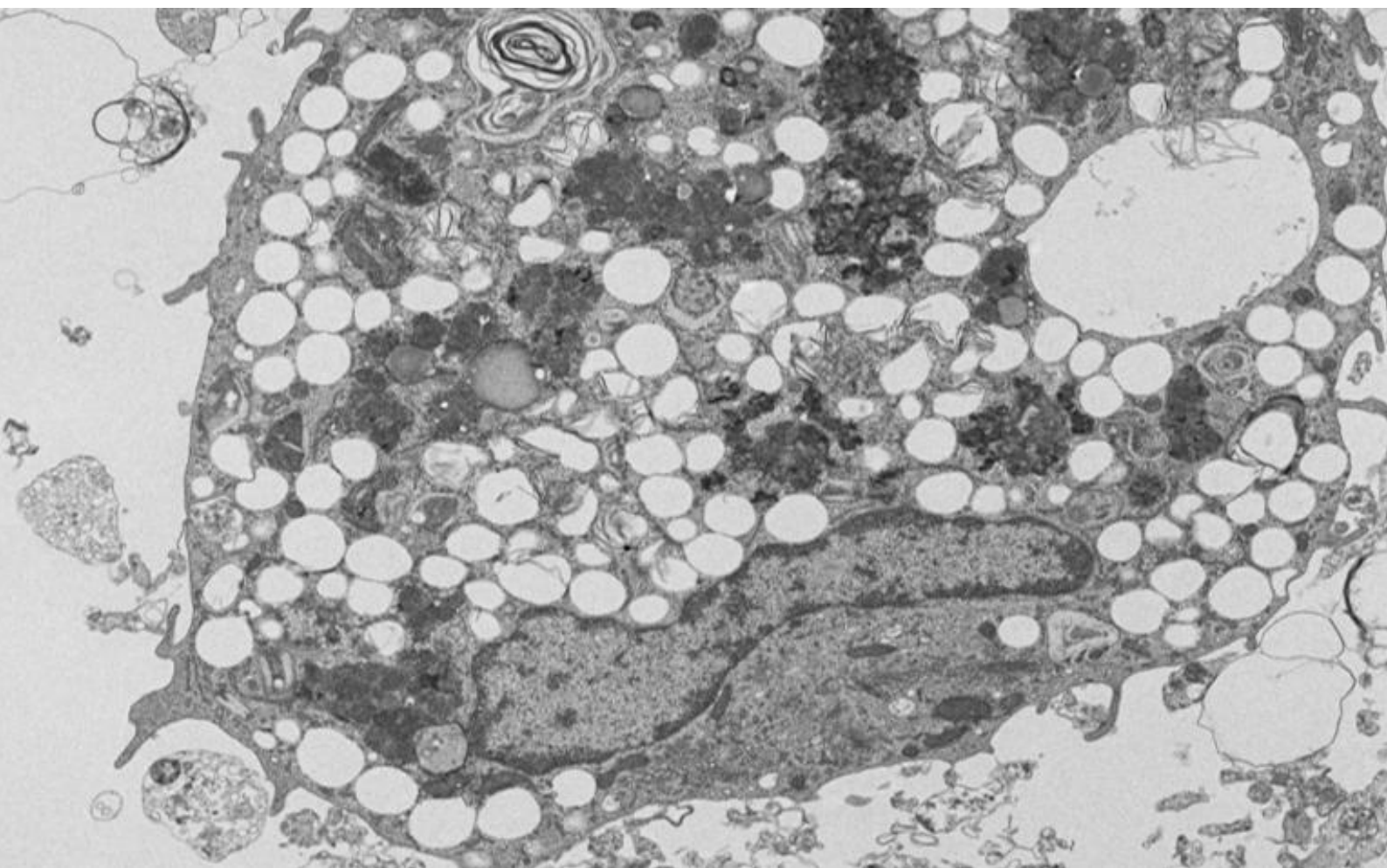


“El mayor misterio del mundo es que resulta comprensible.”

- Albert Einstein.

Adult diffuse gliomas retain therapeutically exploitable pathway dependencies that are reflected in their transcriptional states and can be captured in patient-derived GOs. Therefore, integrating transcriptomic profiling with *in silico* drug prioritization will identify clinically relevant targeted compounds whose antitumoral activity can be functionally validated in GOs and corroborated *in vivo*, enabling rational prioritization of actionable therapies beyond the SOC.

3. OBJECTIVES



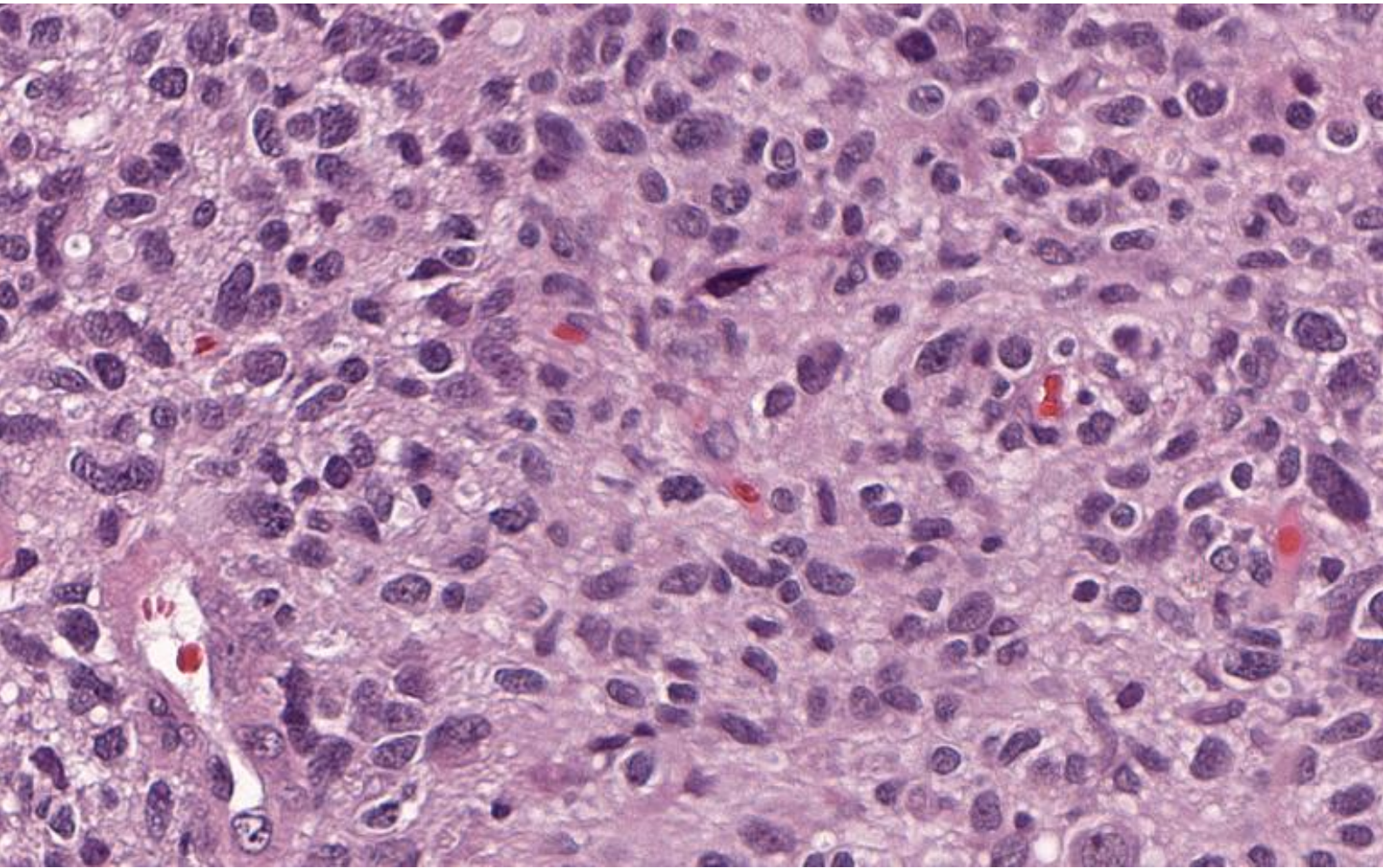
“Todo lo que se emprende con alma y corazón tiene éxito.”

- Miguel de Cervantes.

The main objective of this thesis is to identify and validate actionable therapeutic vulnerabilities in adult gliomas using patient-derived glioma organoids and complementary preclinical models, integrating molecular profiling with functional drug testing to support translational therapeutic prioritization. From this main objective, several subobjectives are derived:

1. Generate, optimize and maintain *in vitro* cultures of patient-derived glioma organoids (GOs) from GBM and IDH-mutant adult glioma samples, establishing a robust experimental workflow.
2. Assess the molecular, histopathologic and phenotypic fidelity of GOs relative to their corresponding parental tumors to confirm their potential as representative tumoral avatars.
3. Characterize the immune microenvironment preserved within GOs, identifying infiltrating immune populations and elucidating whether they replicate the myeloid-dominant profiles of patients.
4. Evaluate responses of GOs to SOC therapies in order to determine whether they reproduce clinically relevant resistance-associated phenotypes of the most prevalent adult gliomas (GBMs).
5. Identify novel therapeutic vulnerabilities in GOs and confirm antitumoral activity across diverse *in vitro* and *in vivo* models, to demonstrate robustness and translational relevance.

4. MATERIALS & METHODS



“Dadme un punto de apoyo y moveré el mundo”

- Arquímedes de Siracusa

4.1 Glioma cohort overview

Tumor specimens were obtained from 61 adult patients undergoing surgical resection for diffuse glioma. Based on histopathological and molecular diagnosis, 49/61 (80%) cases corresponded to GBM, while the remaining 12/61 (20%) comprised IDH-mutant diffuse gliomas spanning WHO grades II–III (Fig. 18, Table 1).

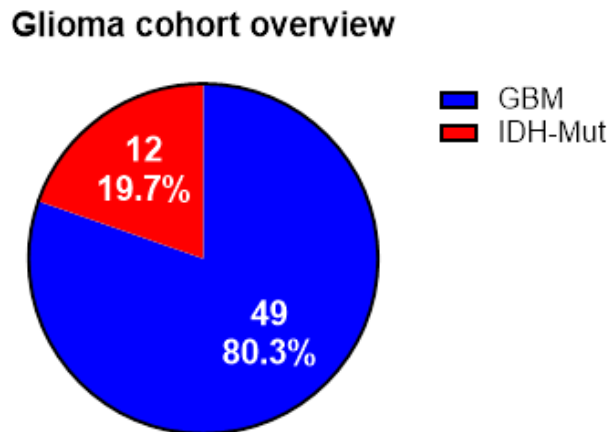


Figure 18: General cohort overview. Pie chart representing adult glioma patient cohort. Blue segment represents patients with GBM (N=49), with red segment corresponding to IDH-mutant gliomas (N=12).

Tumor tissue was collected at Hospital Universitario Puerta de Hierro (Madrid, Spain; biobank), Hospital Universitario Quirónsalud Madrid (Madrid, Spain), and Hospital Universitario 12 de Octubre (Madrid, Spain) immediately after resection for downstream establishment of GOs and molecular analyses.

Ethical approval for the use of these samples was granted by the institutional review board of HM Hospitals (CEIm No: 23.06.2206-GHM) and Hospital 12 de Octubre (CEI 21/480). All patients provided informed written consent prior to enrollment (Annex II), and all procedures were conducted in accordance with the principles of the Declaration of Helsinki.

MATERIALS & METHODS

Table 1: Patient cohort overview:

Patient	Classification	WHO Grade	Hospital	Sufficient tissue/quality	Successful GO derivation
GO1	GBM	IV	Puerta de Hierro	Yes	Yes
GO2	GBM	IV	Puerta de Hierro	Yes	Yes
GO3	IDH-mutant glioma	II	Puerta de Hierro	Yes	Yes
GO4	IDH-mutant glioma	II	Puerta de Hierro	Yes	No
GO5	IDH-mutant glioma	II	Puerta de Hierro	Yes	Yes
GO6	GBM	IV	Puerta de Hierro	Yes	Yes
GO7	IDH-mutant glioma	II	Puerta de Hierro	Yes	Yes
GO8	GBM	IV	Puerta de Hierro	Yes	Yes
GO9	IDH-mutant glioma	III	Puerta de Hierro	Yes	Yes
GO10	IDH-mutant glioma	II	Puerta de Hierro	Yes	No
GO11	GBM	IV	Puerta de Hierro	Yes	Yes
GO12	GBM	IV	Puerta de Hierro	Yes	No
GO13	GBM	IV	Puerta de Hierro	Yes	Yes
GO15	GBM	IV	Puerta de Hierro	Yes	Yes
GO16	GBM	IV	Puerta de Hierro	Yes	Yes
GO17	GBM	IV	Puerta de Hierro	No	N/A
GO18	GBM	IV	Puerta de Hierro	Yes	Yes
GO19	GBM	IV	Puerta de Hierro	Yes	Yes
GO20	GBM	IV	Puerta de Hierro	Yes	Yes
GO21	GBM	IV	Puerta de Hierro	Yes	Yes
GO22	GBM	IV	Puerta de Hierro	No	N/A
GO23	GBM	IV	Puerta de Hierro	Yes	Yes
GO24	GBM	IV	Puerta de Hierro	Yes	No
GO25	GBM	IV	Puerta de Hierro	Yes	Yes
GO26	GBM	IV	Puerta de Hierro	Yes	Yes
GO27	GBM	IV	Puerta de Hierro	Yes	Yes
GO28	GBM	IV	Quirón	Yes	Yes
GO29	IDH-mutant glioma	III	Puerta de Hierro	No	N/A
GO30	GBM	IV	Puerta de Hierro	Yes	No
GO31	IDH-mutant glioma	III	Quirón	Yes	Yes
GO32	GBM	IV	Quirón	Yes	Yes
GO33	IDH-mutant glioma	II	Quirón	Yes	Yes
GO34	IDH-mutant glioma	II	Quirón	Yes	Yes
GO35	GBM	IV	Puerta de Hierro	Yes	Yes
GO36	GBM	IV	Quirón	Yes	Yes
GO37	GBM	IV	Quirón	Yes	Yes
GO38	GBM	IV	Puerta de Hierro	Yes	Yes
GO39	GBM	IV	Puerta de Hierro	Yes	Yes
GO40	GBM	IV	Puerta de Hierro	Yes	Yes
GO41	GBM	IV	Puerta de Hierro	Yes	Yes
GO42	GBM	IV	Puerta de Hierro	Yes	Yes
GO43	GBM	IV	Quirón	Yes	Yes
GO44	GBM	IV	Quirón	Yes	Yes
GO45	GBM	IV	Puerta de Hierro	Yes	Yes
GO46	IDH-mutant glioma	III	Quirón	Yes	Yes
GO47	GBM	IV	Quirón	Yes	Yes
GO48	GBM	IV	12 de Octubre	Yes	Yes
GO49	GBM	IV	12 de Octubre	Yes	Yes
GO50	GBM	IV	12 de Octubre	Yes	Yes
GO51	GBM	IV	12 de Octubre	Yes	Yes
GO52	GBM	IV	Quirón	Yes	Yes
GO53	GBM	IV	La Princesa	Yes	Yes
GO54	GBM	IV	12 de Octubre	Yes	Yes
GO55	GBM	IV	12 de Octubre	Yes	Yes
GO56	GBM	IV	Quirón	Yes	Yes
GO57	GBM	IV	Quirón	Yes	Yes
GO58	GBM	IV	12 de Octubre	Yes	Yes
GO59	GBM	IV	Puerta de Hierro	No	N/A
GO60	GBM	IV	Puerta de Hierro	Yes	Yes
GO61	GBM	IV	12 de Octubre	Yes	Yes
GO62	GBM	IV	Puerta de Hierro	Yes	Yes

4.2 GO establishment

GOs were generated from freshly resected tumor tissue processed within 2 hours of surgical removal. Immediately after resection, tumor fragments were transferred to 50 mL conical tubes containing 5 mL of ice-cold H-GSPA dissection medium (Table 2) and kept at 4 °C until transport to the laboratory.

In a sterile biosafety cabinet, tissue was washed three times with 1X PBS and transferred to a 10 cm glass Petri dish containing 5 mL of cold H-GSPA. Under a stereomicroscope, tissue was coarsely minced using sterile scalpel blades. Fragments showing extensive necrosis, hemorrhage, or low apparent cellularity were excluded. Aliquots of tumor tissue were reserved for downstream molecular and histopathological analyses, and the remaining material was manually dissected into 1 mm fragments (“proto-organoids”) using microdissection scissors. During dissection, medium was replenished with cold H-GSPA to prevent tissue drying.

Proto-organoids were collected using a wide-bore pipette tip into a 15 mL conical tube, washed three times with 1X PBS, and incubated in 1X red blood cell (RBC) lysis buffer for 10 min at RT° with gentle orbital agitation. Following erythrocyte lysis, proto-organoids were washed three times with DMEM/F12 and transferred to 6-well ultra-low attachment plates containing 4 mL of M-GO growth medium (composition in Table 3). Cultures were maintained in a humidified incubator at 37 °C and 5% CO₂. A schematic overview of the workflow is shown in Fig. 19.

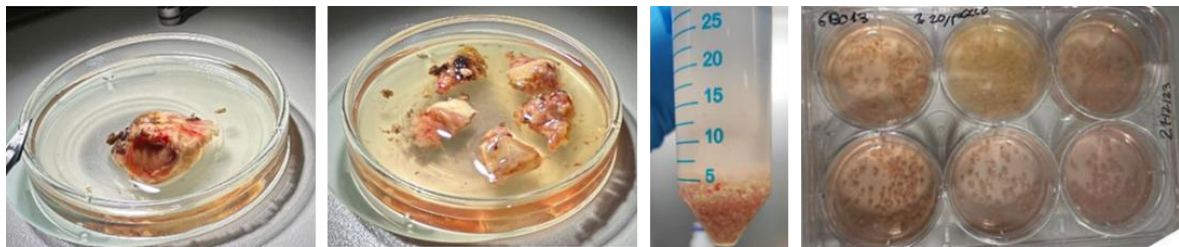


Figure 19: GO establishment overview. Representative images of GO generation protocol. From left to right, resected tumoral fragment placed in glass Petri dish, coarse sectioning and piece selection, GO washing and seeding.

Table 2: H-GSPA dissection media composition:

Component	Volume (mL)	Final concentration
Hibernate-A media base	488	
GlutaMAX Supplement	5	1 X
Penicillin-Streptomycin	2,5	0.5 X
Amphotericin B	5	1 X

Table 3: M-GO organoid maintenance media composition:

Component	Volume (mL)	Final concentration
DMEM-F12	236.25	
Neurobasal	236.25	
NEEAs	5	1 X
N2 Supplement	5	1 X
B27 Supplement	10	1 X
Penicillin-Streptomycin	5	1 X
Human Insulin	0.125	2.5 µg/mL
β-Mercaptoethanol	0.5	1 X

4.3 GO maintenance

GOs were maintained in M-GO growth medium under standard culture conditions in a humidified incubator at 37 °C and 5% CO₂. Medium was refreshed every 48 h by partial medium exchange: 3 mL of spent medium was carefully removed from each well and replaced with 3 mL of fresh, pre-warmed M-GO medium (final volume 4 mL/well). Cultures were visually inspected at each medium change using brightfield microscopy.

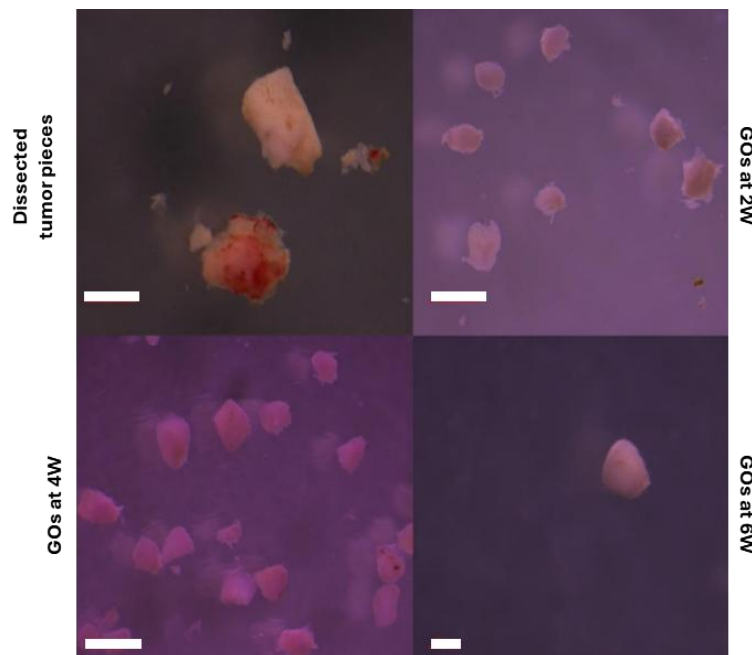


Figure 20: GO progression in culture. Representative brightfield images showing organoid appearance over time (establishment, 2, 4, and 6 weeks). Scale bar: 1 mm.

At 7 days post-establishment, GOs exhibiting poor compaction and persistently low cellular content by brightfield appearance were removed from culture. GOs were passaged every 2 weeks by manual sectioning into 1 mm fragments using sterile microdissection scissors in cold dissection medium. Resulting fragments were

transferred to fresh ultra-low attachment plates containing M-GO medium and returned to standard culture conditions. After successive media changes, GOs appear round and cellular debris is removed from the media (Fig. 20).

GOs were considered successfully established when they adopted a spherical, compact morphology, showed progressive growth relative to their initial diameter, and retained histopathological features consistent with their parental glioma, as assessed on matched GO sections by a blinded pathologist (Fig. 21).

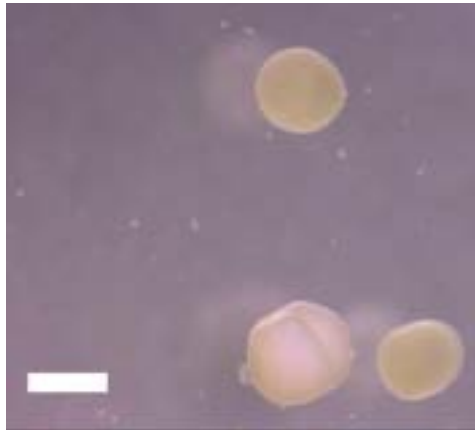


Figure 21: Successful GOs. Representative brightfield image of established GOs at 2 weeks showing compact spherical morphology. Scale bar: 1 mm.

GOs were frequently tested for mycoplasma contamination via PCR.

4.4 GO biobanking and recovery

Successfully established GOs were selected for cryopreservation after two weeks in culture. GOs were prepared by cutting into fragments of 0.1 to 0.5 mm in diameter in a glass Petri dish with 5 mL of ice-cold H-GSPA by using microdissection scissors.

Dissected GOs were then collected in a 15 mL conical tube, washed three times with DMEM/F12 and seeded in a concentration of 10 GOs per well into a 6 well plate with 4 mL of M-GO with Y-27632 10 μ M. These plates were incubated for 1 hour at 37 °C and 5% CO₂ under constant agitation at 90 rpm in an orbital shaker.

Following this incubation, GOs were again collected into 15 mL conical tubes, and resuspended in 10 mL of M-GO with Y-27632 10 μ M and 10% DMSO and incubated for 5 minutes at RT° in a tilt platform shaker. GOs were then placed in cryovials at a density of 20 GOs per vial with 1 mL of fresh M-GO with Y-27632 10 μ M and 10% DMSO. Cryovials were placed in a Freezing Container at – 80°C for 24 hours, at which point they were transferred to permanent storage in liquid nitrogen.

For recovery, GOs were taken from liquid nitrogen storage and quickly thawed in a 37 °C water bath. GOs were transferred to a conical 50 mL tube and 10 mL of M-GO with Y-27632 10 µM was added in drops to dilute DMSO. GOs were centrifuged at 200 x g for 5 min at RT° and the supernatant was aspirated. GOs were then seeded into a 6 well ultra-low attachment plate at a density of 10 GOs per well with 4 mL of fresh M-GO with Y-27632 10 µM. GOs were left O/N and media was fully changed to M-GO. Recovered GOs were then maintained normally.

4.5 Tissue and GO processing and sectioning for staining

Parental tumor fragments and GOs collected at indicated time points were washed three times with 1X PBS and fixed in 4% paraformaldehyde (PFA) in PBS at RT° using excess fixative with gentle orbital agitation O/N. Fixed samples were washed three times with 1X PBS and processed for either paraffin embedding or cryo-embedding, as described below.

4.6 Paraffin embedding and sectioning

Following fixation, samples were transferred to 70% ethanol and stored at 4 °C until processing. Parental tumor fragments were dehydrated, cleared, and paraffin-infiltrated using an automated tissue processor according to the manufacturer's program. GOs were processed manually to avoid sample loss during automated processing. For manual processing, samples were dehydrated through graded ethanol (70%, 96%, and 100%), with two 20 min incubations per concentration, cleared in xylene (two changes, 20 min each), and embedded in paraffin. Paraffin blocks were sectioned using a microtome to obtain 5 µm sections, which were mounted onto SuperFrost glass slides.

4.7 Cryoprotection, OCT embedding, and cryosectioning

For cryosectioning, fixed samples were incubated in 30% sucrose in PBS at 4 °C for 24 h for cryoprotection. Samples were placed in plastic molds, excess sucrose was removed, and molds were filled with OCT compound. Blocks were snap-frozen on a dry ice/isopropanol mixture and stored at -80 °C until sectioning. Sections of 10 µm thickness were cut using a cryostat and mounted onto poly-L-lysine-coated glass slides. Slides were stored at -80 °C until staining.

4.8 Hematoxylin and eosin (H&E) staining and imaging

Paraffin sections were placed in an oven at 80 °C for 15 min to melt paraffin, deparaffinized in xylene (two changes, 5 min each), and rehydrated through a graded ethanol series to 70% ethanol (2 min per step). Slides were rinsed in distilled water (2 min) and stained with H&E as follows: sections were incubated in Harris hematoxylin for

5 min and rinsed in water for 2 min, differentiated in acid alcohol, and blued using a standard bluing reagent. Sections were then counterstained in eosin for 2 min, followed by a brief water rinse to remove excess stain. Slides were dehydrated through increasing ethanol concentrations (2 min per step), cleared in xylene (two changes, 2 min each), and mounted with coverslips using Eukitt mounting medium. Slides were air-dried prior to imaging.

Whole-slide imaging was performed using a Leica Aperio VERSA digital pathology scanner equipped with a 20× objective.

4.9 Immunofluorescence (IF) staining and imaging

Frozen sections were brought to RT and washed in 1X PBS. Antigen retrieval was carried out using a microwave-heated citrate buffer (pH 7.0), with slides incubated in the hot solution for 20 min and then allowed to cool to RT°, followed by permeabilization with 0.1% 100 X Triton in PBS for 10 min. Sections were blocked for 1 h in blocking buffer consisting of 5% normal serum (matched to the host species of the secondary antibody), 5% BSA, and 0.1% 100 X Triton in PBS. Primary antibodies (Table 4) were diluted in blocking buffer and applied to sections overnight at 4 °C.

The following day, sections were washed in 1X PBS (3 times, 5 min) and incubated with fluorophore-conjugated secondary antibodies (Table 5) for 1 h at RT° in the dark. Sections were washed in 1X PBS (3 times, 5 min), counterstained with DAPI (1:10,000 in PBS) for 15 min at RT°, washed in 1X PBS (3 times 5 min), and mounted using Vectashield mounting medium.

Images were acquired using a Leica THUNDER Imaging system equipped with 20X and 40X apochromatic air objectives. Raw images were processed using the THUNDER deconvolution function in Leica LAS X and assembled in Fiji (v2.3.0).

Table 4: List of primary IF antibodies used.

Target	Reference	Host	Dilution
GFAP	Dako (Z0334)	Rabbit (Polyclonal)	1/100
NG-2	Millipore (AB5320)	Rabbit (Polyclonal)	1/100
VEGFa	Abcam (ab52917)	Rabbit (Monoclonal)	1/100
VEGFR2	Santa Cruz (SC-6251)	Mouse (Monoclonal)	1/100
CD31	LSBio (LS-B16832)	Goat (Polyclonal)	1/100
CD90	Abcam (ab9257)	Rabbit (Monoclonal)	1/100
Vimentin	Santa Cruz (SC-6260)	Mouse (Monoclonal)	1/100
Nestin	Millipore (MAB5326)	Mouse (Monoclonal)	1/100
β -Catenin	BD Biosciences (610153)	Mouse (Monoclonal)	1/100
HIF-1 α	GeneTex (GTX127309)	Rabbit (Polyclonal)	1/100
PD-L1	Abcam (AB205921-1001)	Rabbit (Monoclonal)	1/100
IBA1	Santa Cruz (Sc-32725)	Mouse (Monoclonal)	1/100
CD68	Abcam (ab955)	Mouse (Monoclonal)	1/100
CD4	Santa Cruz (SC-19641)	Mouse (Monoclonal)	1/100
CD8	Santa Cruz (SC-1177)	Mouse (Monoclonal)	1/100
SOX2	Santa Cruz (SC-365823)	Mouse (Monoclonal)	1/100
CD44	eBiosciences (14-0441-82)	Rat (Monoclonal)	1/100
TUJ1	Cloud-cloning (MAB870HU21)	Mouse (Monoclonal)	1/100
CD14	Cell Signaling (#91992T)	Rabbit (Monoclonal)	1/100
CD206	Cell Signaling (#56082)	Rabbit (Monoclonal)	1/100
TRMT61b	Sigma Aldrich (HPA026751)	Rabbit (Monoclonal)	1/100
OLIG2	Cell Singaling (#659155)	Rabbit (Monoclonal)	1/100
MTC01	Invitrogen (MA5-60181)	Rabbit (Monoclonal)	1/100

Table 5: List of secondary IF antibodies used.

Target	Reference	Dilution
Goat α Rabbit 488	Cell signaling (4412S)	1/200
Donkey α Rabbit 647	Abcam (ab150075)	1/200
Donkey α Goat 647	Abcam (ab150131)	1/200
Goat α Mouse 488	Invitrogen (A-21402)	1/200
Donkey α Mouse 647	Abcam (ab150107)	1/200
Goat α Chicken 568	Invitrogen (A-11041)	1/200
Goat α Rat 555	Invitrogen (A-21428)	1/200

4.10 Immunohistochemistry (IHC) staining and imaging

Frozen sections were equilibrated to room temperature and washed in 1X PBS. Heat-induced antigen retrieval was performed in citrate buffer (pH 7.0) by boiling the buffer in a microwave and incubating slides in the heated solution for 20 min, followed by cooling to room temperature. Sections were then permeabilized with 0.1% 100 X Triton in PBS for 10 min at RT^o and blocked for 1 h in blocking buffer containing 5% normal serum

(selected according to the host species of the secondary antibody), 5% BSA, and 0.1% 100 X Triton in PBS. Primary antibodies (Table 6) were diluted in blocking buffer and applied overnight at 4 °C.

Table 6: List of primary IHC antibodies used.

Target	Reference	Host	Dilution
GFAP	Dako (Z0334)	Rabbit (Polyclonal)	1/100
IDH1 R132H	Dianova (DIA-H09)	Mouse (Monoclonal)	1/100
γH2AX	Abcam (ab81299)	Rabbit (Monoclonal)	1/100
ALK	Roche (790-4794)	Rabbit (Monoclonal)	1/100
OLIG2	Cell Singaling (#65915S)	Rabbit (Monoclonal)	1/100

The next day, slides were washed in 1X PBS three times for 5 min and incubated with biotinylated secondary antibodies (Table 7) for 1 h at RT°. After washing three times (1X PBS; 5 min), signal amplification was performed using the VECTASTAIN® Elite® ABC Kit for 1 h. Slides were washed again for 5 min (1X PBS; 3 times) and endogenous peroxidase activity was quenched for 30 min. Sections were developed using the Vector DAB substrate kit for 5 min and counterstained with Harris hematoxylin for 1 min, rinsed in running tap water, dehydrated through graded ethanol, cleared in xylene, and mounted using Eukitt mounting medium.

Table 7: List of secondary IHC antibodies used.

Target	Reference	Dilution
Goat α Mouse Bt	Vector (BA9200)	1/200
Goat α Rabbit Bt	Vector (BA1000)	1/200

Brightfield images were acquired using a Nikon Eclipse Ci microscope equipped with a Gryphax camera system and 4X and 20X air objectives.

4.11 Bromodeoxyuridine (BrdU) staining in GOs

Two-week-old GOs were transferred to 6-well ultra-low attachment plates at a density of 10 organoids per well in M-GO medium supplemented with BrdU (20 μM) and incubated overnight at 37 °C and 5% CO₂ on an orbital shaker set to 90 rpm. Following labeling, GOs were washed three times in 1X PBS, fixed, cryoprotected, embedded in OCT, and cryosectioned as described previously. BrdU incorporation was detected by IF using the previously described protocol with DNA denaturation performed by 2N HCl treatment for 30 min at 37°C prior to antibody incubation. Sections were then incubated with an anti-BrdU primary antibody (Agilent, M0744).

Images were acquired using a Leica THUNDER Imager equipped with 20X and 40X air objectives.

4.12 Transmission electron microscopy (TEM)

Paired parental tumoral pieces 1 mm³ in volume and two-week old GOs were collected into 15 mL conical tubes, washed with 1X PBS three times and fixed O/N at 4°C in a solution containing 2% PFA and 2.5% glutaraldehyde (electron microscopy grade). Sample processing and imaging was performed by the Electron Microscopy Unit at CNB (Madrid, Spain). Fixed samples were washed and post-fixed for 1 hour at 4°C with osmium tetroxide 1% in potassium ferricyanide 0.8% and incubated with 2% aqueous uranyl acetate for 1 hour at 4°C. Samples were then dehydrated with successive increasing concentrations of acetone and embedded in epoxy resin TAAB 812. Polymerization was carried out for 2 days at 60°C. Resin blocks were trimmed and ultrathin 70 nm sections were obtained with the Leica UC6 ultramicrotome, transferred to 200 mesh nickel grids and stained with saturated aqueous uranyl acetate (25 min) and lead citrate 0.2% (1 min) at RT. Sections were visualized on a JEOL JEM 1400 Flash electron microscope (operating at 100 kV). Micrographs were taken with a Gatan One View digital camera at various magnifications.

4.13 Periodic acid-Schiff (PAS) staining

Two-week old GOs were collected and subjected to fixation, dehydration, paraffin embedding and sectioning as described above. Following IHC staining, after DAB development, slides were washed. Washed slides were oxidized with 0.5% periodic acid for 5 min and rinsed by successive changes in dH₂O. Slides were incubated for 15 min in Schiff's reagent and developed for 10 min in warm tap water. Developed sections were counterstained with Mayer's hematoxylin for 1 min, rinsed in tap water for 5 min and washed in dH₂O. Sections were then dehydrated, mounted with Eukitt mounting medium and imaged with a Nikon Eclipse Ci microscope equipped with a Gryphax camera system and 4X and 20X air objectives.

4.14 Measurement of GO growth

To quantify GO growth dynamics in culture, GOs were seeded immediately after establishment (day 0) into 6-well ultra-low attachment plates at three densities: low (10 GOs/well), medium (20 GOs/well), and high (40 GOs/well). GOs were maintained in 4 mL of M-GO medium per well. Medium was refreshed every 48 h and GOs were not passaged (cut) for the duration of the experiment.

Growth was monitored for 10 weeks by weekly brightfield imaging using a stereomicroscope equipped with a digital camera; the full well was captured in a single field of view. Two wells were analyzed per condition, and the same wells were imaged longitudinally such that individual GOs were followed over time. GO size was quantified in Fiji (v2.3.0) by measuring the long and short axes of each GO and calculating diameter as the mean of both axes. Relative growth was computed by normalizing each organoid diameter to its corresponding day 0 value.

4.15 Quantitative PCR (qPCR) of parental tumors and GO

Paired fragments of parental tumor tissue and corresponding GOs were mechanically dissociated using a tissue lyser in the presence of the lysis buffer provided with the Rneasy Mini Kit, and total RNA was extracted according to the manufacturer's instructions. For each sample, 1 µg of RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit following the manufacturer's protocol.

Table 8: List of qPCR primers:

Gene	Forward Sequence	Reverse Sequence
<i>Nestin</i>	GAGAGGGAGGACAAAGTCCC	TCCCTCAGAGACTAGCGGCAT
<i>OCT 3/4</i>	CAAAAACCCTGGCACAAACT	CCTGTCTCCGTCACCACTCT
<i>BMI-1</i>	GGAGACCAGCAAGTATTGTCCTTTG	CATTGCTGCTGGGCATCGTAAG
<i>GFAP</i>	CTTTCCTCCACTCCTTCCT	CAGCATTTGTCTTTATTTTCC
<i>S100B</i>	TTAAATGCGTTCCTCATCCA	TGCGAGTTCTGATGGAGTTG
<i>TUBB3</i>	GCCTGACAATTCATCTTTGGT	GCAGGCAGTCGCAGTTTT
<i>MAP2</i>	CCTGTGTTAAGCGGAAAACC	AGAGACTTTGTCCTTTGCCTGT
<i>PDGFR2</i>	CCACCTGAGTGAGATTGTGG	TCTTCAGGAAGTCCAGGTGAA
<i>CNP</i>	AGAAGGAGCTGCGACAATTC	TCCAAAGTAGGTGACCAAGTCC
<i>CD31</i>	TGTGCTCATCATGCTCTGTG	ACGCTTGTCACACTGCTTTG
<i>VEGFa</i>	CTAACACTCAGCTCTGCCC	ACACACAAATACAAGTTGCCAA
<i>VEGFR2</i>	GCTCGCCTCCCTTTGAAAT	ATTTCCACAGCAAAACACC
<i>PD-L1</i>	CCATACAGCTGAATTGGTCATC	CAGAATTACCAAGTGAGTCCTTTCA
<i>B-Actin</i>	TTCTACAATGAGCTGCGTGTG	GGGGTGTGAAGGTCTCAAA

qPCR was performed in optical 96-well plates using a Bio-Rad CFX Connect Real-Time PCR system with Takara Bio SYBR Green master mix. Each sample was run across 2 replicate reactions. Reactions were set up in a final volume of 20 µL, using 2 µL cDNA input per reaction and primers at a final concentration of 2 µM (Table 8). β-actin was used as a reference gene for normalization using the $2^{-\Delta\Delta Ct}$ method.

4.16 IDH1 R132H detection via droplet digital PCR (ddPCR)

Genomic DNA was extracted from GOs using the Dneasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions and quantified by NanoDrop. IDH1 R132H

mutation presence was assessed using the Bio-Rad ddPCR Mutation Assay (IDH1 R132H) together with Bio-Rad ddPCR Supermix. Reactions were prepared in a final volume of 20 μ L, using DNA at 10 ng/ μ L (200 ng DNA input per reaction). Droplets were generated using a QX200 Droplet Generator (Bio-Rad) and the corresponding Bio-Rad droplet generation oil, according to the manufacturer's instructions. Plates were sealed using a PX1 PCR Plate Sealer (Bio-Rad) and amplified on a C1000 Touch Thermal Cycler (Bio-Rad). Droplets were read using a QX200 Droplet Reader (Bio-Rad), and data were analyzed with QuantaSoft™ software version 1.7.

Each sample was run in two technical replicates. Assay controls included a no-template control, WT gDNA, and IDH1 R132H-positive gDNA from a confirmed mutant patient sample.

4.17 IDH1 G395 detection via Sanger-seq

Genomic DNA was extracted from GOs using the Dneasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA concentration was measured by NanoDrop, and the targeted IDH1 region was amplified by conventional PCR using Paq5000 DNA polymerase. Each reaction contained 1 μ L of gDNA and forward and reverse primers at 2 μ M final concentration (FW: CGGTCTTCAGAGAAGCCATT; RV: GCAAAATCACATTATTGCCAAC).

PCR products were resolved by 2% agarose gel electrophoresis, and the specific amplicon band was excised and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were submitted for bidirectional Sanger sequencing to the Genomics Service, Instituto de Investigaciones Biomédicas Alberto Sols (Madrid, Spain). Sequence traces were aligned to the IDH1 reference sequence (GRCh38). Chromatograms were visually reviewed using FinchTV (v1.4.0).

4.18 D-2-Hydroxyglutarate (D-2HG) measurement

D-2HG was quantified in GO samples and matched culture supernatants collected after 48 h of incubation. GOs were dissociated using TrypLE dissociation reagent combined with mechanical dissociation through a 21G needle mounted on a 1 mL syringe. In parallel, conditioned culture medium was collected from the same wells. Both dissociated GO samples and conditioned media were centrifuged at 10,000 $\times$ g for 5 min at 4 °C to remove cellular debris, and the remaining supernatants were used for D-2HG measurement.

D-2HG levels were measured using the BioVision PicoProbe D-2HG Assay Kit following the manufacturer's protocol. Fluorescence was measured on a Tecan Spark microplate reader with excitation and collection at 535 and 587 nm, respectively.

4.19 Immunophenotyping of immune populations by flow cytometry

Paired parental tumor tissue fragments and GOs after two weeks in culture from three independent patients were dissociated into single cell suspensions using a combination of enzymatic dissociation with the Neural Tissue Dissociation Kit (Miltenyi Biotec) and mechanical mincing.

Single cell suspensions were washed twice with 1X PBS (centrifugations at 500 X g for 5 min at 4°C) and passed through a 70 µm cell strainer to remove debris. Filtered samples were resuspended in RBC lysis buffer 1X for 10 min at RT° for erythrocyte removal. RBC lysis buffer was washed twice with 1X PBS.

Cells were resuspended in 100 µL of FACS Buffer containing Ca²⁺ and Mg²⁺ free PBS, 0.5% BSA and 0.05% sodium azide and stained with either a cell population panel (Table 9) or an IC panel (Table 10) of fluorochrome conjugated primary antibodies for 30 min at RT° in the dark. Samples were then washed twice, resuspended in 500 µL of FACS Buffer and kept on ice until analysis.

Table 9: Immune cell population flow cytometry antibody panel.

Antibody	Reference	Dilution
CD45-VioBlue	Miltenyi Biotec (130-110-637)	1/200
CD3-VioGreen	Miltenyi Biotec (130-113-142)	1/200
CD19-PE	Miltenyi Biotec (130-113-646)	1/200
CD4-APC	Miltenyi Biotec (130-113-222)	1/200
CD8-APC-Vio770	Miltenyi Biotec (130-110-681)	1/200

Table 10: Immune checkpoint flow cytometry antibody panel.

Antibody	Reference	Dilution
CD279-PE	Miltenyi Biotec (130-120-382)	1/200
CD274-VioBright-515	Miltenyi Biotec (130-122-813)	1/200
CD152-PE-Vio770	Miltenyi Biotec (130-130-455)	1/200
CD86-APC-Vio770	Miltenyi Biotec (130-116-163)	1/200
CD80-APC	Miltenyi Biotec (130-117-719)	1/200

Flow cytometry was performed on a Miltenyi MACSQuant 10 flow cytometer equipped with the standard 3 laser and 8 detector configuration. Voltages and compensation setting were carried out with single-stained samples, with fluorescence-minus-one

controls for setting the signal threshold and non-labeled samples for autofluorescence determination. Data analysis was performed in FlowJo software (version 10).

4.20 Tumor and GOs RNA extraction, sequencing and analysis

11 paired parental tumor tissue fragments and GOs after two weeks in culture were collected, washed in 1X PBS three times and stored in RNeasy lysis solution at 4°C for 24 hours, before being moved to -80°C until processing. RNA extraction was performed with the Qiagen RNeasy Mini Kit following the manufacturer's instructions. Extracted and purified RNA was kept at -80°C for long term storage.

For quality control before sequencing, RNA concentration was measured by NanoDrop spectrophotometry, and integrity was assessed by capillary electrophoresis in an Agilent Bioanalyzer system. Samples with concentrations higher than 10 ng/μL, a total RNA mass of over 20 μg and RIN value above 7 were selected for subsequent sequencing.

RNA sequencing was performed by BGI TECH SOLUTIONS using the DNBSEQ platform, with 150 bp paired-end reads and a sequencing depth of 20 million clean reads per sample. Sequencing quality control, sequence alignment and annotation was performed by BGI using their custom bioinformatics pipeline.

RNA-seq results were analyzed with the Dr. Tom bioinformatics platform provided by BGI. To assess the statistical significance of clustering patterns in heatmaps, we used a multiscale bootstrap resampling approach implemented in the pvclust R package (230). Clustering was performed using Pearson correlation distance ($1 - r$) and average linkage, consistent with the metrics used to generate the heatmaps. The resulting Approximately Unbiased (AU) p-values provide a robust and statistically validated measure of cluster reliability, as it estimates the probability that a given cluster is supported by the data beyond random chance. Clusters with AU $\geq 95\%$ were considered statistically significant ($p < 0.05$).

4.21 GBM molecular subtype classification

GBM molecular subtype classification into mesenchymal, classical and proneural subtypes as defined by Verhaak et al. (97) was performed from our RNA-seq cohort using the GlioVis application (231), which integrates three complementary classification algorithms: support vector machines (SVM), k-nearest neighbors (k-NN), and gene set enrichment analysis (GSEA) and using the refined gene signature described by Wang et al. (232). Each sample received an individual prediction from each method, and a consensus subtype was assigned when at least two of the three classifiers agreed.

4.22 scRNA-seq GBM datasets analysis

Two independent GBM scRNA-seq datasets were downloaded from GEO (GSE117891) (233) and GBmap (234). All analyses were conducted in R (v4.4.1) using the Seurat package (v5.3.0) (235).

After merging raw matrices, cells with <1 000 UMIs, <250 genes, or >15 % mitochondrial reads were discarded. Mitochondrial, ribosomal (RPL/RPS) and hemoglobin genes were removed to reduce technical noise. Doublets (DoubletFinder) (236) and ambient-RNA-contaminated cells (decontX) (237) were excluded; genes expressed in <1 % of cells were filtered out.

Data were log-normalised, the 2000 most variable genes were selected, and counts were scaled. We computed 30 principal components, corrected patient-specific batch effects with Harmony (238), built a shared-nearest-neighbour graph and applied Louvain clustering; and UMAP for 2-D visualization.

Malignant versus non-malignant cells were identified with SCEVAN (239), which infers large-scale copy-number alterations from transcriptomes. Cells with GBM-typical aneuploidies were labelled “tumor”, diploid cells “non-tumor”. Broad lineages (tumor-associated macrophages, microglia, glia, vasculature, T cells) were assigned by SingleR (240) label transfer from multiple reference atlases, then harmonized at cluster level by majority rule.

4.23 In silico drug sensitivity screening via DiSCoVER

To predict therapeutic sensitivities from transcriptomic profiles, RNA-seq expression data was analyzed using the DiSCoVER platform (v1.1) (241). For each parental tumor and paired GO sample, a gene-level expression file containing TPM values was provided as input. As a non-malignant reference, bulk RNA-seq expression data from healthy brain samples from the GTEx portal (242) were used; because the platform requires a single reference expression value per gene, GTEx brain samples were pooled by averaging TPM values per gene to generate one reference profile.

DiSCoVER returned separate drug response predictions per sample based on compound–response associations from CTRP, GDSC, and CCLE (179,243,244), with a sensitivity score ranging from –1 to 1, where higher values indicate greater predicted sensitivity and negative values indicate predicted resistance. For guided screening, predicted sensitivity scores were extracted for a manually curated list of compounds comprising SOC glioma agents and selected alternative regimens. For *de novo* screening, for each sample we selected the 20 compounds with the highest predicted

sensitivity scores. Candidate compounds were then aggregated across samples, and the six most recurrently selected compounds were retained as the final candidate panel for parental tumors and for matched GOs, respectively. The three compounds overlapping between the parental tumor and GO panels were selected for subsequent functional validation.

4.24 Maintenance of GBM38 GSCs and U87, LN229 and U373 cells

GBM38 cells were generated and maintained as previously described (194). In short, cells were derived from an adult GBM patient and grown as GSCs neurospheres in tissue culture flasks. Cells were maintained with serum-free M21 media (Table 11) supplemented with heparin at 2 $\mu\text{g/mL}$, human epidermal growth factor (hEGF) at 20 ng/mL and human fibroblast growth factor (hFGF) at 20 ng/mL. Cells were passaged via manual dissociation into a single-cell suspension with a thin-bore glass Pasteur pipette.

Table 11: M21 GSCs growth media composition.

Component	Volume (mL)	Final concentration
DMEM/F-12	470 ml	
MEM NEAA	5 ml	1 x
Hepes	1.9 ml	3.8 mM
D-(+)-Glucose solution	6 ml	0.54%
BSA FractionV	667 μl	0.01%
Sodium Pyruvate	5 ml	1 mM
L-Glutamine	5 ml	1 X
Antibiotic-Antimycotic	2 ml	0.4 X
N2 Supplement	5 ml	1 X
Hydrocortisone	150 μl	0.3 $\mu\text{g/mL}$
Tri-iodotironina	150 μl	0.03 $\mu\text{g/mL}$

On the other hand, U87, U373 and LN229 established cell lines were maintained as 2D monolayer cultures with DMEM/F12 supplemented with FBS 10% and penicillin-streptomycin. Cellular passaging was performed by enzymatic dissociation via TrypLE Express and re-plated at the required density for each assay. All cultures were maintained at 37°C with 5% CO₂.

4.25 Metabolic activity assessment after drug treatment via MTS assay

Metabolic activity following drug treatment was quantified using a colorimetric MTS assay. For both SOC agents and *de novo* selected compounds, drug concentrations were set to the mean IC₅₀ value reported for each compound across glioma cell lines in the GDSC database (179).

For each condition, 10 GOs were cultured in individual wells of a 6-well ultra-low attachment plate containing 4 mL of M-GO medium supplemented with the indicated compound or DMSO vehicle control. Plates were placed on an orbital shaker at 37 °C for 1 hour immediately after seeding and then transferred to standard culture conditions (37 °C, 5% CO₂) for 48 h. Following treatment, GOs were dissociated to single cells by incubation with TrypLE dissociation reagent for 15 min, followed by mechanical separation using P1000 and P100 pipette tips. Cell suspensions were filtered through 70 µm cell strainers, washed twice in 1XPBS, resuspended in 600 µL of M-GO medium, and plated in technical triplicates into 96-well plates.

GBM38, U87, U373, and LN229 cells were seeded in 96-well plates in 100 µL of their respective growth media at a concentration of 3000 cells per well and allowed to adhere/recover for 24 h. Drug treatments were prepared at twice the desired final concentration, added at 100 µL per well to a final volume 200 µL per well, and plates were incubated for 48 h under standard culture conditions.

After treatment, 30 µL of MTS reagent was added per well and plates were incubated for 2 h at 37 °C in the dark. Absorbance was measured at 492 nm (formazan peak) with 620 nm as a reference wavelength to correct for non-specific absorbance. Background signal was corrected by subtracting absorbance from media-only wells. Relative metabolic activity was calculated by normalizing each condition to its corresponding vehicle control.

4.26 IC50 determination in GOs and glioma cell lines and GOs time-course analysis

Half-maximal inhibitory concentrations (IC₅₀) for alectinib, ruxolitinib and dabrafenib were determined in GOs and glioma cell lines using the MTS assay workflow described previously, testing serially increasing concentrations of each compound. Relative metabolic activity values were calculated by normalization to vehicle-treated controls for each model. Dose–response curves were fitted in GraphPad Prism software version 10 using a four-parameter nonlinear regression model (variable slope), and IC₅₀ values were extracted from the fitted curves.

To evaluate whether sustained treatment further reduced GO metabolic activity, 10 GOs per condition were treated with alectinib, ruxolitinib, or dabrafenib and assayed by MTS after 48, 96, and 120 h of exposure using the same workflow. All experiments were performed with two biological replicates, each measured in technical triplicates.

4.27 Annexin V/PI apoptosis assay in GOs

To assess apoptosis following drug treatment, Annexin V/propidium iodide (PI) staining was performed and analyzed by flow cytometry. For each condition, 10 GOs were seeded and treated for 48 h, then dissociated into single-cell suspensions as described previously. Cell suspensions were passed through a 70 μm cell strainer to remove aggregates and washed in 1 $\times$ PBS. Cells were resuspended in 100 μL Annexin V Binding Buffer (BioLegend) and stained with 5 μL Annexin V–FITC (BioLegend) and 5 μL PI (Sigma-Aldrich) for 15 min at RT° in the dark. Samples were then diluted with 400 μL of Annexin V Binding Buffer, kept on ice, and acquired immediately on a MACSQuant 10 flow cytometer.

Compensation was performed with single-stained samples, while photomultiplier voltages were adjusted using a mixture of untreated cells and cells subjected to heating at 95°C for 10 min as a positive control. Unstained samples were used to assess autofluorescence. Data was analyzed using Kaluza (v2.1) and reported as relative cell fractions.

4.28 GOs transwell migration assay

To evaluate migration capabilities of cells within GOs, seven similarly sized GOs per condition were dissociated into single-cell suspensions as explained above. 10000 cells from the resulting suspension were resuspended in 300 μL of M-GO with the corresponding treatment and added to an 8 μm pore transwell insert, which was placed in a 24-well plate containing 1 mL of M-GO medium supplemented with heparin (2 $\mu\text{g}/\text{mL}$), hEGF (20 ng/mL), and hFGF (20 ng/mL) to generate a chemoattractant gradient; growth factors were added only to the lower chamber. Cells were allowed to migrate for 6 h under standard culture conditions.

After incubation, transwells were washed with 1X PBS and non-migrating cells were removed from the transwell via cotton swab. Transwells were fixed in 4% PFA for 15 min at RT° and stained with 0.1% crystal violet for another 15 min. Transwell membranes were cut from the insert and mounted on a standard microscope slide with Eukitt mounting medium. Images of migrated cells in the center of the well were captured immediately using a Nikon Eclipse microscope and manually quantified using Fiji. GOs from 3 independent patients were used, with 3 images per patient and condition acquired.

4.29 U373 and LN229 wound-healing migration assay

To assess 2D migratory behavior of U373 and LN229 glioma cells, 500,000 cells per well were seeded in 6-well plates in 3 mL of DMEM/F-12 supplemented with 10% FBS and incubated overnight to reach full confluency. A linear scratch was generated using a 200 µL pipette tip by drawing a straight line across the cell monolayer. Wells were gently washed twice with 1× PBS to remove detached cells and debris, and fresh medium containing the indicated drug concentration was added.

Wound areas were imaged immediately after scratching and again after 24 h using a Leica THUNDER Imager equipped with a motorized stage, enabling re-imaging of the same fields of view at both time points. For each well, three fields along the wound were acquired, and each condition was performed in duplicate wells. Wound closure was quantified using an automated image analysis macro (245) in Fiji to minimize bias introduced by manual region selection.

4.30 *In vivo* experiments

For *in vivo* drug studies, athymic nude female mice were used. For GO xenografts, 10 GOs per mouse flank were resuspended in 0.2 mL of ice-cold liquid Matrigel and placed and implanted subcutaneously through a small surgical flank incision using a pre-chilled wide-bore P1000 tip; the wound was closed by suture. On the other hand, for U87 xenografts, 10⁶ cells per flank were resuspended in cold liquid Matrigel and subcutaneously injected with a 25G needle and a 1 mL syringe. Mice were anesthetized using 5% isoflurane for induction and maintained at 2% during the procedure. Mice were given 0.3 mL of meloxicam at 0.2 mg/mL as analgesia following the procedure.

Tumor growth was monitored daily with caliper measurements with the previously described formula $V = \frac{DI \cdot Ds^2}{2}$, where DI and Ds represent the long and short tumoral diameters, respectively (246,247). Treatments commenced when tumors reached a volume of approximately 200 mm³. Mice were treated daily via intraperitoneal injections of vehicle, alectinib (25 mg/kg), ruxolitinib (50 mg/kg) or dabrafenib (20 mg/kg). Mice were euthanized via cervical dislocation either when tumors reached 1500 mm³ or when tumor burden became apparent, in accordance with ethical guidelines. Flank tumors were removed and separated from remaining skin for imaging and volume measurement

These experiments were performed under PROEX 142.8/24 in compliance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, as well as other applicable national and institutional

regulations, ensuring the highest standards of animal welfare and ethical research practices.

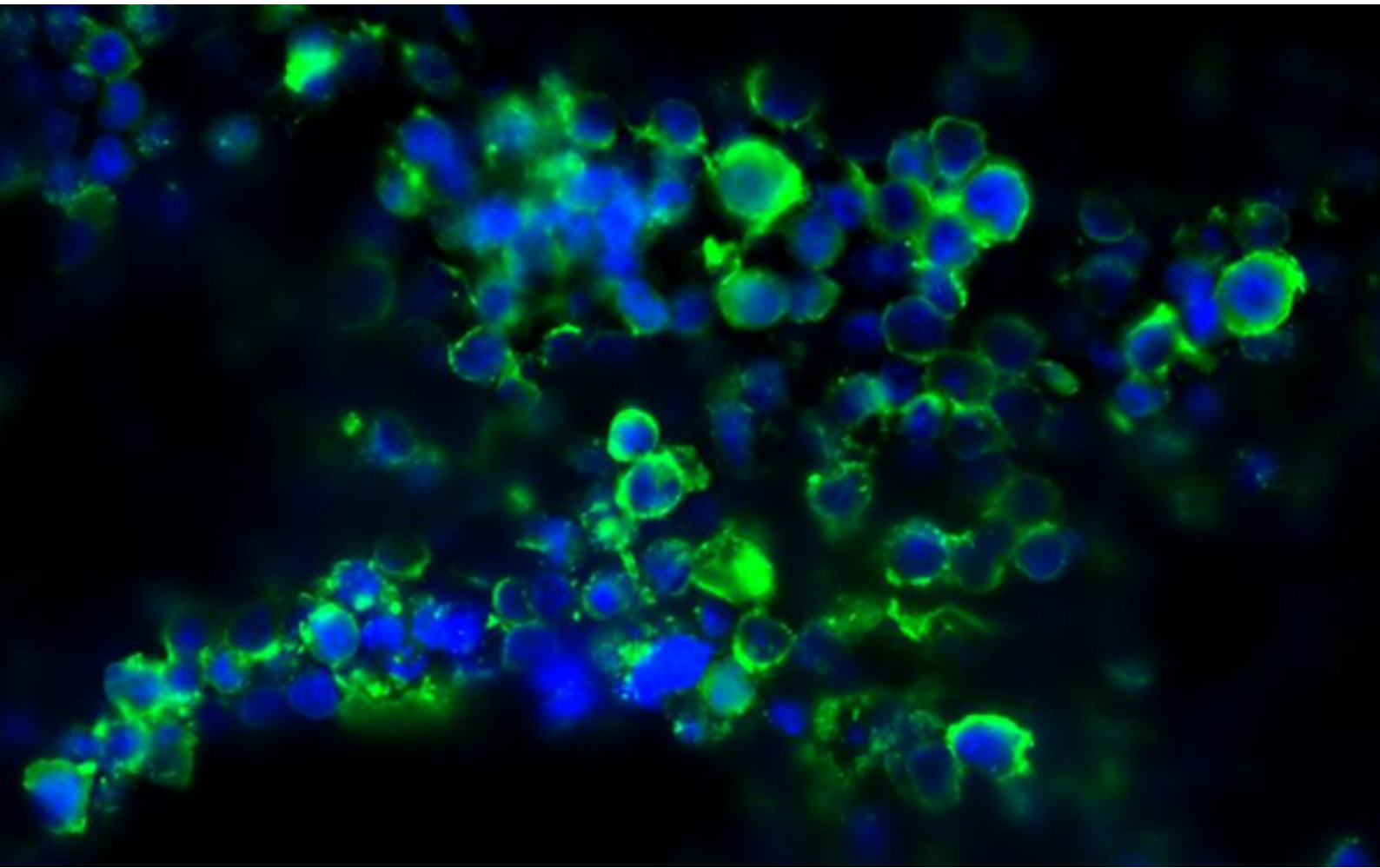
4.31 Statistical analysis of results

All statistical analyses were performed using GraphPad Prism software (version 10). Sample normality was assessed with either the Shapiro-Wilk or Kolmogorov-Smirnov test, depending on sample size. For normally distributed data, comparisons among groups were conducted using either one-way or two-way ANOVA with Welch's correction, while non-parametric data were analyzed with the Kruskal-Wallis test. Statistical significance was represented as follows: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Differences between groups were considered statistically significant at $p < 0.05$.

4.32 Illustrations

All illustrations were created in BioRender (<https://BioRender.com>).

5. RESULTS



“El entusiasmo y la perseverancia hacen milagros”

- Santiago Ramón y Cajal

5.1 Glioma organoids (GOs) establishment performance

5.1.1 High success rates in GO establishment

From our original cohort, we discarded 4 samples upfront due to either insufficient sample volume or low cellularity and generated GOs from the remaining 57 samples. Successful GO establishment was defined by survival beyond two weeks in culture, progressive increase in GO size, and preservation of key histological features of the corresponding parental tumors, as confirmed by an attending pathologist. Within the overall cohort, we had an efficient generation rate of 91% with 52 successful cases and only 5 failed GOs. When stratified by tumor type, GBM derived GOs (GOWts) presented a slightly higher success rate at 93%, with 43 successful GOs and 3 failed examples. On the other hand, IDH-mutant GOs (GOMuts) dropped to 81%, as 2 out of 11 samples did not yield satisfactory GOs (Fig. 22).

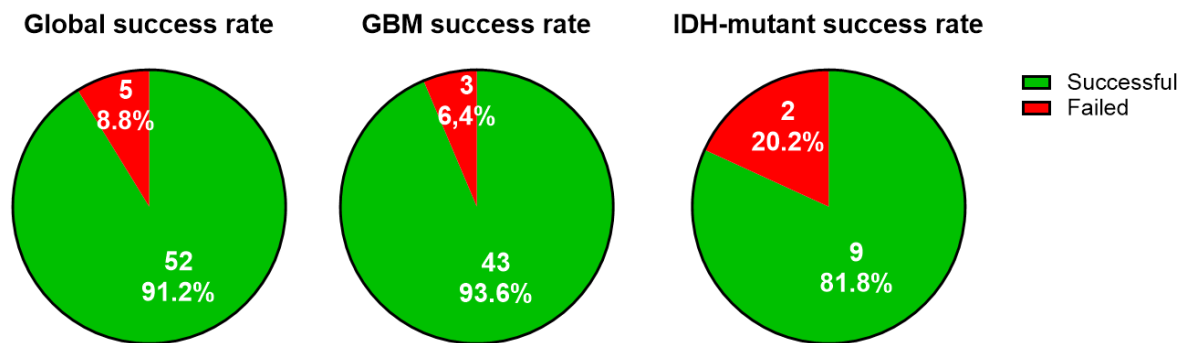


Figure 22: Efficient generation of GOs. Pie charts representing GO generation success rates. Green segments correspond to successful GOs, while red segments represent failed cultures. From left to right we have the overall cohort (N=57), GBM patients (N=46) and IDH-mutant patients (N=11). Samples excluded prior to culture initiation (N=4) are not included.

5.2 GO characterization and model validation

5.2.1 GOMuts conserve the clinically relevant IDH1 R132H mutation

Since mutations in the *IDH1* gene constitute the only clinically relevant alteration in the molecular diagnosis of gliomas (13,16), we began our characterization by evaluating whether the most common mutation (*IDH1* R132H) was conserved in GOs derived from IDH-mutant gliomas utilizing different complementary approaches. Sanger sequencing confirmed the IDH1 c.395G>A substitution in IDH-mutant-derived GOs (GO7 and GO9), whereas this variant was absent in an IDH-wildtype control GO (GO8) (Fig. 23).

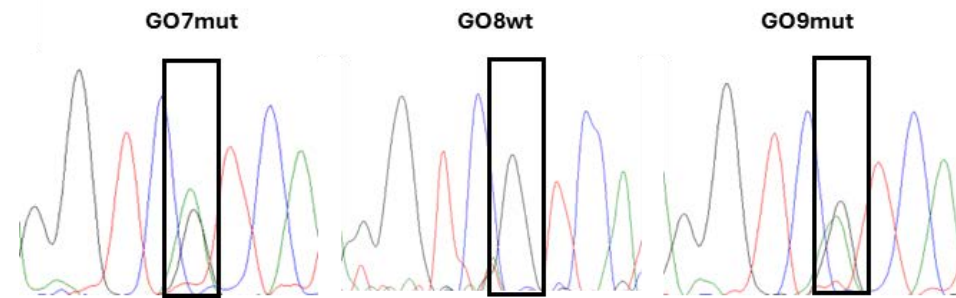


Figure 23: Sanger sequencing confirms IDH1 c.395G>A (R132H) in IDH-mutant-derived GOs. Representative Sanger chromatograms from GO7 and GO9 (IDH-mutant-derived) and GO8 (IDH-wildtype control). The c.395G>A substitution site is highlighted. Base colors: T (red), C (blue), G (black), A (green).

Retention of mutant *IDH1* was further evaluated by ddPCR on genomic DNA, which detected the *IDH1* R132H allele and supported its persistence in GOs across extended culture up to eight weeks (Fig. 24).

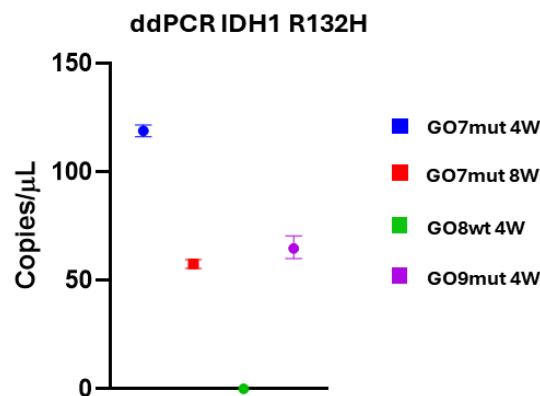


Figure 24: ddPCR detects IDH1 R132H mutant alleles in GOMuts. ddPCR results for IDH1 R132H mutation in 4 GOs at different growth times. GO7 and GO9 are representative GOMuts samples, with GO8 representing an IDH-WT sample. GO7 is assessed 4 and 8 weeks after establishment. Mean copies/μL values are indicated by colored points, with bars representing SD.

In addition, IHC using an IDH1 R132H-specific antibody confirmed protein-level detection of mutant IDH1 in matched IDH-mutant tumors and derived GOs, but not in an IDH-wildtype GBM control (Fig. 25).

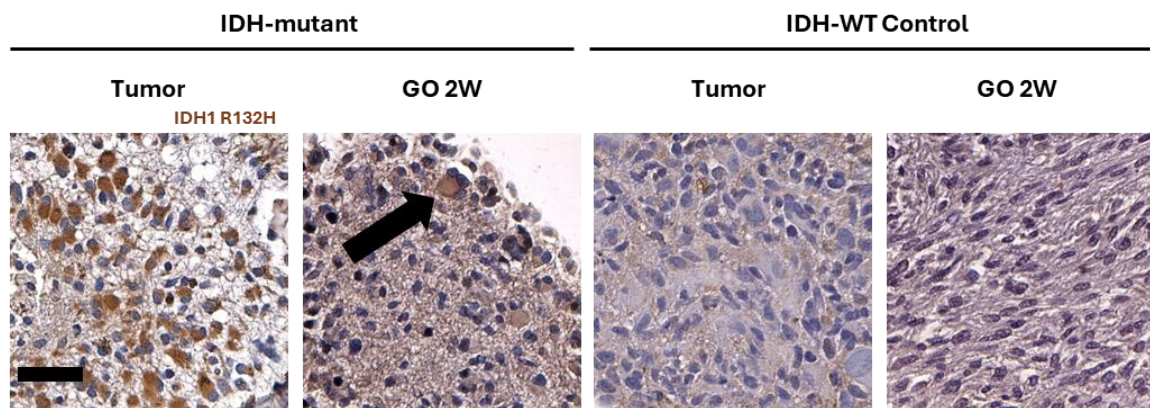


Figure 25: IHC of IDH1 R132H mutation is positive in GOMuts. Representative IHC images of IDH1 R132H mutation (brown) in GO7mut (left) and an IDH-WT patient (right). Black arrow indicates IDH1 mutant cells in the GO. Scale bar: 100 μ m.

Finally, we quantified the oncometabolite D-2HG, a hallmark metabolic product of mutant IDH1, in GOs and culture supernatants using an enzymatic assay. Consistent with the previous results, D-2HG levels were elevated in GOMuts and their supernatants relative to GOWts controls (Fig. 26).

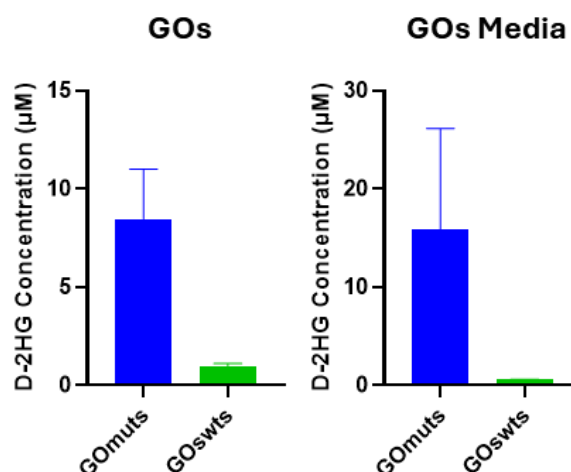


Figure 26: Oncometabolite D-2HG levels are higher in GOMuts than GOWts. Bar graph of D-2HG concentration (μ M) in GOs (left) and their respective culture media (right). Blue represents GOMuts and green GOWts, bar height represents mean value with error bars indicating SD.

5.2.2 GOs preserve histological characteristics of parental tumors

Histology of successfully established GOs was compared with their matched parental tumor specimens using H&E staining. Both GBM-derived and IDH-mutant glioma-derived GOs closely resembled their corresponding tumors, as assessed by an attending pathologist. GOWts maintained pleomorphic cells with nuclear atypia, increased cellularity, microvascular proliferation and necrotic areas (Fig. 27A), while GOMuts, in contrast, presented less aggressive proliferation, no microvascular proliferation and reduced necrosis with more differentiated cellular phenotypes (Fig. 27B), mimicking their respective parental tumors.

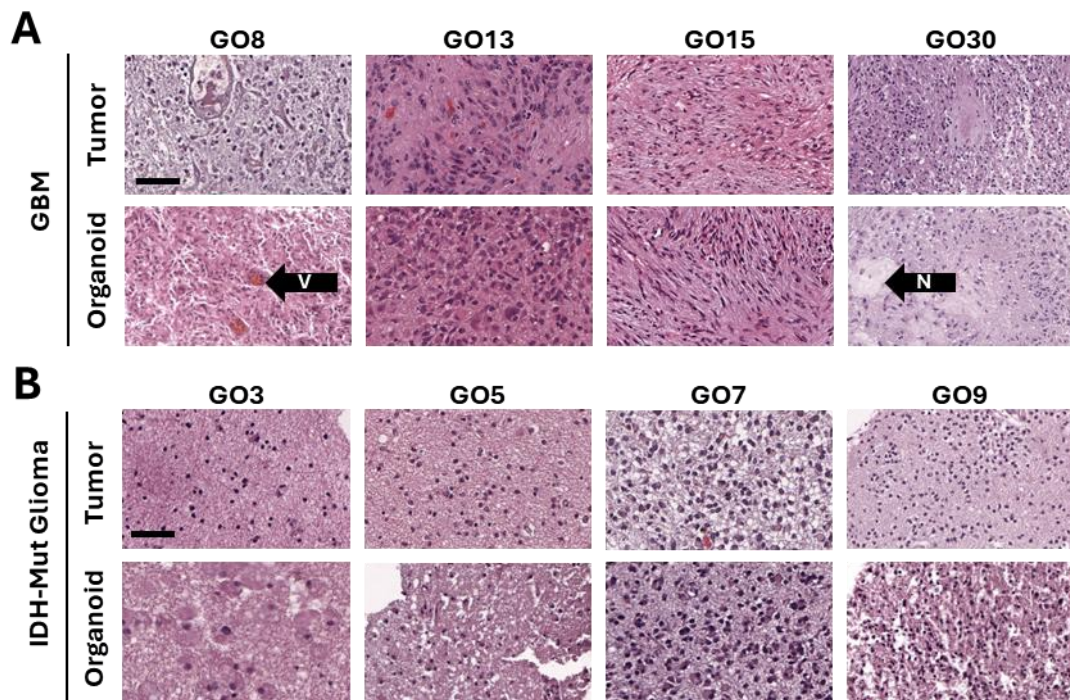


Figure 27: GOs preserve histological features of parental tumors. H&E staining of representative sections from matched parental tumor tissue (top) and derived GOs (bottom) for GBM (A) and IDH-mutant glioma (B). Scale bar: 100 μ m. Regions of microvascular proliferation are indicated by arrows labeled “V”, and necrotic regions by arrows labeled “N.”

5.2.3 GOs conserve parental tumor gene expression patterns

Following morphological characterization, we assessed transcript-level concordance between parental tumors and GOs using a targeted qPCR gene panel. For each gene, mean expression values were computed across all samples within each group (GBM tumors vs GOWts; IDH-mutant tumors vs GOMuts) and compared by linear regression. This gene-wise mean expression analysis showed strong concordance, with $R^2 = 0.79$ for GBM and $R^2 = 0.83$ for IDH-mutant samples (Fig. 28).

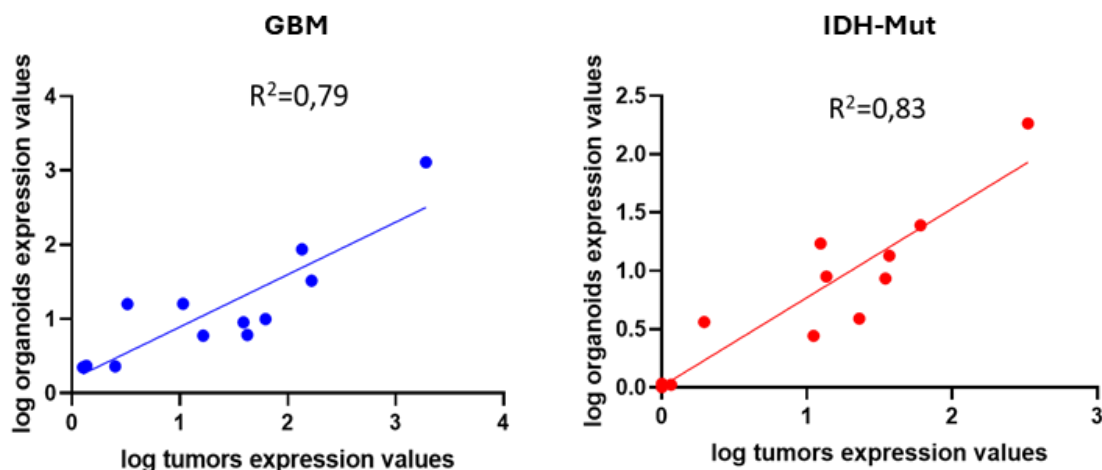


Figure 28: GOs and parental tumors present high correlation levels in gene expression. Linear regression comparing gene-wise mean log-transformed qPCR expression values between parental tumor tissue and derived GOs after two weeks in culture, shown separately for GBM (left) and IDH-mutant glioma (right). Each point represents one gene from the targeted qPCR panel plotted as the mean across all samples in each group. R^2 is shown on each plot. $N = 3$ samples per group.

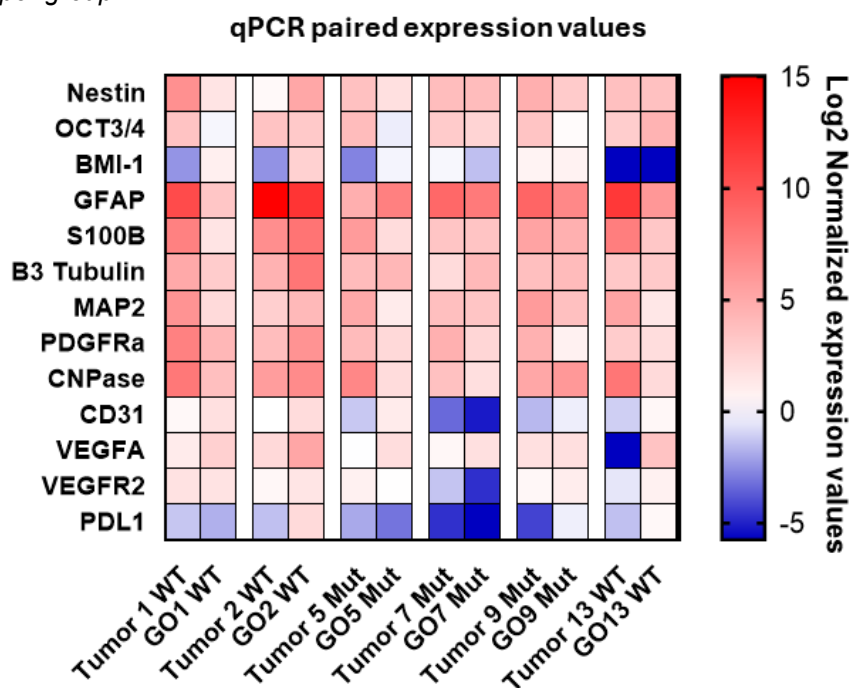


Figure 29: GOs recapitulate mRNA expression patterns of parental tumors. Heatmap of $\log_2$ -normalized qPCR expression values for the assessed genes, with each pair of columns representing parental tissue (left) and the corresponding GO after two weeks in culture (right). $N = 6$ paired samples.

To assess concordance across individual matched samples, log-transformed qPCR expression values were represented in a heatmap. Across the assessed gene panel,

RESULTS

GOs broadly preserved relative expression patterns observed in their corresponding parental tumors. Moreover, vascular markers presented lower expression values in IDH-mutant samples (Fig. 29).

5.2.4 GOs retain stemness-associated programs

To further characterize GOs, we assessed expression of markers associated with stem-like and mesenchymal-like tumor cell states that are frequently observed in GBM. IF imaging detected CD90, vimentin, and nestin expression in GOs (Fig. 30).

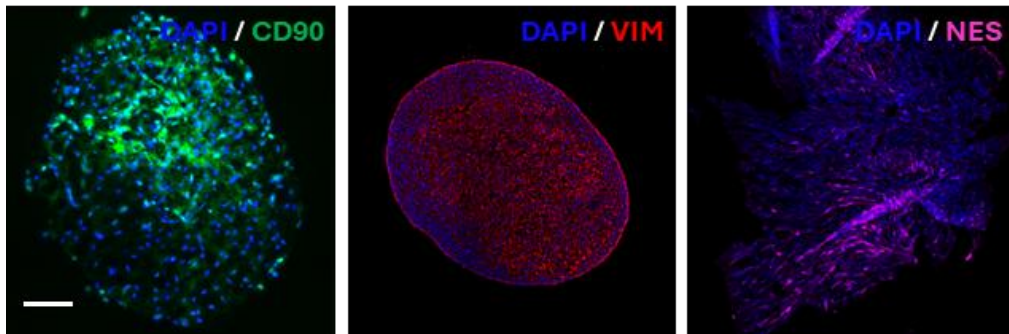


Figure 30: Stemness- and mesenchymal-associated markers in GOs. Representative IF images of GOs after two weeks in culture showing CD90 (green), vimentin (red), and nestin (magenta), with nuclei counterstained with DAPI (blue). Scale bar: 200 μ m.

qPCR analysis further detected nestin transcripts in both GBM-derived and IDH-mutant-derived GOs, together with OCT3/4 (Fig. 31). BMI1 transcripts were consistently detectable in GOs and were low or undetectable in matched parental tumor samples (Fig. 31).

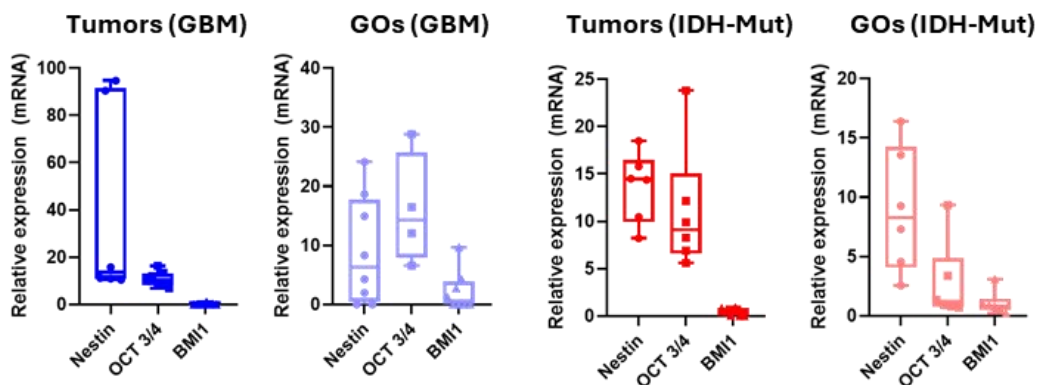


Figure 31: qPCR expression of stemness-associated markers in parental tumors and matched GOs. Violin plots showing qPCR expression levels of Nestin, OCT 3/4 and BMI1 in matched parental tumors and derived GOs, stratified by diagnosis (GBM vs IDH-mutant). Expression is shown as $2^{-\Delta\Delta C_t}$. Overlay indicates median and IQR; points represent individual samples. N = 3 paired GBM samples and 3 paired IDH-mutant samples.

5.2.5 Endothelial-associated features are detectable in GOs

We next evaluated whether GOs retained vascular-associated features, crucial to mirroring the different GBM tissular niches. IF imaging detected the presence of VEGFA and its receptor VEGFR2 in GOs after two weeks in culture, together with CD31 positive vessel-like structures (Fig. 32).

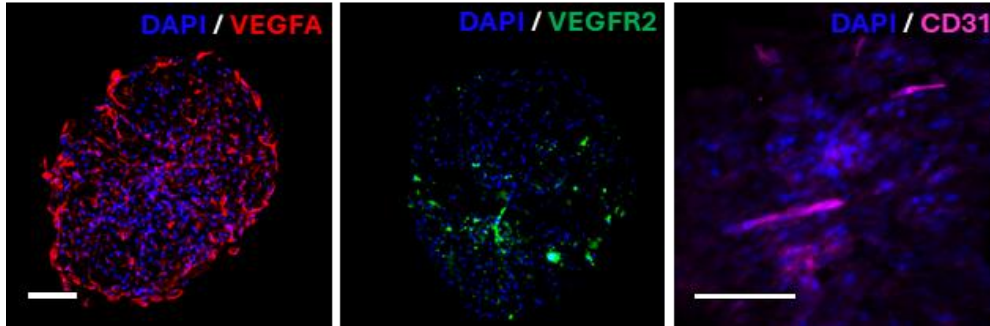


Figure 32: Vascular-associated features in GOs. Representative IF images of GOs after two weeks in culture showing VEGFA (red), VEGFR2/KDR (green), and CD31 (magenta), with nuclei counterstained with DAPI (blue). Scale bar: 200 μ m.

Expression of VEGFA, VEGFR2, and CD31 was further assessed at the transcript level by qPCR in GOs and matched parental tumors from both GBM and IDH-mutant cases (Fig. 33). All three markers were detectable across conditions. Notably, GBM samples presented higher expression on vascular markers, and VEGFA mRNA levels appeared higher in GOwts relative to matched tumors, whereas this pattern was not as pronounced in IDH-mutant-derived GOs (Fig. 33).

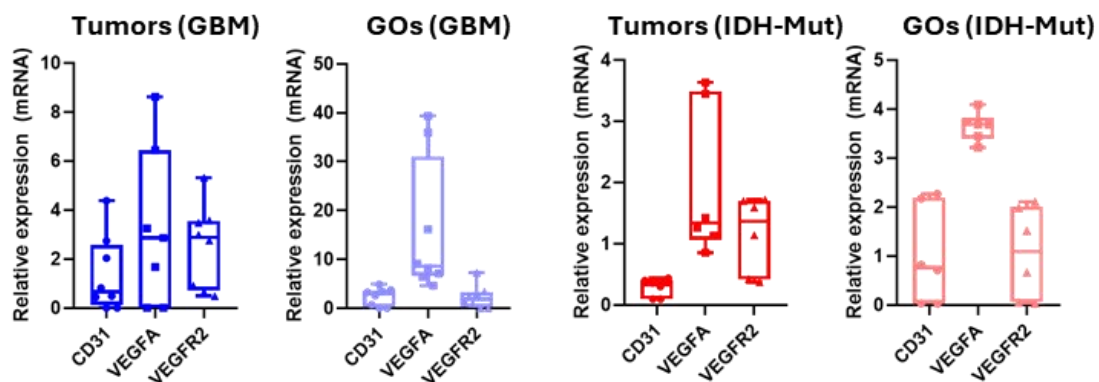


Figure 33: qPCR expression of vascular-associated markers in parental tumors and matched GOs. Violin plots showing qPCR expression levels of VEGFA, VEGFR2, and CD31 in matched parental tumors and derived GOs, stratified by diagnosis (GBM vs IDH-mutant). Expression is shown as $2^{-\Delta\Delta C_t}$. Overlay indicates median and IQR; points represent individual samples. $N = 3$ paired GBM samples and 3 paired IDH-mutant samples.

RESULTS

Having determined that both GOMuts and GOWts closely replicate histological features and expression patterns of their parental tumors, we decided to focus the rest of the experiments on GOWts, as GBMs constitute the most common tumors with the most challenging clinical management.

5.2.6 GOs culture at medium density and smaller sizes optimizes growth while cryopreservation of GOs maintains histological characteristics

We next sought to optimize GO culture conditions by determining culture density, growth times and maximum organoid size.

In order to characterize culture density and establish growth dynamics, GOs were cultured at three different seeding densities: low (10 GOs per well), medium (20 GOs per well), and high (40 GOs per well) in six-well plates (well surface area: 9.6 cm²). GO growth was monitored for 10 weeks following establishment, and relative changes in GO diameter were quantified over time.

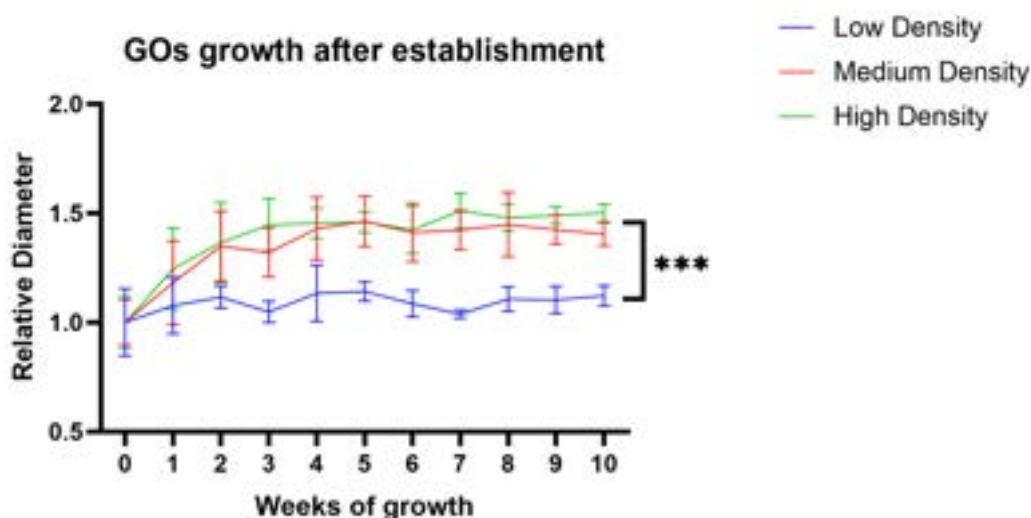


Figure 34: GO seeding optimization. Line plot showing longitudinal growth curves of GOs cultured at low (10 GOs per well), medium (20 GOs per well), or high (40 GOs per well) seeding densities. Data points represent the mean relative organoid diameter normalized to the time of establishment (week 0), with error bars indicating standard deviation (SD). Blue, red, and green lines correspond to low, medium, and high-density conditions, respectively. Statistical significance is indicated (** $p < 0.0001$).

GOs cultured at low seeding density exhibited significantly reduced growth compared with those cultured at medium and high densities. In contrast, no significant differences in growth were observed between medium and high-density conditions. Notably, GO

growth under both medium and high-density conditions plateaued after approximately four weeks in culture (Fig. 34).

Based on these observations, a seeding density of 2 organoids per cm² and a culture duration of two weeks post-establishment were selected for subsequent experiments.

Histological assessment of GOs after prolonged culture via H&E confirmed the preservation of histological characteristics up to 10 weeks in culture (Fig. 35).

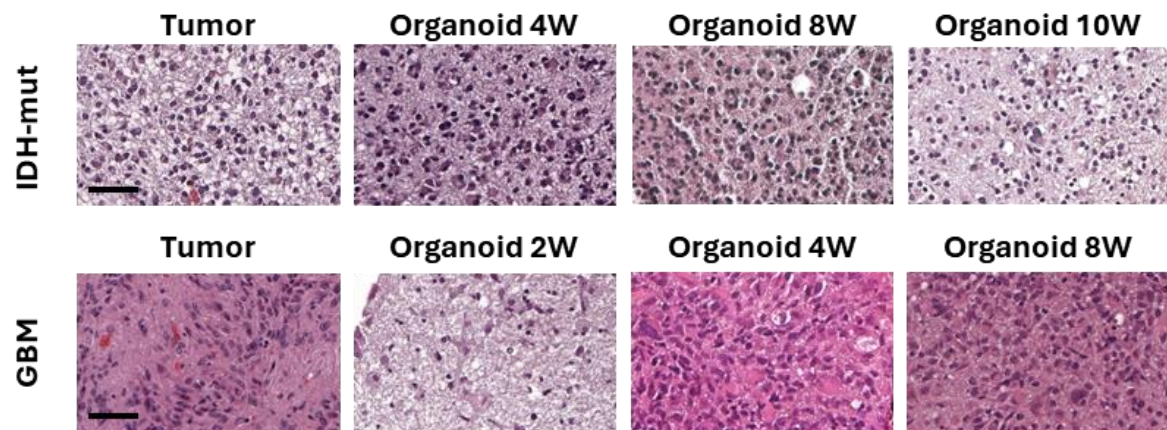


Figure 35: GOs preserve histological features of parental tumors in prolonged culture. H&E staining of representative IDH-mutant (top) and GBM (bottom) parental tumor section (left) and its derived GOs. Scale bar: 200 μ m.

We next examined GOs at larger sizes, when they reached a diameter of approximately 3 mm, IHC analysis revealed that expression of the proliferation marker Ki-67 was largely restricted to a subset of cells located at the GO periphery. In contrast, the core of the GO displayed ample cellularity but no Ki-67 signal (Fig. 36).

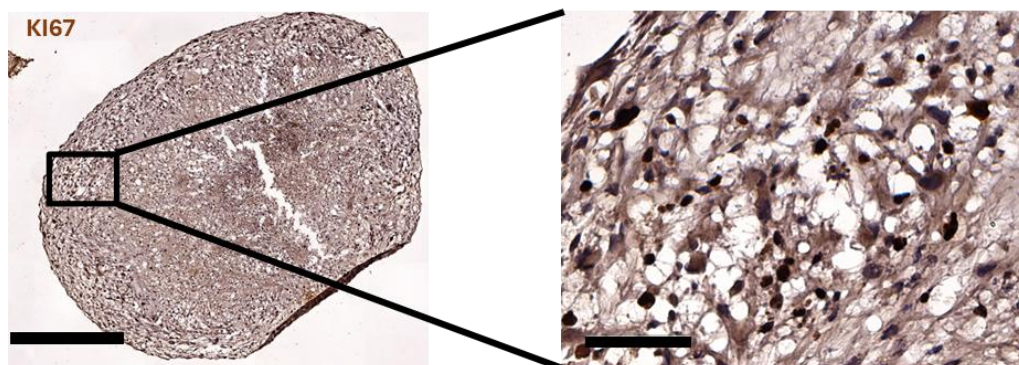


Figure 36: Proliferation only occurs in the periphery of larger GOs. Immunohistochemistry (IHC) of a large 3 mm wide GO. Brown nuclei are stained for proliferation marker Ki67, with nuclei counterstained by hematoxylin. Left image depicts a general overview (scale bar is 1000 μ m), with an amplified section of the proliferating periphery (scale bar is 100 μ m).

RESULTS

This core to periphery dichotomy was also evident for vimentin, a marker associated with mesenchymal identity and cellular plasticity, which was predominantly expressed in peripheral cells of large GOs in stark contrast to the more homogeneous distribution in smaller GOs (Fig. 37).

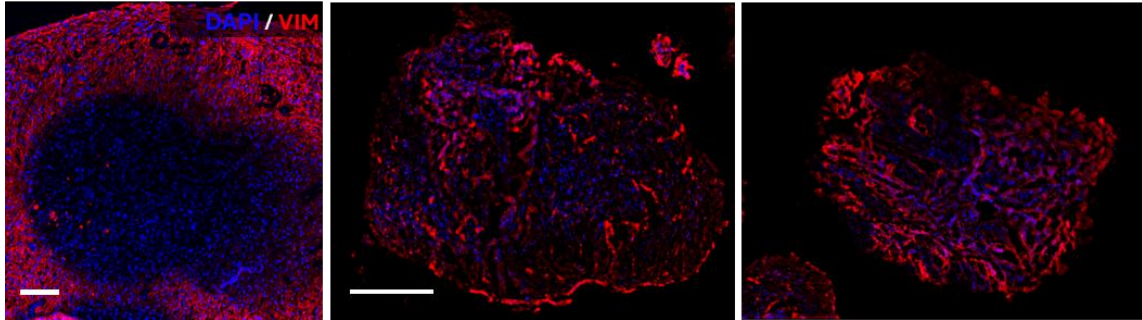


Figure 37: Vimentin distribution varies depending on GO size. Representative Vimentin (red) IF image in larger GO (left) and two different normal sized GOs (center and right). Nuclei are counterstained with DAPI in blue. Scale bar: 200 μ m.

In contrast, β -catenin (CTNNB1) did not display a comparable spatial distribution and was more uniformly observed throughout the GO (Fig. 38). Similarly, expression of the hypoxia-associated factor HIF-1 α was detected across both core and peripheral regions (Fig. 38). Qualitative assessment indicated that overall cellularity was comparable between the core and periphery of large GOs. Therefore, we established GO diameter of less than 3 mm for subsequent analysis.

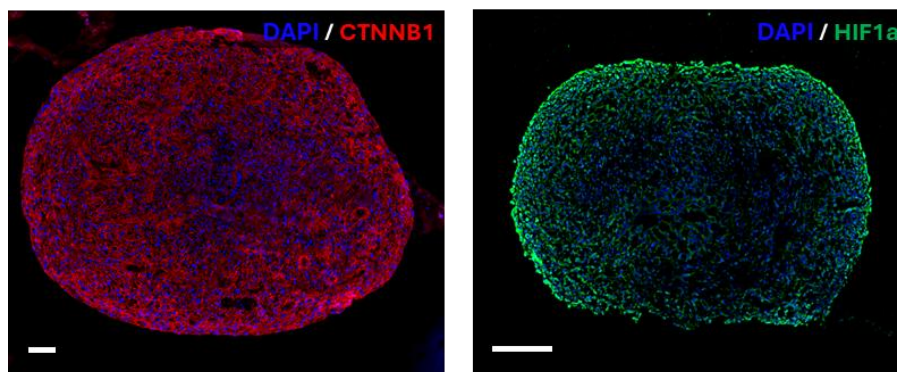


Figure 38: CTNNB1 and HIF1a appear uniformly in larger GOs. (A) Representative CTNNB1 (red) IF image in larger GO. Scale bar: 100 μ m. (B) Representative HIF1a (green) IF image in larger GO. Scale bar: 100 μ m.

We therefore established a medium culture density to promote efficient growth, an experimental window of two weeks post-establishment to avoid culture stagnation and a size of less than 2 mm for GOs for all subsequent experiments.

To evaluate the feasibility of long-term storage for downstream applications, GOs were cryopreserved and subsequently recovered in culture. Recovered GOs preserved histological characteristics of both parental tumors and freshly derived GOs as assessed by H&E staining (Fig. 39).

In contrast, monitoring of GO diameter following recovery indicated minimal growth relative to the recovery time point (week 0), suggesting impaired post-thaw growth under the conditions tested (Fig. 40).

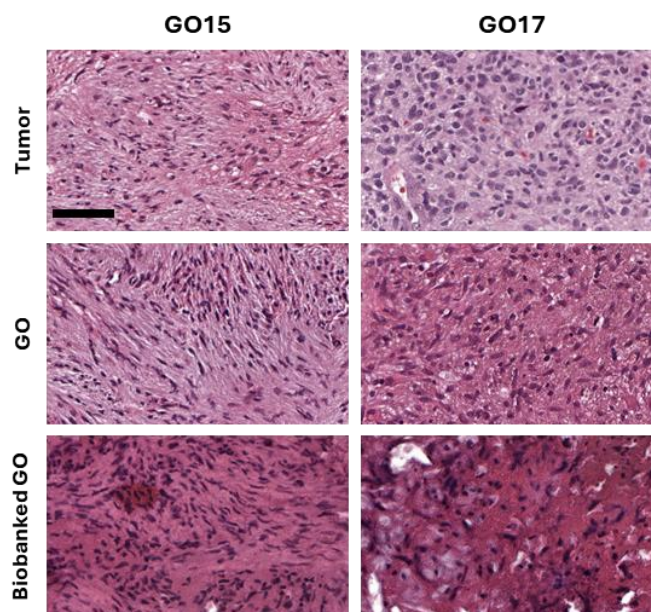


Figure 39: Preservation of histological features after GO cryopreservation and recovery. H&E staining of parental tumors (top), freshly derived GOs (middle) and cryopreserved and recovered GOs (bottom) from two representative patients. Scale bar: 200 μ m.

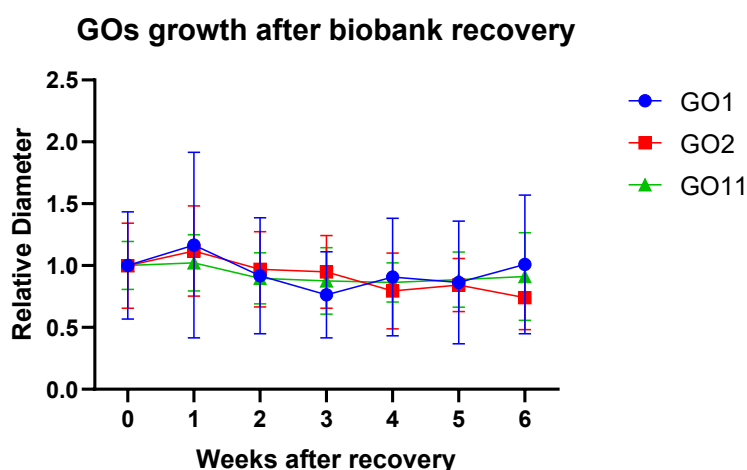


Figure 40: Growth of GOs is impaired after cryopreservation. Line plot showing longitudinal growth curves of GOs from three representative patients (one line color each). Data points represent the mean relative GO diameter normalized to the time of recovery (week 0), with error bars indicating SD. N = 10-20 GOs per patient.

5.2.7 GOs retain proliferative cell populations

To determine whether GOs were actively proliferating in our tissue culture conditions, we first observed macroscopic increases in GO size (Fig. 34). In addition to these observations, we performed IF for the DNA intercalating agent BrdU, which intercalates DNA during cellular replication. BrdU-positive cells were detected throughout two-week-old GOs, indicating the presence of actively proliferating cell populations within these 3D cultures. This finding suggests that GOs maintain sustained proliferative activity *in vitro*, consistent with a key biological feature of parental tumors (Fig. 41).

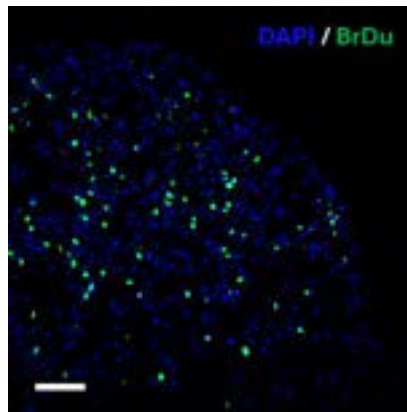


Figure 41: GOs conserve proliferating cellular populations. *BrdU IF in a representative two-week-old GO. BrdU-positive proliferating nuclei are shown in green, with all nuclei counterstained with DAPI in blue. Scale bar: 100 μ m.*

5.2.8 TEM reveals ultrastructural features of GOs

To further characterize GOs composition at the ultrastructural level, TEM was performed after two weeks in culture. Myelin sheath-like structures were detected in GOs. However, compared with matched parental tumors, these appeared markedly deteriorated, as evidenced by a much thinner and disorganized appearance (Fig. 42).

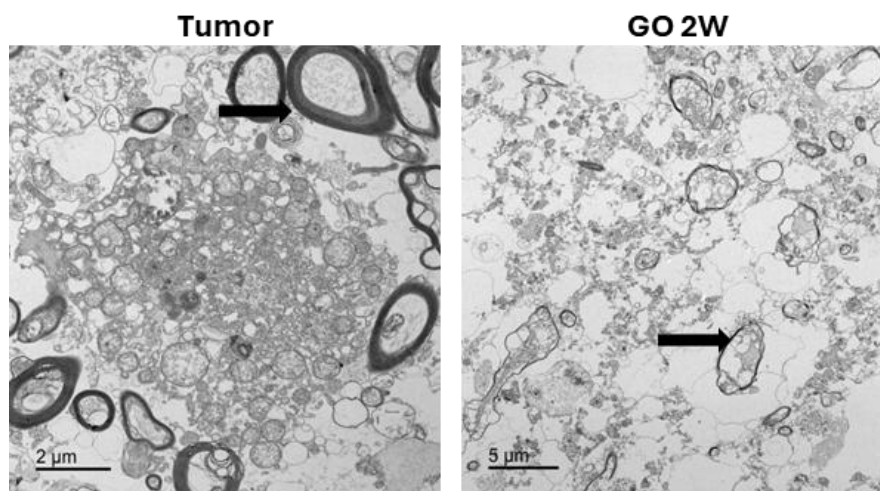


Figure 42: Myelin sheath-like structures are detectable in GOs but appear deteriorated in culture. Representative TEM images from parental tumors (left) and paired GO (right) after two weeks in culture. Black arrows indicate myelin sheath-like structures. Scale bars: 2 and 5 μm, respectively.

Ultrastructural features consistent with edema-like alterations and necrotic regions, which are characteristic of GBM, were also observed in both parental tumors and paired GOs (Fig. 43).

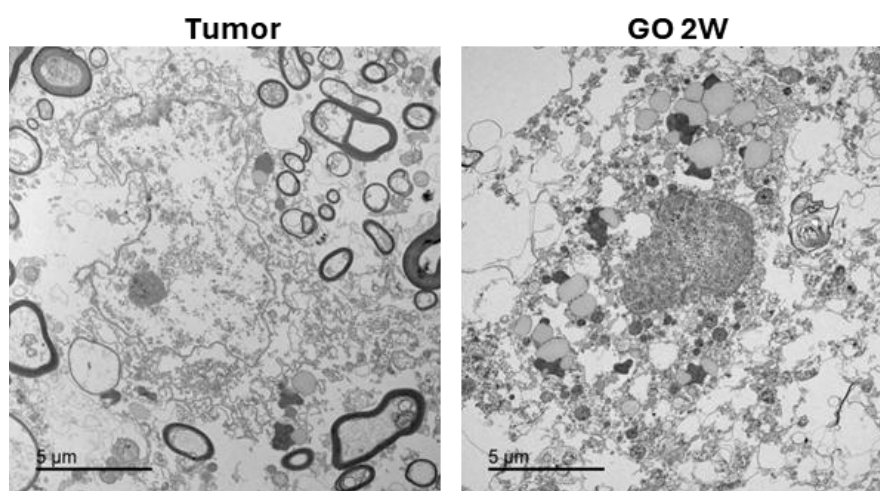


Figure 43: GOs present signs of edema and necrosis. Representative TEM images from parental tumors (left) and paired GO (right) after two weeks in culture. Images show regions consistent with edema-like alterations and necrosis. Scale bar: 5 μm.

In addition, extracellular collagen fibers were detected in GOs, indicating the presence of extracellular matrix (ECM) components within the model (Fig. 44).

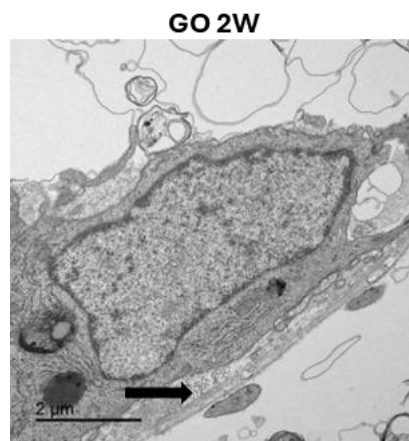


Figure 44: Extracellular collagen fibers are detectable in GOs. Representative TEM images from a GO after two weeks in culture. Black arrow indicates extracellular collagen fibers. Scale bar: 2 μm .

In addition to these observations, we identified multiple instances of cellular projections loaded with mitochondria (Fig. 45A), which may correspond to tumor microtubes. Amongst the total of cellular projections, we quantified around 7% loaded with mitochondria, with no significant differences between tumors and paired GOs (Fig. 45B); in terms of number of mitochondria in these loaded projections, we quantified about 6 mitochondria per μm^2 of projection in 2D TEM section, again with no significant variation between tumors and GOs (Fig. 45B).

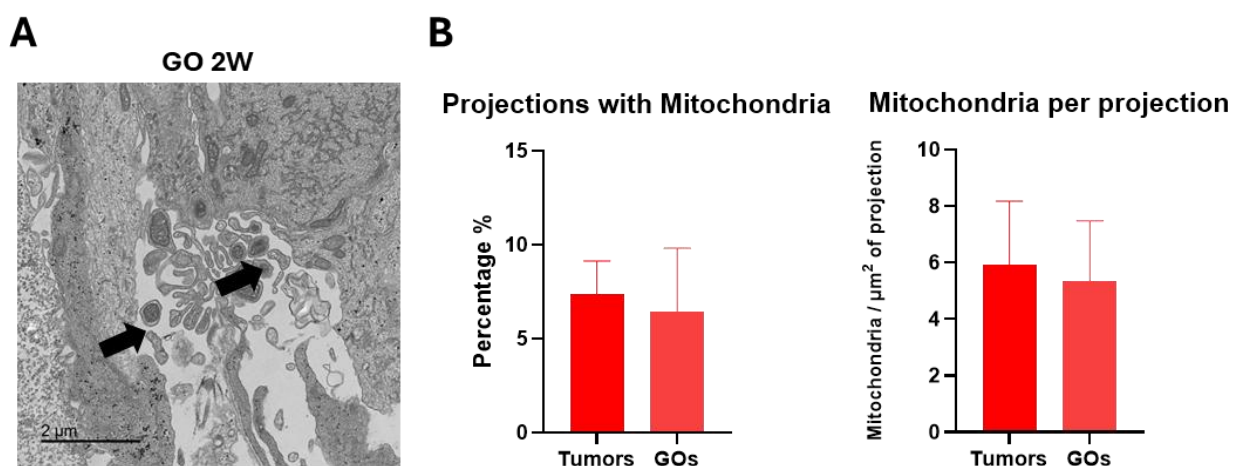


Figure 45: Mitochondria-containing cellular projections in GO. (A) Representative TEM image showing cellular projections containing clustered mitochondria (black arrows). Scale bar: 2 μm . (B) Proportion of analyzed cellular projections containing clustered mitochondria (left) and mitochondria density within mitochondria-containing projections (right), expressed as number of mitochondria per μm^2 of projection area (2D TEM section), in parental tumors and paired GO. Bars represent mean $\pm$ SD. $N = 2$ patients, with 5 random images per patient. $p > 0.05$.

5.2.9 GOs conserve neuronal cells

To evaluate whether GOs conserved a diverse representation of cellular populations like the original tumors, we combined TEM with IF and IHC imaging. Neurons were readily identified via TEM in both tumors and paired two week old GOs thanks to their ultrastructural features, with their nuclei appearing as large, round, with ample euchromatin, electron lucent and with a prominent nucleolus; while their cytoplasm was also not electron dense and loaded with an ample endoplasmic reticulum (ER) network and healthy mitochondria (Fig. 46).

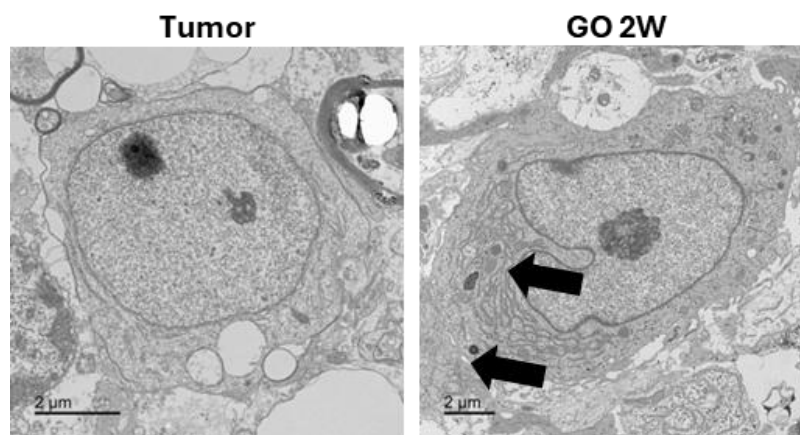


Figure 46: Neurons are maintained in GOs after culture. Representative TEM images from a parental tumor (left) and paired GO (right) after two weeks in culture depicting neurons. Black arrow indicates ER network and mitochondria. Scale bar: 2 μ m.

These observations were corroborated by IF imaging, where we detected the neuronal Tuj1 marker in cells that did not express the classical glioma stem-like marker CD90 in GOs. Moreover, these TUJ1 positive cells also were positive for the mitochondrial marker TRMT61B (Fig. 47).

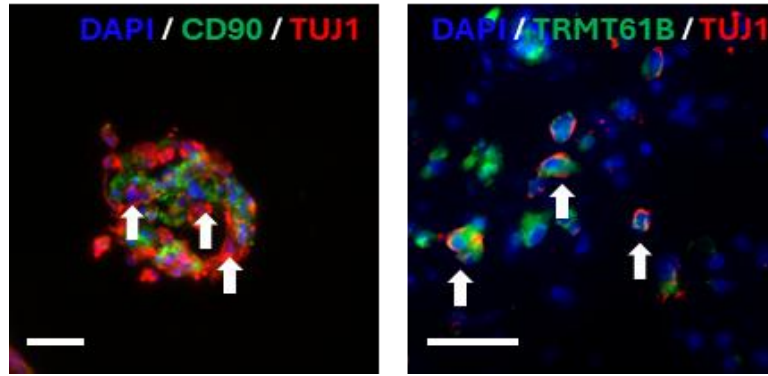


Figure 47: Validation of neurons in GOs. Representative IF images of GOs after two weeks in culture. Left, double IF of neuronal marker TUJ1 (red) and glioma stemness marker CD90 (green), counterstained with DAPI (blue). Scale bar: 200 μ m. White arrows indicate TUJ1 positive and CD90 negative cells. Right double IF of neuronal marker TUJ1 (red) and mitochondrial marker TRMT61B (green), counterstained with DAPI (blue). Scale bar: 50 μ m. White arrows indicate TUJ1 neurons with mitochondria.

5.2.10 GOs retain non-tumoral glial populations

Having identified neurons in our GOs, we investigated the presence of glial cells. These cells were also identified in GOs by TEM, based on their electron-dense cytoplasm and chromatin and their frequent localization adjacent to myelin sheath-like structures (Fig. 48 and Fig. 49).

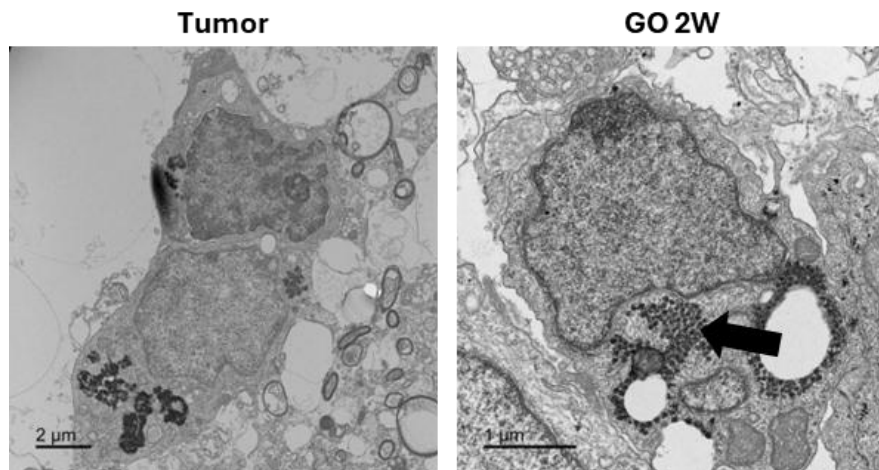


Figure 48: Glial cells in GOs contain abundant glycogen deposits. Representative TEM images from a parental tumor (left) and paired GO (right) after two weeks in culture depicting glial cells. Black arrow indicates electron-dense glycogen deposits. Scale bar: 2 μ m and 1 μ m, respectively.

Within these glial cells, electron-dense glycogen granules and lipid droplets were commonly observed (Fig. 48 and Fig. 49). Approximately 30% of glial cells contained prominent glycogen deposits and approximately 50% contained lipid droplets, with comparable proportions between parental tumors and paired GOs (Fig. 50).

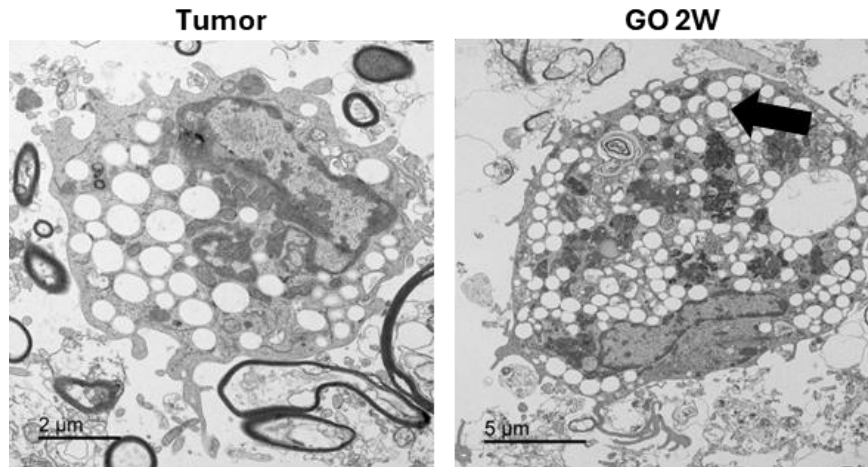


Figure 49: Glial cells in GOs contain ample lipid droplets. Representative TEM images from a parental tumor (left) and paired GO (right) after two weeks in culture depicting glia. Black arrow indicates lipid deposits. Scale bar: 2 μm and 5 μm, respectively.

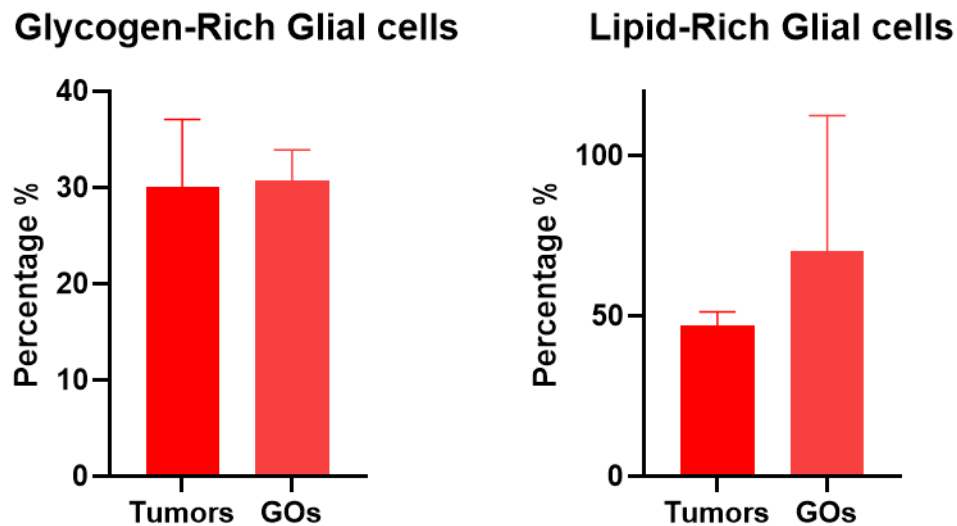


Figure 50: Quantification of glial cells characteristics. Bar graphs depicting percentages of glycogen-rich (left) and lipid-rich (right) glial cells in parental tumors (dark red) and paired GOs after two weeks culture (light red). Bar represents mean value of 5 different fields from 2 different patients together with SD.

RESULTS

These glial cells also exhibited intact nuclear membranes and well-preserved organelles, as well as cellular projections containing abundant ribosomes, discarding phagocytic processes (Fig. 51).

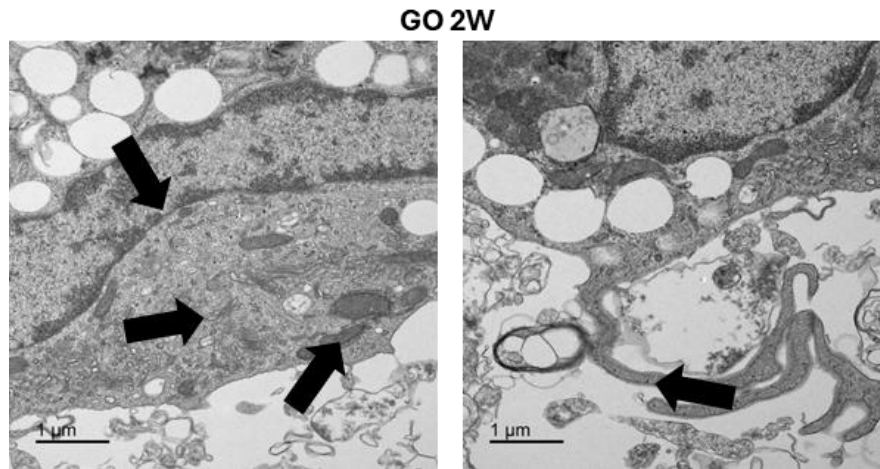


Figure 51: Glial cells in GOs contain well-preserved organelles. TEM images from a glial cell in a representative GO after two weeks in culture. Left depicts intact nuclear membrane and organelles, indicated by black arrows. Right shows a cellular projection loaded with ribosomes, as indicated by a black arrow. Scale bar: 1 µm.

In addition, cell–cell contacts between glial cells and neurons were detected in multiple instances (Fig. 52).

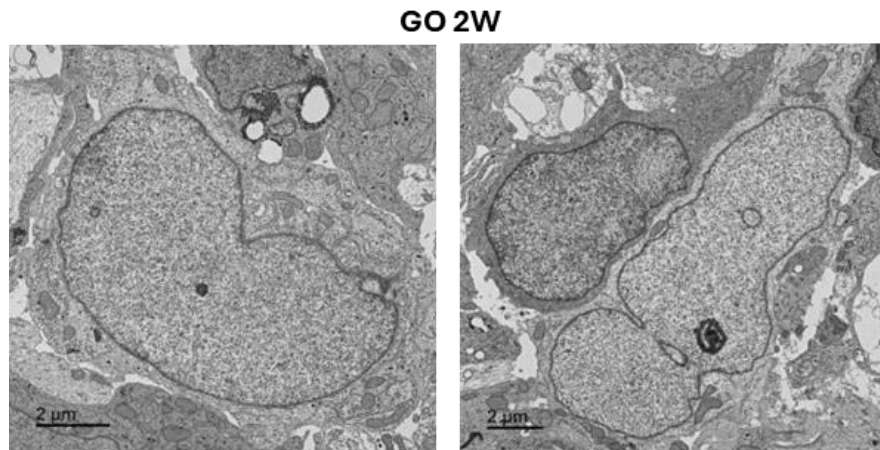


Figure 52: Neuron-glial cell interactions in GOs. Representative TEM images from glial cells interacting with neurons in GOs cultured for two weeks. Scale bar: 2 µm.

To assess whether these glycogen deposits were located in astrocytes or oligodendrocytes periodic acid–Schiff (PAS) staining was combined with immunohistochemistry for GFAP (astrocytes) and OLIG2 (oligodendrocyte lineage).

PAS-positive glycogen deposits were observed in both GFAP-positive and OLIG2-positive cells, with a higher frequency in GFAP-positive cells (Fig. 53).

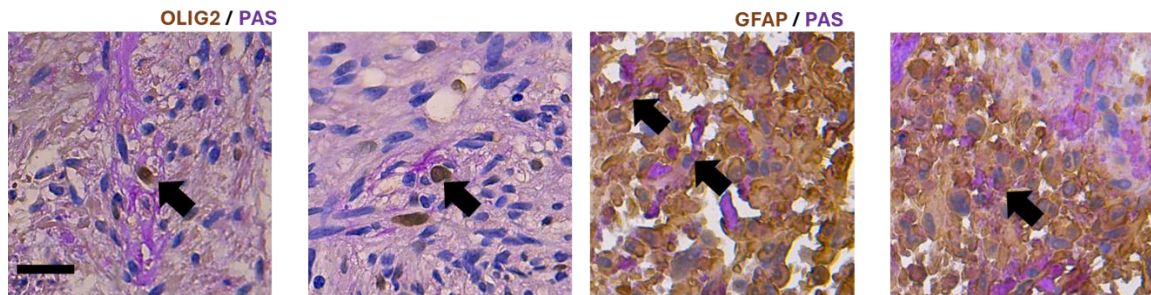


Figure 53: Oligodendrocytes and astrocytes in GOs present glycogen deposits: IHC of OLIG2 (top) and GFAP (bottom) in brown together with PAS stain (magenta) in two-week-old GOs. Black arrows point to oligodendrocytes (top) and astrocytes (bottom) positive for glycogen. Scale bar: 50 μ m.

Finally, double immunofluorescence staining indicated the presence of GFAP-positive and OLIG2-positive populations with no SOX2 signal, consistent with glial-lineage cells distinct from SOX2 tumor cell populations (Fig. 54).

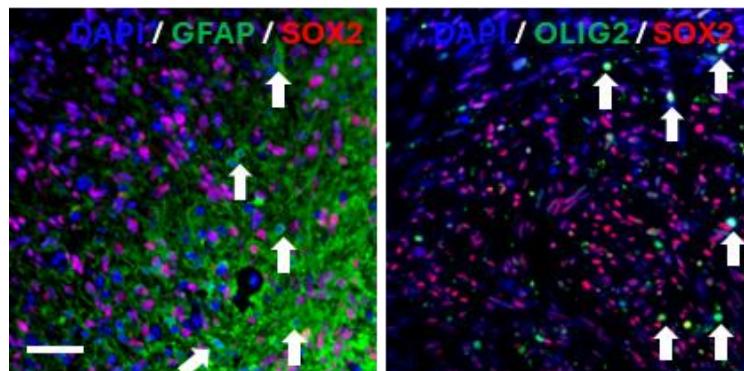


Figure 54: GOs contain non-tumoral astrocytes and oligodendrocytes. Representative IF images of GOs after two weeks in culture. Left, double IF of astrocyte marker GFAP (green) and glioma stemness marker SOX2 (red), counterstained with DAPI (blue). White arrows indicate non-tumoral astrocytes. Right, double IF of oligodendrocyte marker OLIG2 (green) and glioma stemness marker SOX2 (red) counterstained with DAPI (blue). White arrows indicate non-tumoral oligodendrocytes. Scale bar: 200 μ m.

5.2.11 Tumor cells in GOs display abundant, morphologically intact mitochondria

After evaluating the microarchitecture of TME cells, we decided to characterize tumoral cells within GOs using TEM. These cells were identified by their pleomorphic morphology and prominent dilation of endoplasmic reticulum cisternae (Fig. 55).

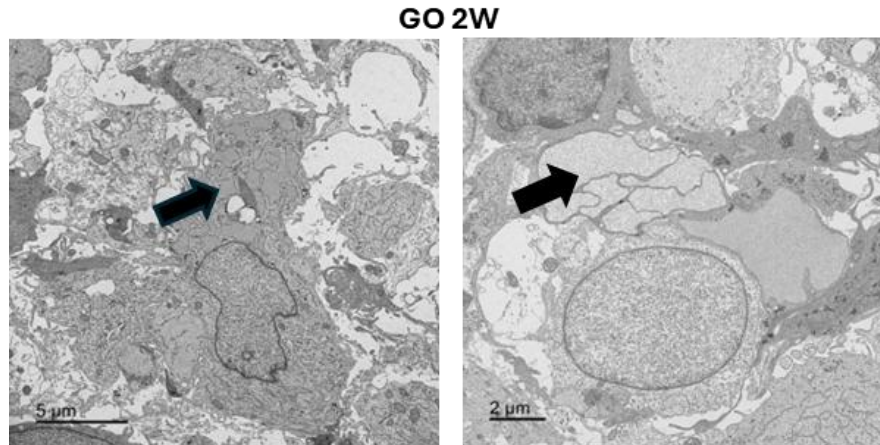


Figure 55: Tumor cells in GOs exhibit dilated endoplasmic reticulum. Representative TEM images of glioma organoids after two weeks in culture showing pleomorphic cells with enlarged rough ER cisternae (black arrows). Scale bar: 2 μm .

These cells frequently presented larger sizes than normal glial cells and contained abundant mitochondria that appeared morphologically intact, without overt ultrastructural abnormalities such as swelling or disrupted cristae (Fig. 56).

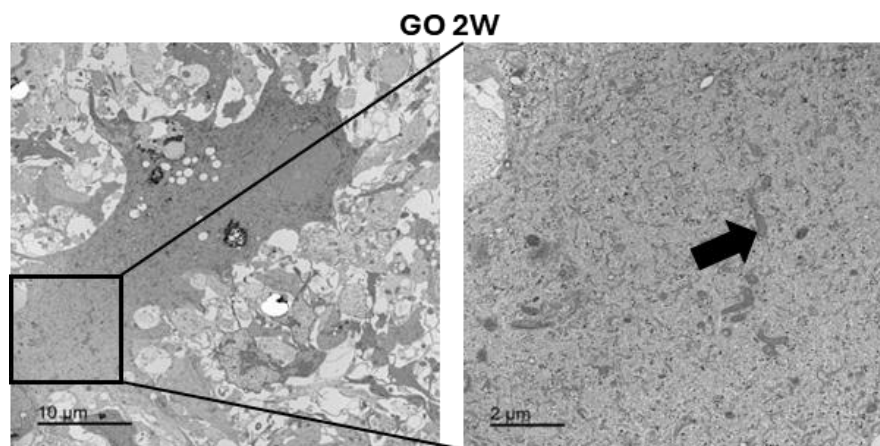


Figure 56: Tumoral cells in GOs contain abundant mitochondria with preserved ultrastructural morphology. Representative TEM images of glioma organoids after two weeks in culture. Left, overview of a large pleomorphic cell. Right, higher-magnification view indicating mitochondria with a black arrow. Scale bars: 10 μm (left) and 2 μm (right).

IF imaging further supported mitochondrial enrichment within the SOX2-positive tumoral population, as indicated by co-detection of SOX2 and the mitochondrial marker MTCO1 in GOs after two weeks in culture (Fig. 57).

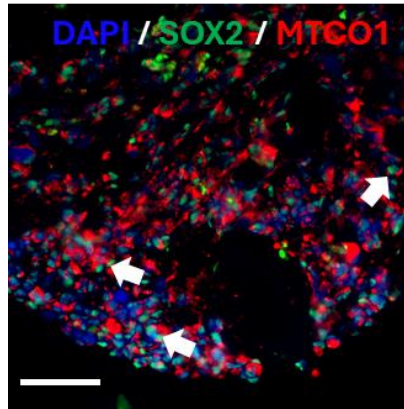


Figure 57: Tumoral cells within GOs contain mitochondria. Representative double IF images of tumoral stem marker SOX2 (green) and mitochondrial marker MTCO1 (red), counterstained with DAPI (blue) of GOs after two weeks in culture. White arrows indicate tumoral cells positive for mitochondria. Scale bar: 200 μm .

5.3 Molecular characterization of GOs

5.3.1 GOs and parental tumors share most gene expression complement

To further evaluate molecular concordance between GOs and their parental tumors, we performed RNA-seq on 11 matched GOWt and tumor pairs. Data analysis revealed that almost all genes (over 96%) were detectable in both samples, highlighting the molecular fidelity of GOs. However, there was a small fraction of genes that were only expressed by either parental tumors or GOs, representing 287 (1.6%) and 396 (2.2%), respectively, of the total detected gene complement (Fig. 58).

Gene ontology analysis of these unique gene subsets revealed that they mostly consisted of genes involved in signaling processes, extracellular and membrane components as well as both innate and adaptive immune responses (Fig. 59).

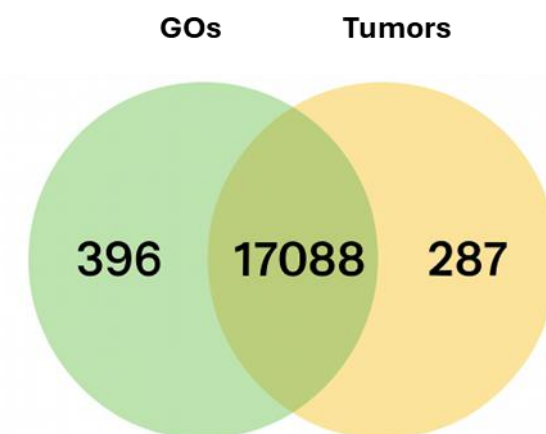


Figure 58: Tumors and GOs express unique subsets of genes. Venn diagram depicting genes uniquely expressed in GOs (salmon) and parental tumors (green).

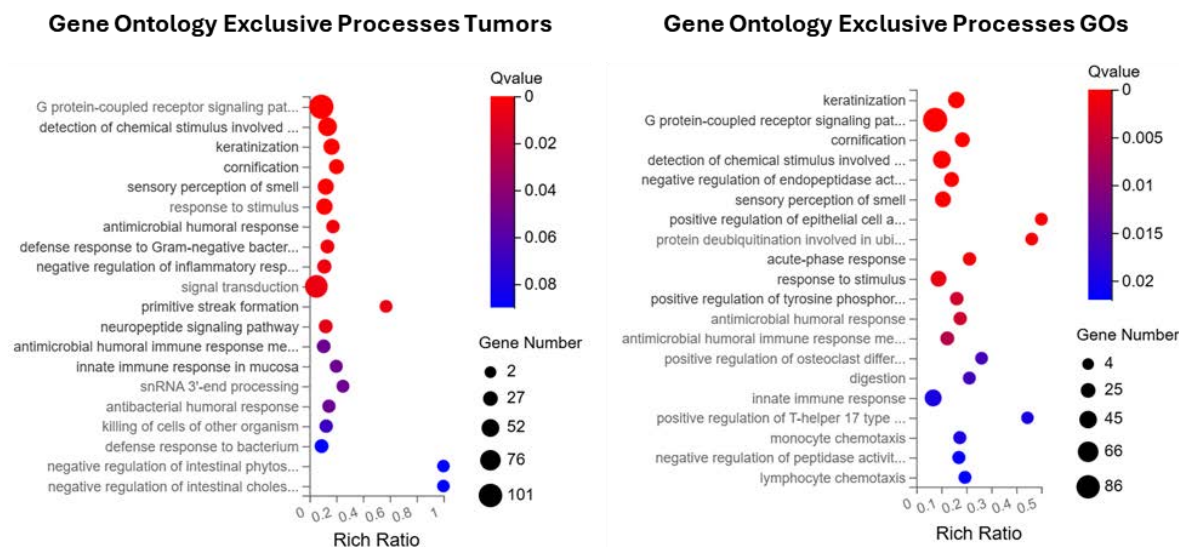


Figure 59: Classification of tumor and GO exclusive genes. Gene Ontology enrichment bubble plots showing enriched biological process of genes exclusively expressed in tumors (left) or GOs (right). Each bubble represents an enriched GO term (y-axis). The x-axis shows the rich ratio. Bubble size indicates the number of input genes mapping to each term. Bubble color denotes the adjusted significance (q-value), with dark red indicating more significant enrichment.

Moreover, Pearson correlation of gene expression profiles between each tumor and its paired GO showed high concordance, with most pairs exhibiting r values higher than 0.9, with the lowest correlation observed being 0.8 for pair GO 54 (Fig. 60), further solidifying the idea of GOs being accurate avatars of GBM.

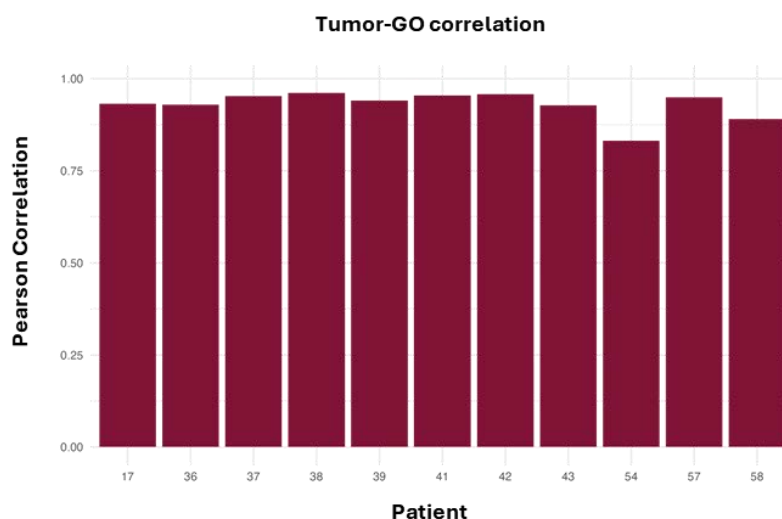


Figure 60: GOs gene expression is highly correlated with their original tumor. Bar graph of Pearson correlation coefficient (R) values of matched tumor-GO pairs. Each bar denotes a different patient. $N = 11$ paired samples.

5.3.2 RNA-seq demonstrates high transcriptomic similarity between tumors and derived GOs

Following RNA-seq analysis, we next examined expression patterns of selected gene sets representing stromal-associated markers (Fig. 61) and cell activity-associated markers (Fig. 62). Across both panels, GOs broadly preserved the relative expression patterns observed in their matched parental tumors.

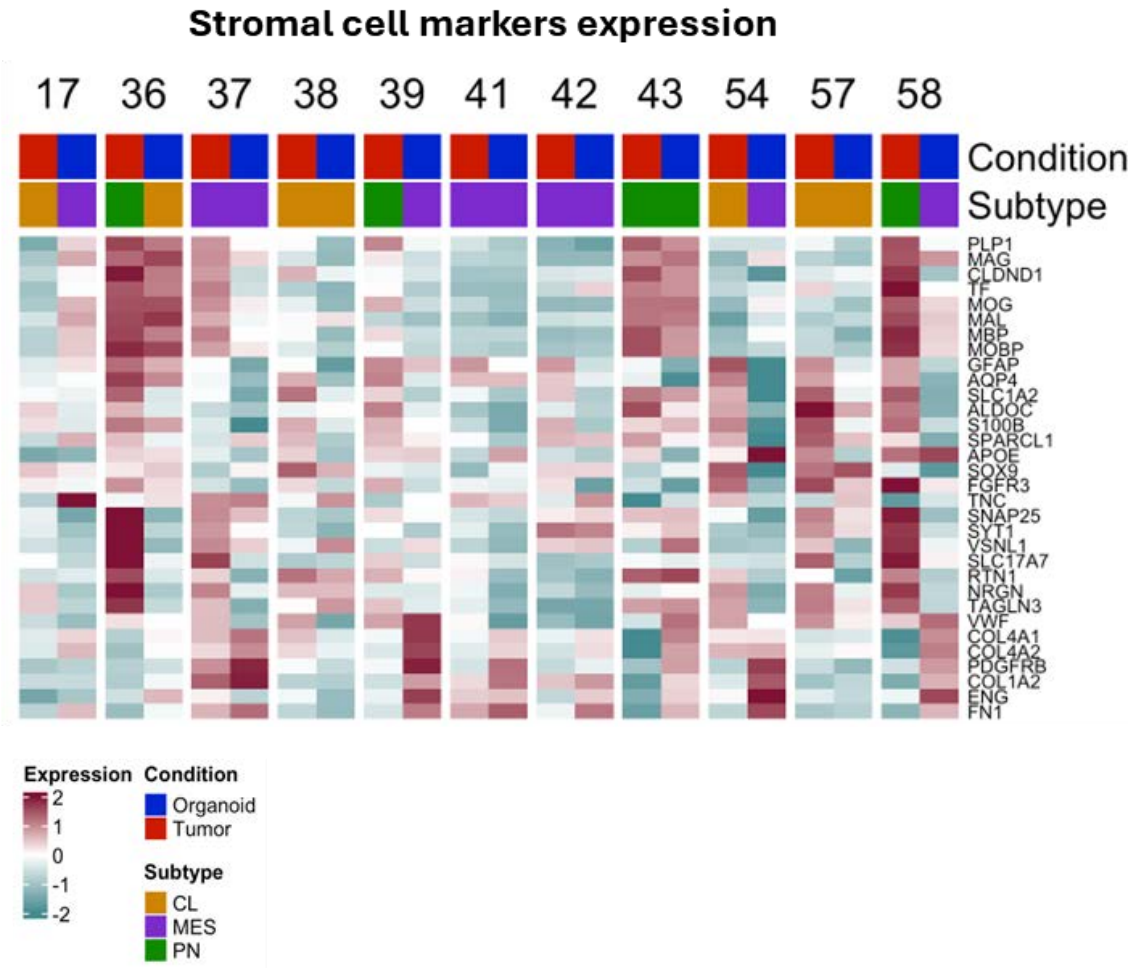


Figure 61: GOs replicate unique stromal cell expression patterns of parental tumors. Heatmap displaying z-score normalized expression of stromal cell markers in matched parental tissues (red) and GOs (blue). Samples are color coded according to their molecular subtype: classical (yellow), mesenchymal (purple) and proneural (green). TPM values were scaled by gene-wise z-score normalization (range -2 to 2) to standardize the expression levels across samples. N=11 paired samples.

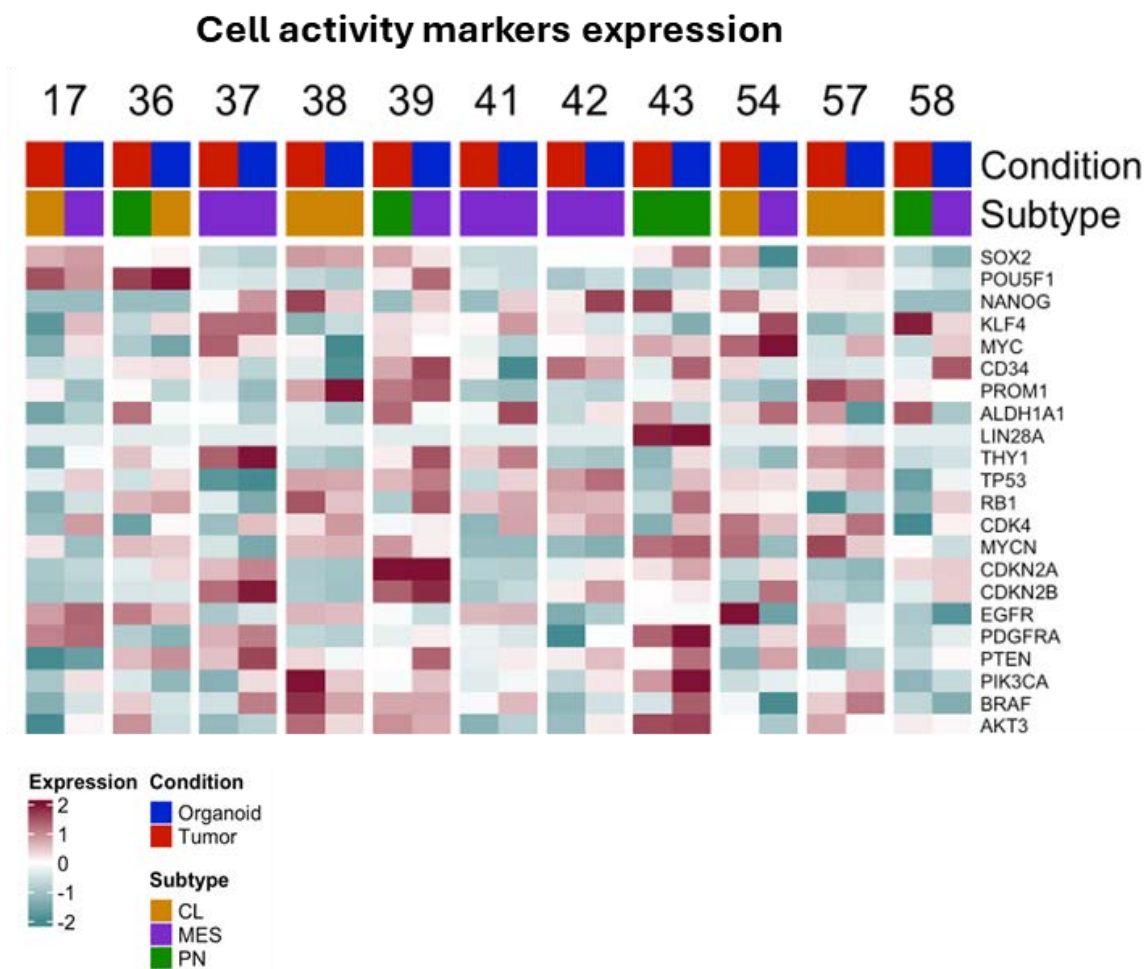


Figure 62: GOs replicate unique cell activity expression patterns of parental tumors.

Heatmap displaying z-score normalized expression of cell activity markers in matched parental tissues (red) and GOs (blue). Samples are color coded according to their molecular subtype: classical (yellow), mesenchymal (purple) and proneural (green). TPM values were scaled by gene-wise z-score normalization (range -2 to 2) to standardize the expression levels across samples. N=11 paired samples.

Consistent with this, sample-wise correlation analyses based on each gene set showed that tumors and their matched GOs tended to group together (Fig. 63).

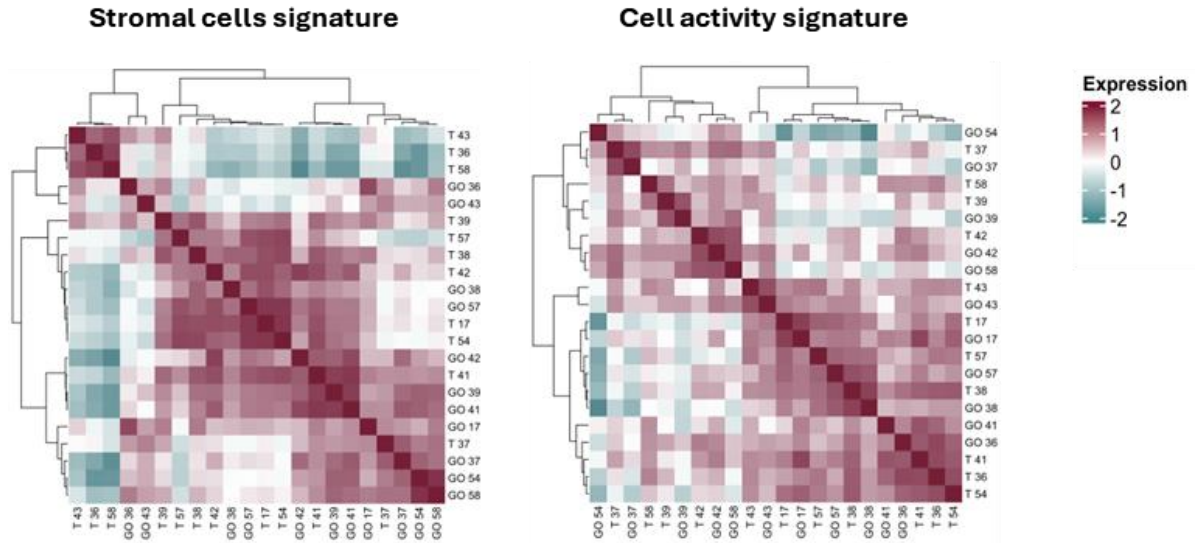


Figure 63: GOs and parental tumors expression values cluster together. Sample-wise correlations of stromal cell markers (left) and cell activity markers (right) in matched parental tissues and GOs. TPM values were scaled by gene-wise z-score normalization (range -2 to 2) to standardize the expression levels across samples. N=11 paired samples.

To evaluate the robustness of these clusters, we performed hierarchical clustering with Approximately Unbiased (AU) p-values computed using pvclust (Fig. 64). We observed a very close pairing between respective tumor-GO pair in the cellular activity signature, whole tumors and GOs seemed to form two separate clusters in the stromal cell signature.

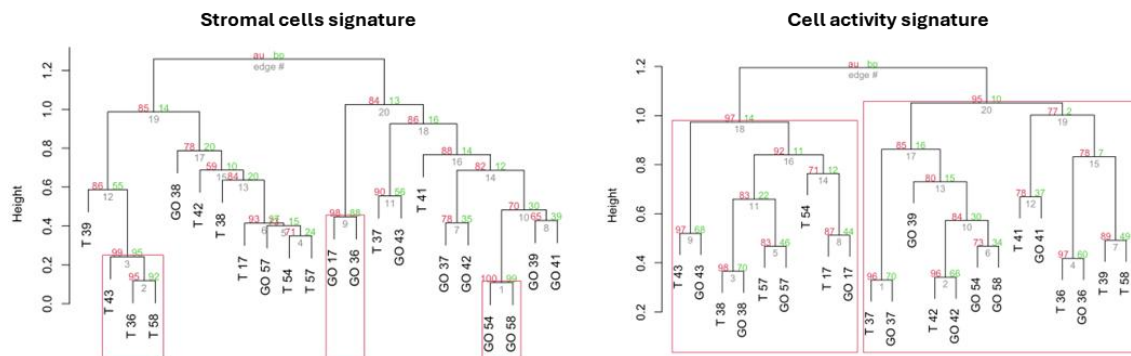


Figure 64: GO-tumor pairs cluster together within sample dendrograms. Hierarchical clustering dendrograms with Approximately Unbiased (AU) p-values computed by pvclust of stromal cell markers (top) and cell activity markers (bottom) in matched parental tissues and GOs. Major clusters are highlighted in red boxes. N=11 paired samples.

5.3.3 RNA-seq identifies differentially expressed gene programs in GOs

Differential expression analysis of matched tumor–organoid RNA-seq pairs ($n = 11$) identified subsets of genes upregulated and downregulated in GOs relative to parental tumors (Fig. 65).

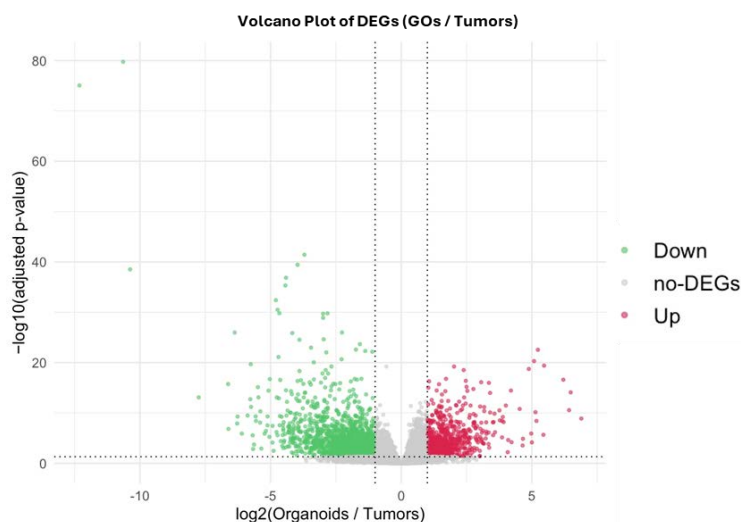


Figure 65: GOs present DEG patterns in respect to parental tumors. Volcano plot highlighting upregulated (red) and downregulated (green) genes in GOs compared to their parental tumors. Gene expression is represented as $\log_2$ fold change, with statistical significance shown as $-\log_{10}$ q -value.

GSEA on the ranked transcriptome (GO vs tumor) revealed significant enrichment of cancer hallmark pathways related to protein secretion, glycolysis, and hypoxia in GOs (Fig. 66), whereas no gene sets were significantly enriched among downregulated genes at the selected FDR threshold.

RESULTS

Upregulated signatures in GOs

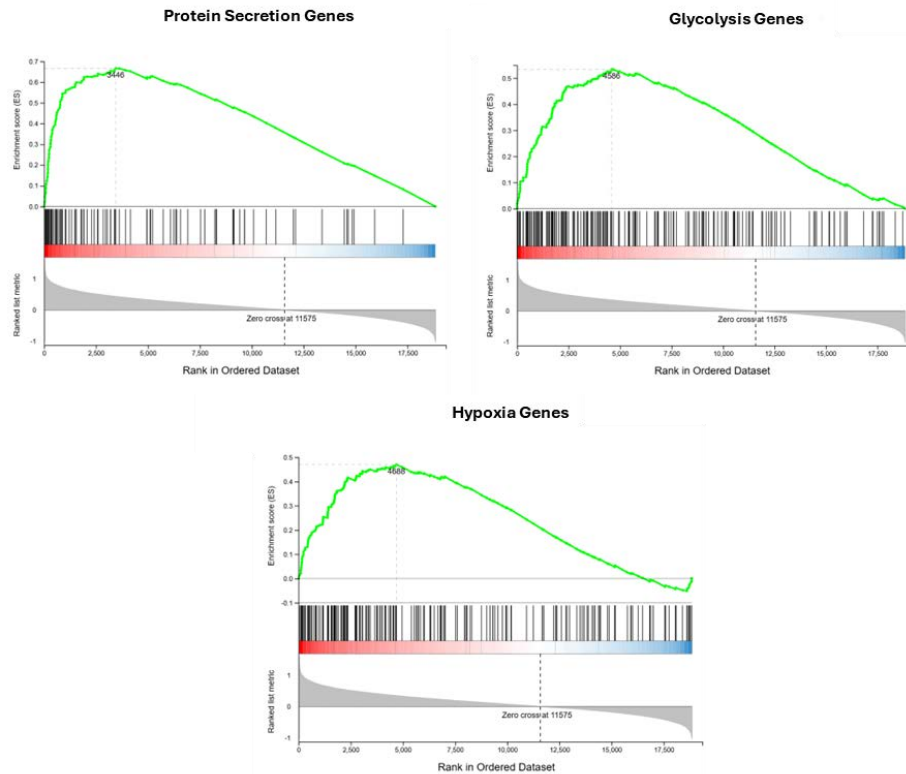


Figure 66: GOs present differentially upregulated gene signatures to tumoral samples. GSEA plots of differentially upregulated gene pathways in GOs. Top left, protein secretion genes; top right, 91 glycolysis genes and bottom, hypoxia genes. Genes were ranked by signal-to-noise represented by red to blue color gradient. The green line shows the running enrichment score (ES) across the ranked gene list; the maximum deviation from zero corresponds to the peak ES (dashed line). Black vertical ticks indicate the positions of gene set hits within the ranked list. The lower gray panel shows the distribution of ranking metric scores across the ordered dataset.

Subsequently, KEGG pathway classification of DEGs in GOs indicated enrichment across cellular communication/signaling, protein transport, environmental adaptation, and multiple metabolic pathways (Fig. 67).

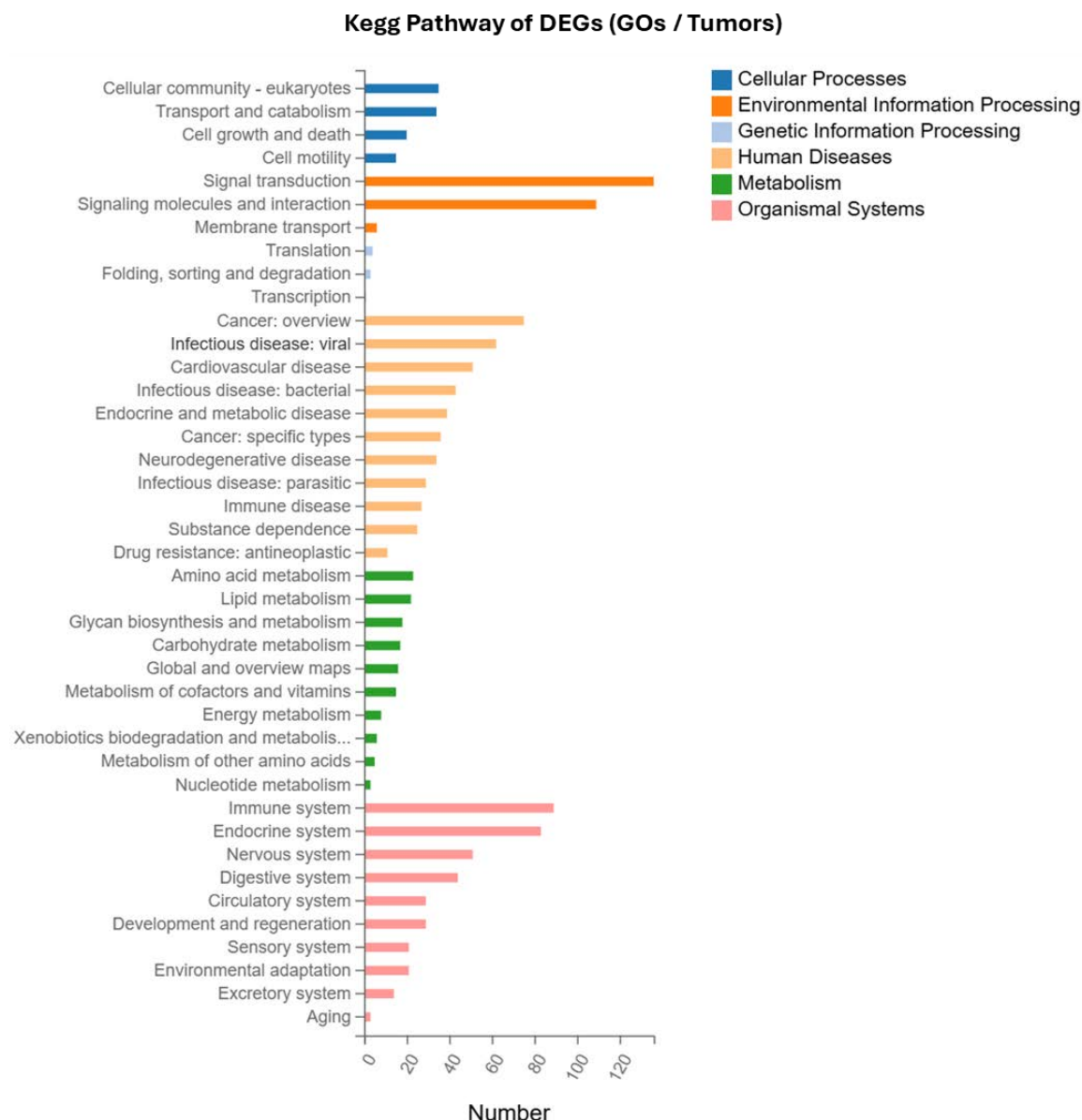


Figure 67: GOs present DEG patterns in respect to parental tumors. Bar graph of KEGG pathway classifications of upregulated genes in GOs, with broad categories color-coded as follows: Cellular processes in dark blue, Environmental information processing in dark orange, Genetic information processing in light blue, Human diseases in light orange, Metabolism in green and Organismal systems in pink. X-axis represents the number of genes in each pathway.

5.3.4 GOs maintain parental tumor molecular subtype

We next evaluated the conservation of the GBM molecular subtype in our GOs as defined by Verhaak et al. (97) (Fig. 68). Overall, GOs retained subtype-associated expression patterns similar to their parental tumors, although subtype classification was not uniformly conserved across pairs.

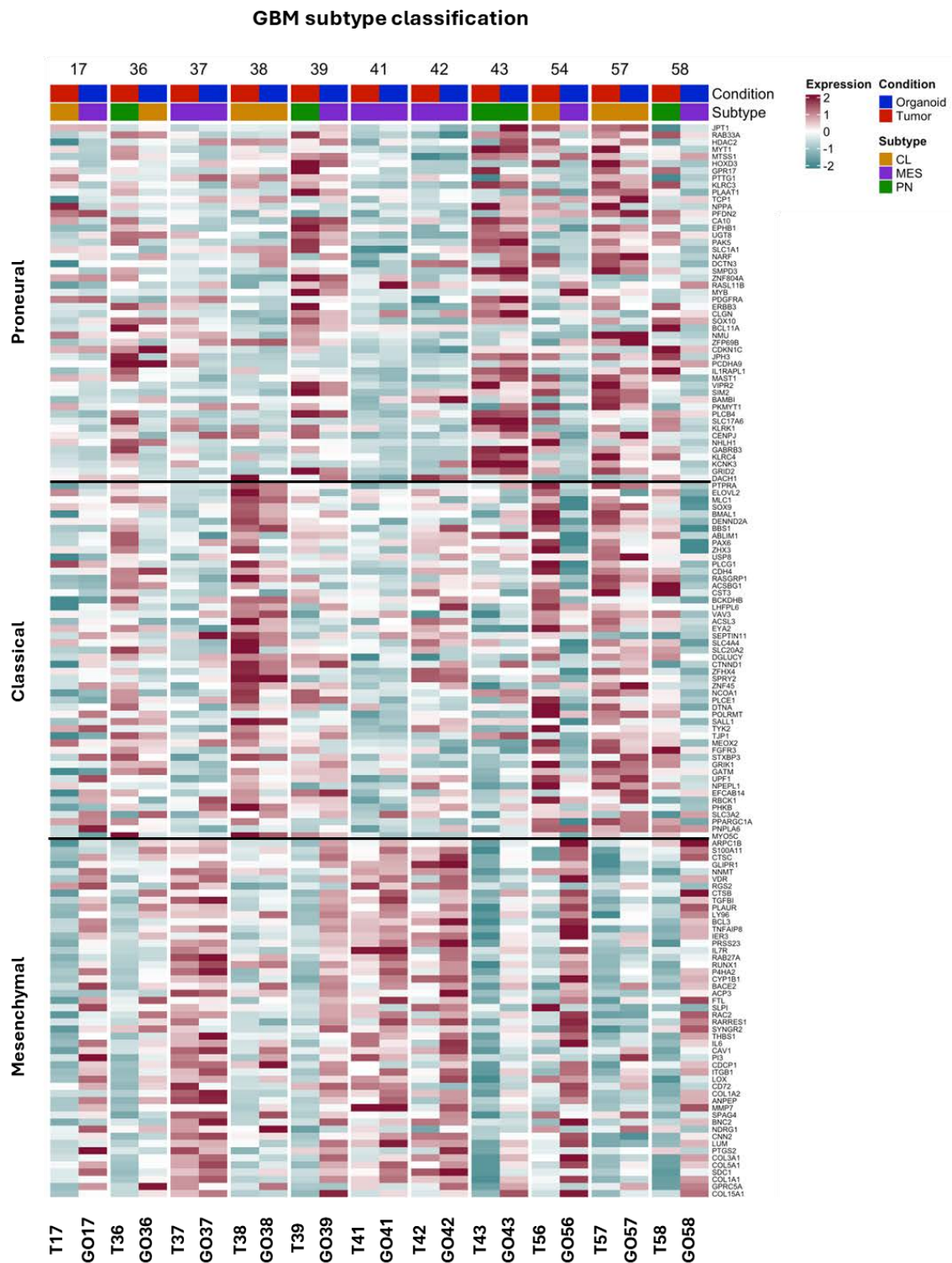


Figure 68: GOs maintain GBM molecular subtypes. Heatmap displaying z-score normalized expression of GBM molecular subtype genes in matched parental tissues (red) and GOs (blue). Samples are color coded according to their molecular subtype classification: classical (yellow), mesenchymal (purple) and proneural (green). TPM values were scaled by gene-wise z-score normalization (range -2 to 2) to standardize the expression levels across samples. N=11 paired samples.

Across the cohort, 6 out of 11 GOs matched the subtype assignment of their corresponding tumors, whereas 5 out of 11 showed a different subtype classification (Fig. 68). Specifically, GO17 and GO54 shifted from classical in the tumor to mesenchymal in the matched organoid; GO36 shifted from proneural to classical; and GO39 and GO58 shifted from proneural to mesenchymal.

Hierarchical clustering based on the subtype gene set grouped many matched tumor–GO pairs together, particularly among pairs with conserved subtype assignments, and major clusters were supported by high Approximately Unbiased (AU) values computed using pvclust (Fig. 69).

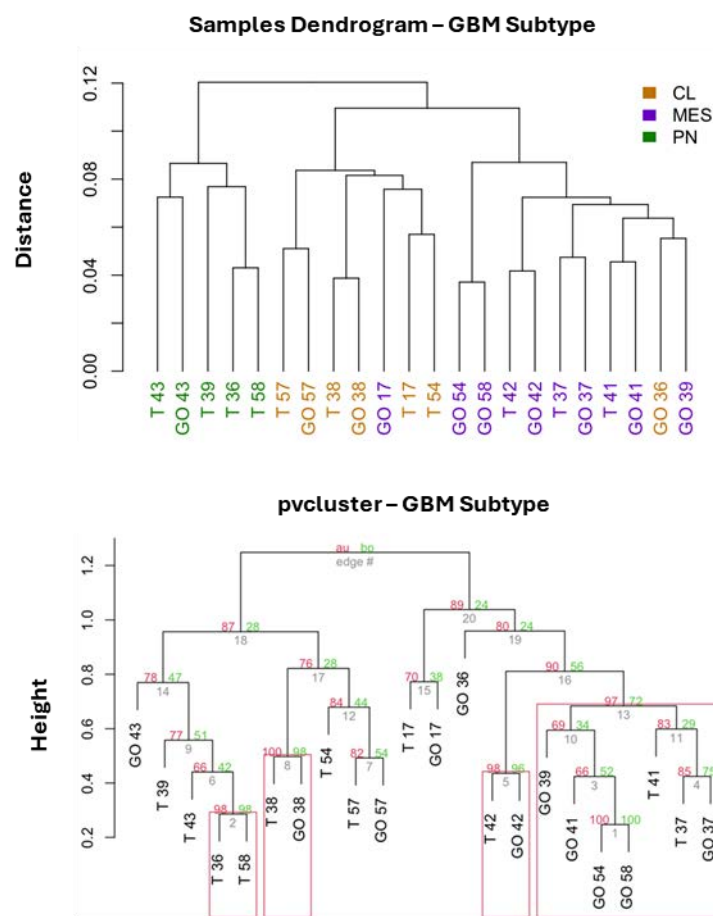


Figure 69: Hierarchical clustering of GOs based on molecular subtype. Left, dendrogram of tumors and GOs, color coded according to molecular subtype classical (yellow), mesenchymal (purple) and proneural (green). Right, approximately unbiased (AU) p-values computed by pvclust of GBM molecular subtype gene signature. Major clusters are highlighted in red boxes. N=11 paired samples.

5.4 Evaluation of immune system landscape in GOs

5.4.1 Myeloid immune populations are abundant in GOs and parental tumors

To explore the immune TME components retained in GOs, we began by examining the myeloid compartment. First, we utilized our RNA-seq data with a curated marker panel for myeloid/microglia-associated genes, polarization-associated markers, and myeloid-associated immune checkpoint genes (Fig. 70).

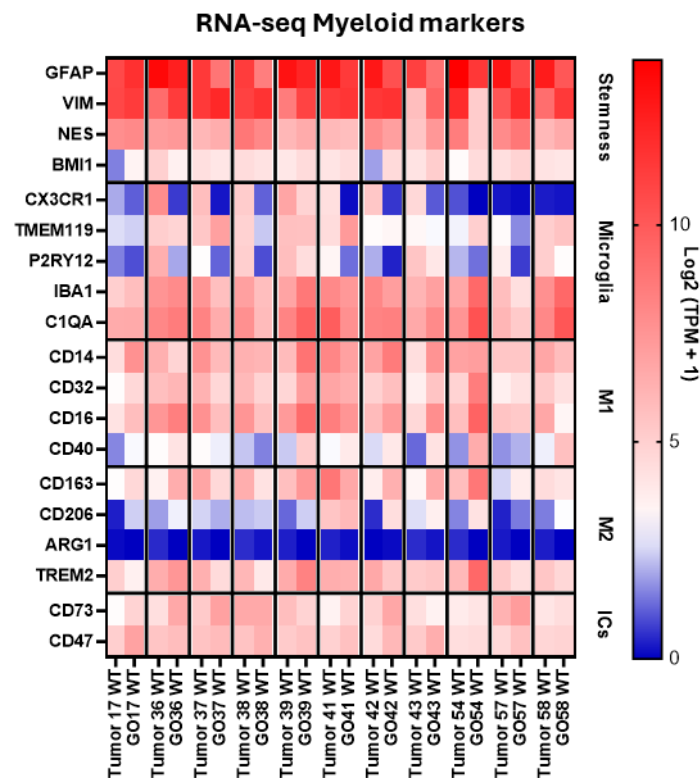


Figure 70: RNA-seq of myeloid-associated genes. Heatmap of RNA-seq expression values for myeloid marker panel in parental tumors (left) and matched GOs (right) ($n = 11$ pairs). Color indicates $\log_2(\text{TPM} + 1)$ (blue = low, red = high). Genes are grouped by functional category (tumor/stemness, microglia/myeloid, polarization-associated markers, and immune checkpoint genes).

Expression of myeloid markers like AIF1 (IBA1) and C1QA was readily apparent, hinting at their conservation within GOs. These observations were supported by IF for CD68 and IBA1 (Fig. 71), which confirmed the presence of myeloid cells in GOs.

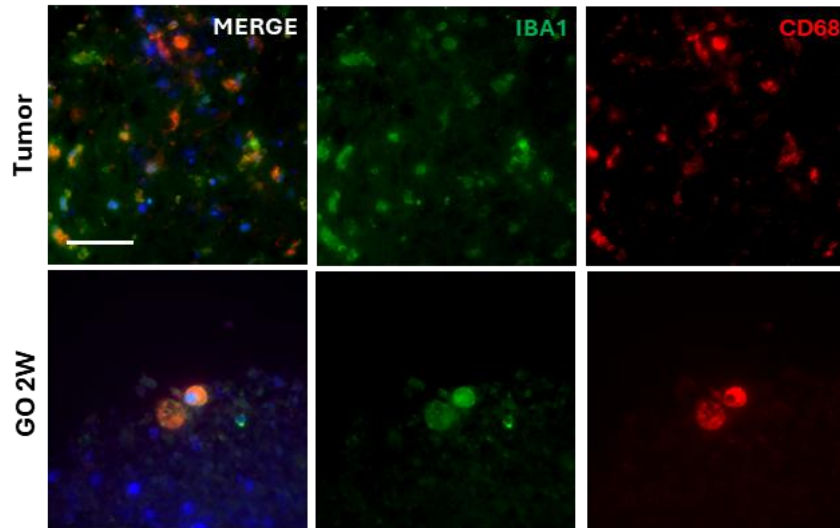


Figure 71: GOs contain myeloid-lineage populations. Representative double IF images of IBA1 (green) and CD68 (red) in GBM tumor (top) and paired GO after 2 weeks in culture (bottom). Counterstained with DAPI. Scale bar: 100 μ m.

In regard to polarization, both M1 and M2 markers were expressed at similar levels, apart from the M2 marker ARG1, which was almost undetectable. These observations were also confirmed by IF imaging in GOs (Fig. 72).

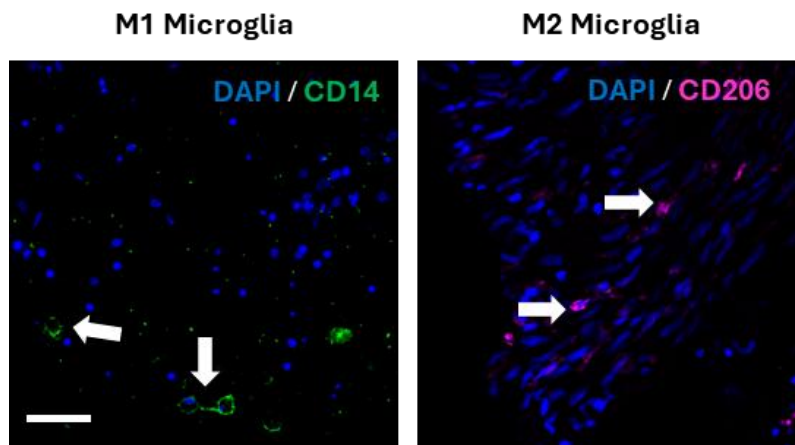


Figure 72: GOs contain both M1 and M2 microglial cells. Representative IF for M1 marker CD14 (left, green) and M2 marker CD206 (right, magenta) in GOs after 2 weeks in culture. Nuclei are counterstained with DAPI. Scale bar: 50 μ m.

Finally, myeloid-associated immune checkpoint genes, NT5E/CD73 and CD47 were very prominent. Globally, relative expression patterns across the full marker panel were remarkably preserved between parental tumors and their matched GOs (Fig. 70).

5.4.2 Lymphoid cells are scarcely represented in GOs and parental tumors

We continued analyzing the immune TME by focusing on the lymphoid population. RNA-seq data revealed a very apparent lack of lymphoid marker expression in stark contrast to the previous myeloid results, with both T-cell and associated immune checkpoints at very low levels (Fig. 73).

IF staining for CD4 and CD8 also did not reveal detectable levels of CD4 or CD8 positive cells in either GOs or their paired tumoral samples, further arguing against their presence in our cohort (Fig. 74). We additionally verified both CD4 and CD8 primary antibodies in murine spleen samples as positive controls to confirm their reactivity (Fig. Annex IA).

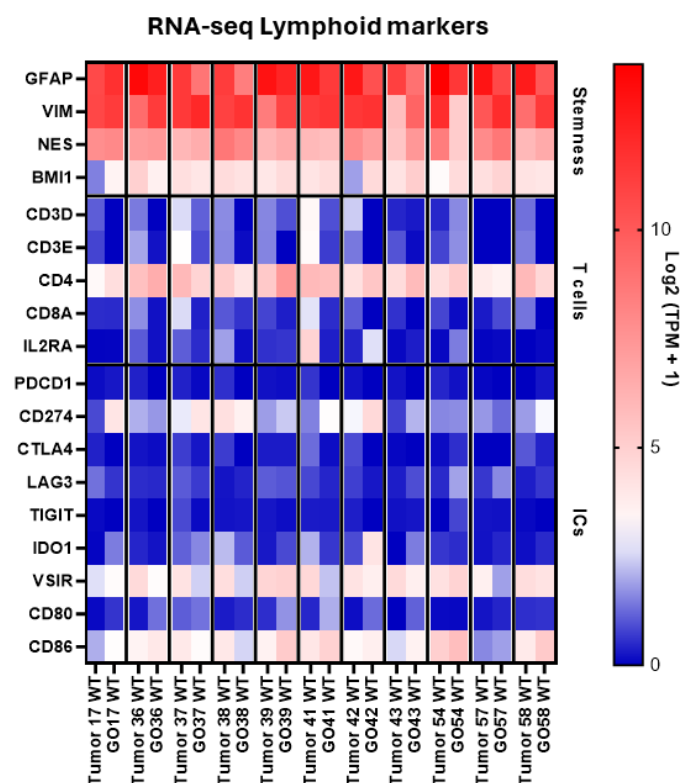


Figure 73: RNA-seq of lymphoid-associated genes. Heatmap of RNA-seq expression values for lymphoid marker panel in parental tumors (left) and matched GOs (right) ($n = 11$ pairs). Color indicates $\log_2(\text{TPM} + 1)$ (blue = low, red = high). Genes are grouped by functional category (tumor/stemness, T-cells and immune checkpoint genes).

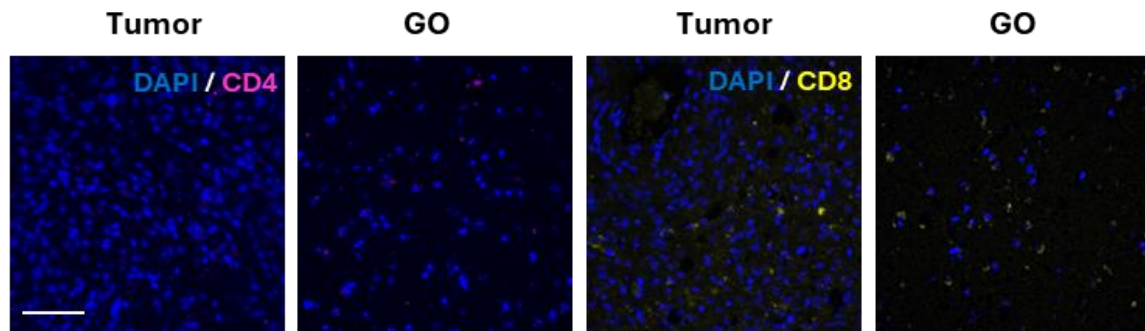


Figure 74: CD4- and CD8-positive cells are not detected by IF in tumors and matched GOs. Representative IF images for CD4 (magenta) and CD8 (yellow) in parental tumors (left) and derived GOs (right) after 2 weeks in culture. Counterstained with DAPI in blue. Scale bar: 100 μ m.

To more accurately quantify lymphoid populations, flow cytometry was performed on three fresh GBM tumors and their matched GOs after two weeks in culture. Across samples, CD45-positive immune cells represented approximately 5% of live cells in both tumors and GOs. Within the CD45-positive compartment, ~15–20% of cells were CD3-positive T cells, whereas CD19-positive B cells were rare or undetectable. Among CD3-positive cells, CD4-positive cells represented <5%, and CD8-positive cells were near or below the detection threshold (Fig. 75). Together, these data support a low abundance of lymphoid infiltrates in the analyzed GBM samples and their derived GOs.

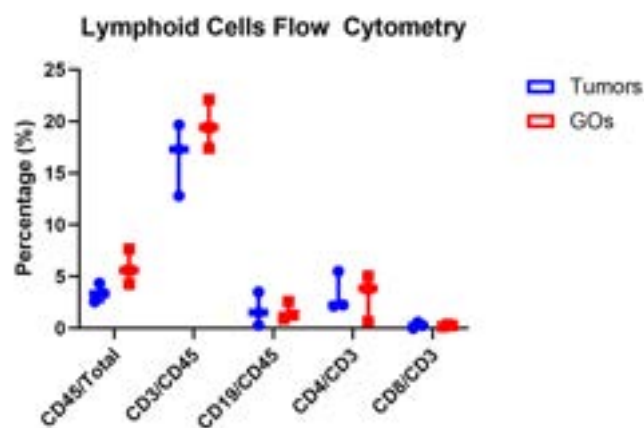


Figure 75: Flow cytometry confirms low levels of lymphoid infiltrates in tumors and matched GOs. Scatter plot showing the proportions of indicated immune populations in tumors (blue) and matched GOs (red). Each point represents one patient ($n = 3$). Horizontal bars indicate mean values, with error bars showing SD.

RESULTS

Focusing on T-cell-associated immune checkpoints, flow cytometry analysis of the well-established PD-1, PD-L1, CTLA-4, CD80, and CD86 checkpoints showed low frequencies of checkpoint-positive cells across the analyzed samples, with no marker exceeding 0.8% of total live cells. PD-L1 and CTLA-4 signals were near or below the detection threshold in most samples, whereas CD80 showed the highest detectable frequency among the assessed markers (Fig. 76). Overall, tumors and matched GOs exhibited comparable checkpoint marker frequencies.

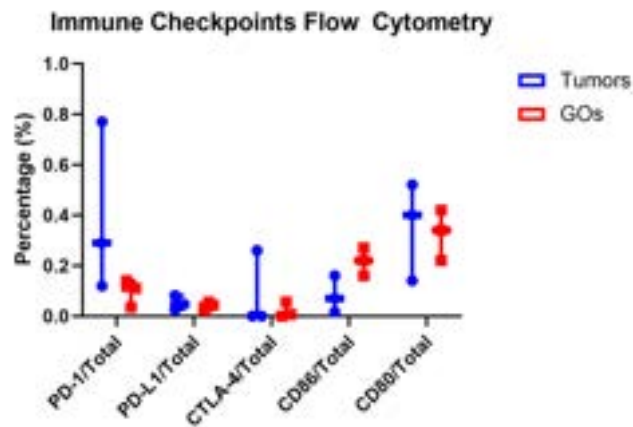


Figure 76: Flow cytometry indicates low frequencies of immune checkpoint–positive cells in tumors and matched GOs. Scatter plot showing the proportion of cells positive for PD-1, PD-L1, CTLA-4, CD80, or CD86 in tumors (blue) and matched organoids (red). Each point represents one patient ($n = 3$). Horizontal bars indicate the mean and error bars indicate SD.

To further assess the PD-1/PD-L1 axis, we examined PD-L1 by IF and did not detect PD-L1 immunoreactivity in tumor or GO sections (Fig. 77A). Antibody performance was confirmed using a PD-L1-positive human control sample (Fig. Annex IB). These results were further confirmed by qPCR, where we observed low levels of expression of *PD-L1* in both GOs and their parental tumors (Fig. 77B).

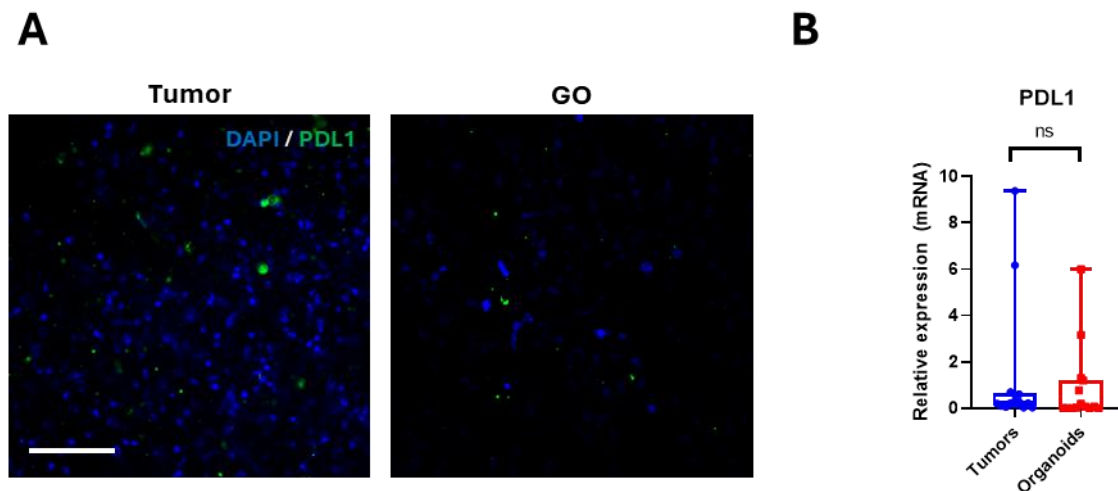


Figure 77: PD-L1 is not detectable in parental tumors and derived GOs. (A) Representative IF images of PD-L1 in green counterstained with DAPI in blue in parental tumors (left) and corresponding GOs after 2 weeks in culture (right). Scale bar: 100 μ m. (B) Bar graph of relative mRNA expression of PD-L1 in parental tumors (blue) and derived GOs (red). N = 6 independent patients.

5.4.3 Public scRNA-seq datasets support low T-cell abundance in GBM

To confirm that our myeloid-dominant profile was consistent with other studies, we analyzed two publicly available sc-RNAseq cohorts, the CGGA (233) and Bhaduri cohorts (234) (Fig. 78).

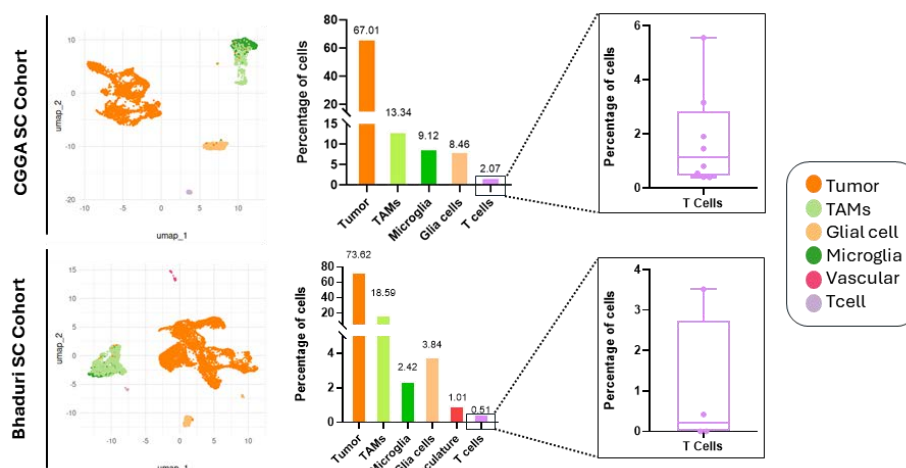


Figure 78: Independent scRNA-seq GBM cohorts contain abundant myeloid cells but sparse lymphoid infiltration. scRNA-seq analysis of immune cell populations in glioma datasets from the CGGA (top) and Bhaduri (bottom) cohorts. UMAP plot of cell-type distribution (left), bar graph of immune cell proportions (middle), and box plot of T-cell percentages per patient (right).

RESULTS

Our analysis indicates that T-cells are extremely sparse in these cohorts, with 2% and 0.5% of the total cells, respectively. On the contrary, myeloid populations including microglia and TAMs are much more abundant at around 20% of the tumoral composition (Fig. 78).

5.5 Guided drug-response profiling of GOs using clinically relevant glioma therapies

5.5.1 Transcriptomic-guided screening predicts low TMZ sensitivity scores and higher gefitinib sensitivity scores across tumor–GO pairs

To evaluate whether GOs could support preclinical therapy screening, we first performed a transcriptomics-based *in silico* drug-response prediction using the DiSCoVER platform (241) on our paired RNA-seq cohort. Using a guided approach, we assessed predicted sensitivity scores for a curated panel of six compounds, including the GBM SOC agent TMZ, as well as irinotecan (248,249), vincristine (250,251), etoposide (252,253), doxorubicin (254,255), and the EGFR inhibitor gefitinib (256,257), which have been explored in GBM treatment contexts as monotherapies or in combination.

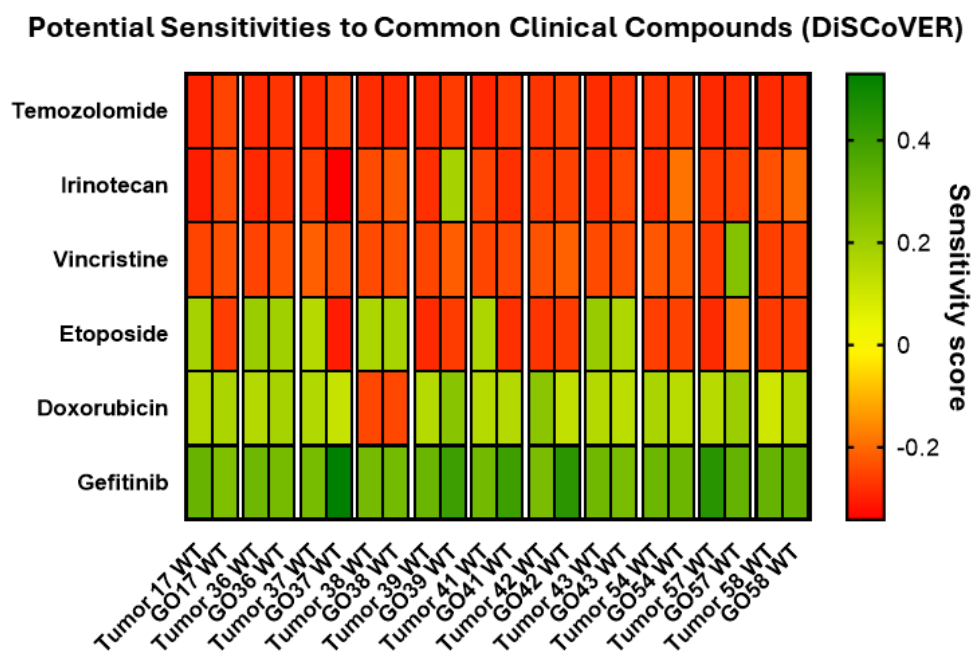


Figure 79: *In silico* screening of common chemotherapeutic compounds predicts GOs resistance to SOC compounds but sensitivity to gefitinib. Heatmap of predicted drug sensitivities to SOC therapeutic agents. Drugs are listed on the left, and each pair of rows represents a matched parental tumor and derived GO. Color corresponds to sensitivity score, with dark green representing the potential best responders, and dark red the most resistant samples.

Across our matched tumor and GO pairs, TMZ, irinotecan, vincristine, and doxorubicin showed uniformly low or negative predicted sensitivity scores. Etoposide also showed low predicted sensitivity, with only modest positive scores in a subset of samples. In contrast, gefitinib consistently yielded the highest predicted sensitivity scores across the

RESULTS

cohort with all sensitivity scores above 0.2, reaching 0.4 in some samples, nominating this inhibitor as a candidate compound for subsequent experimental validation (Fig. 79). Moreover, parental tumor sensitivity scores were closely matched by their corresponding GOs.

5.5.2 *In vitro* assays validate predicted drug response patterns in GOs

To test whether transcriptomics-based drug-response predictions translated into measurable effects *in vitro*, we treated freshly established GOwts with a panel of compounds and assessed responses using an MTS metabolic assay and Annexin V/PI flow cytometry after 48 hours. Drug concentrations were selected based on reported IC₅₀ ranges for glioma cell lines from the Genomics of Drug Sensitivity in Cancer database (179).

In the MTS assay, only gefitinib produced a significant reduction in metabolic activity relative to vehicle control (***p* < 0.001), with over 50% decrease in signal. Doxorubicin and irinotecan showed non-significant trends toward reduced metabolic activity, whereas TMZ, vincristine, and etoposide did not produce discernible effects (Fig. 80).

GOwts manually selected drug screening

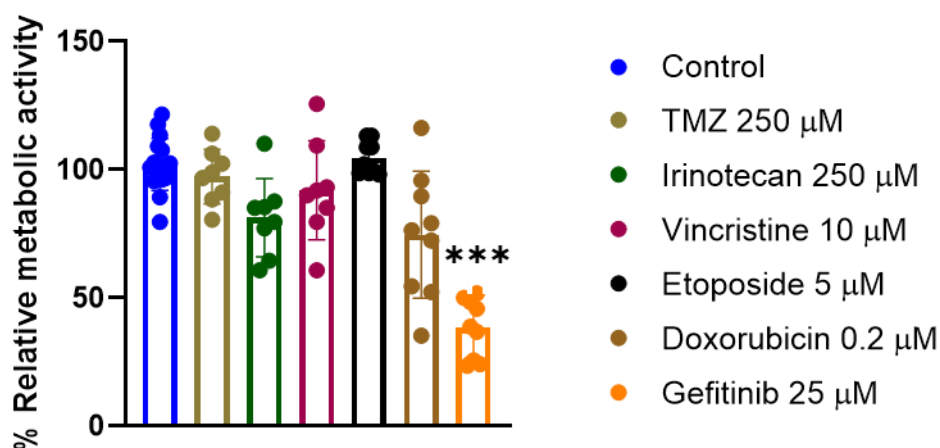


Figure 80: MTS assay detects reduction in metabolic activity of GOs treated with gefitinib. Bar graph of MTS results on GOwts after 48 hours of treatment with the indicated compounds, represented by color coding, and concentrations. Bar height indicates average relative metabolic activity as normalized to the control. Each dot represents the average values of an independent GO (3 technical replicates), with error bars for SD. Statistical comparisons were performed using ANOVA, *** *p* < 0.001. GOs from *N* = 8 independent patients.

Consistent with these findings, Annexin V/PI analysis showed that gefitinib-treated GOs yielded significantly lower recovered cell counts (cells per mL) compared with controls ($***p < 0.001$), as well as an FC increase of 4.67 in the number of late apoptosis/dead population. The rest of compounds did not yield any notable changes in apoptotic stages, except for a slight reduction in necrotic fractions in the case of irinotecan and doxorubicin (Fig. 81).

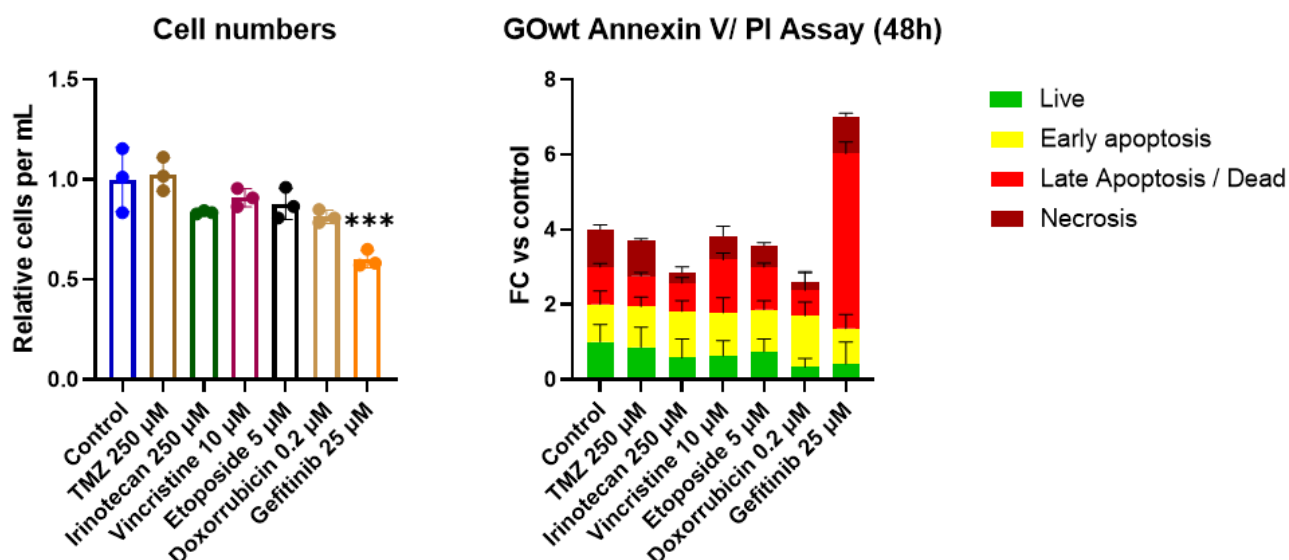


Figure 81: Gefitinib reduces recovered cell counts and increases late apoptotic/dead fraction in GOs. Left, bar graphs of relative number of cells per mL after 48 hour treatment of the indicated compounds as determined by flow cytometry. Bar height indicates mean cell counts, with individual GOs shown. Right, segmented bar graph of fold change of number of cells in each apoptotic stage, each segment is color coded in the following manner: live cells, green; early apoptotic cells, yellow; late apoptotic/dead cells, red and necrotic cells as brown. Segment height indicates average fold change value. Error bars indicate SD. Statistical comparisons were performed using ANOVA, $***p < 0.001$. GOs from $N = 3$ independent patients.

5.5.3 Drug treatment of GOs induces DNA damage without broad changes in GFAP/vimentin patterns

We next investigated whether these compounds produced alterations in GOs that were not captured by our metabolic and apoptosis assays (Fig. 82).

First, qualitative assessment via H&E staining highlighted a great degree of similarity in cellularity, morphology or histopathological characteristics relative to the vehicle control. This was also true for the Vimentin and GFAP markers, where all treatments displayed comparable levels of integrated intensity normalized to nuclei per field. On the other hand, DAPI positive nuclei counts were significantly reduced in both TMZ and gefitinib treated samples (** $p < 0.001$), while TMZ, gefitinib, etoposide and doxorubicin treated samples presented increased levels of DNA damage (** $p < 0.001$), with TMZ and etoposide reaching almost 100% of nuclei (Fig. 83). Taken together, these results confirm our previous observations, showing reduced nuclei and increased DNA damage in gefitinib-treated GOs. However, they also show increases in DNA damage levels for samples treated with TMZ, etoposide or doxorubicin that, in turn, do not translate into decreases in metabolic activity or increases in apoptosis after 48 hour treatment (Fig. 83).

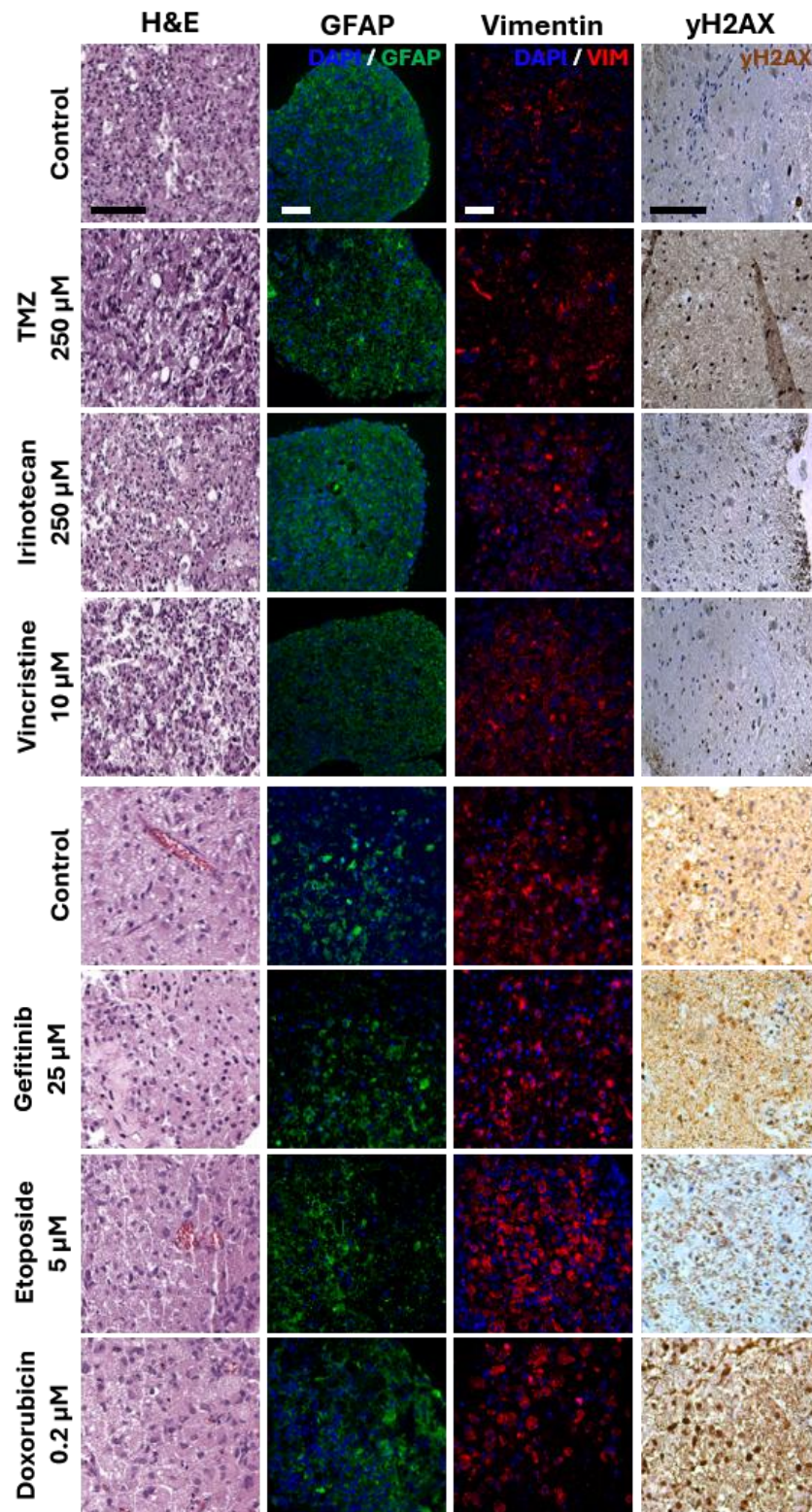


Figure 82: Histology and marker evaluation in treated GOs. From left to right, representative images of: H&E, GFAP IF (green), Vimentin IF (red) and γH2AX IHC (brown) images in two-week-old GOwts after 48 h treatment. IF images are counterstained with DAPI in blue. Scale bar: 100 μm.

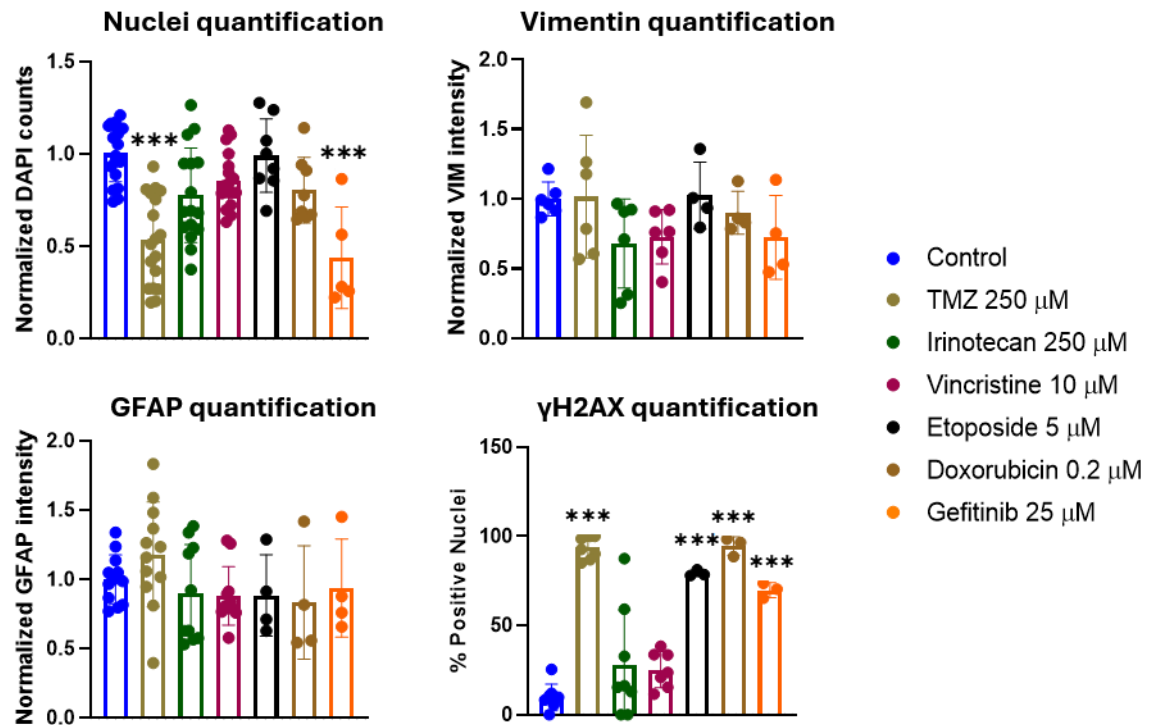


Figure 83: Quantification of markers after 48-hour treatment in GOs. Bar plots showing DAPI-positive nuclei per field, GFAP and vimentin signal quantified as mean fluorescence intensity per nucleus; and the fraction of γH2AX-positive nuclei. Bar height represents average mean values, with each point the average value per patient. Error bars indicate SD. Statistical comparisons were performed using ANOVA, *** $p < 0.001$. GOs from $N = 4-8$ independent patients, depending on condition.

5.5.4 Clinical follow-up of the RNA-seq cohort shows frequent recurrence after SOC therapy

To contextualize GO predictions with clinical outcomes, we reviewed follow-up data for patients included in the GBM RNA-seq cohort. Despite receiving SOC treatment (maximal safe resection followed by RT with concomitant and adjuvant TMZ), 8/11 (around 70%) of patients developed radiographic progression/recurrence within 12 months of surgical intervention (defined here as treatment start point) (Fig. 84). These observations are consistent with the well-recognized limited durability of TMZ therapy in GBM and provide clinical context for the low TMZ sensitivity scores observed in our GO analyses.

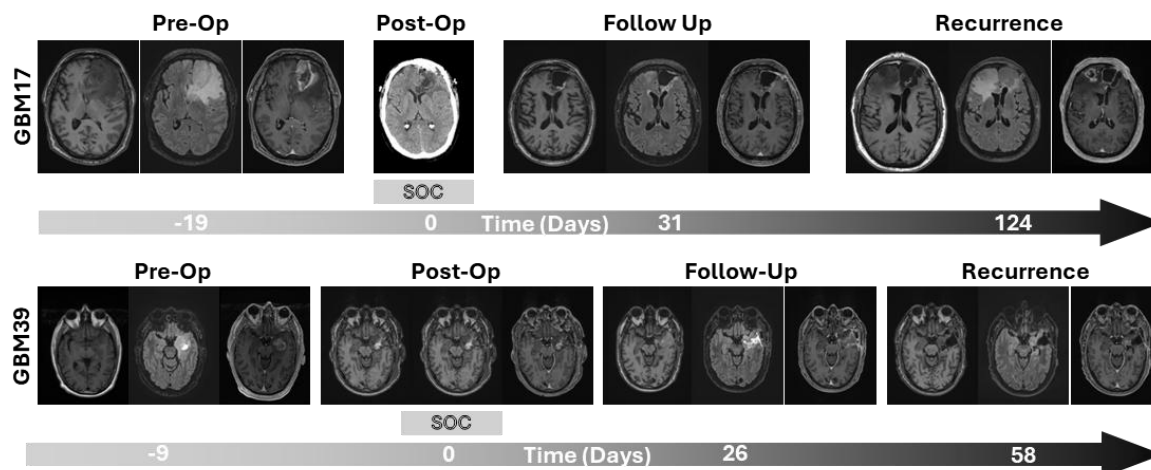


Figure 84: Representative longitudinal MRI scans illustrating GBM recurrence after SOC therapy. Representative MRI images depicting the clinical progression of patients GBM17 (top) and GBM39 (bottom) following SOC therapy. Sequential scans illustrate tumor evolution across key clinical stages: pre-operative baseline, post-operative status, follow-up period, and recurrence. Timepoints are annotated.

5.6 Unbiased screening and validation of non-glioma compounds in GOs

5.6.1 Unguided *in silico* screening identifies dabrafenib, alectinib, and ruxolitinib as promising candidate drugs in GOs

To identify additional candidate compounds beyond clinically used glioma therapies, we repeated the DiSCoVER based *in silico* drug screening in an unbiased mode, allowing the platform to rank compounds across its full library by predicted sensitivity. We prioritized compounds with the highest predicted sensitivity scores and broad consistency across the 11 matched tumor–GO pairs.

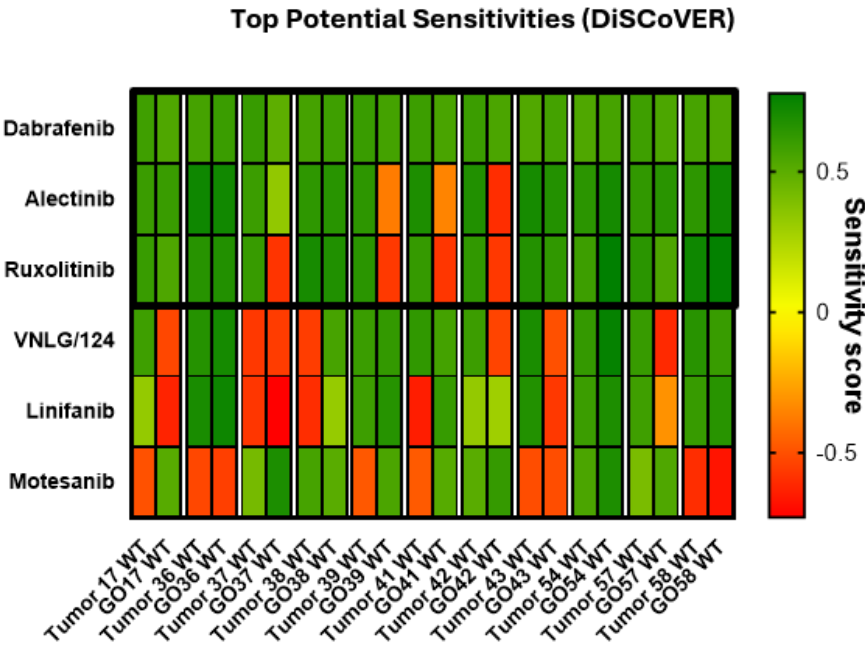


Figure 85: Unbiased *in silico* screening identifies dabrafenib, ruxolitinib and alectinib as potential GBM therapies. Heatmap of DiSCoVER-predicted sensitivity scores for the top-ranked compounds from the full DiSCoVER library across matched parental tumors and derived GOs. Drugs are listed on the left, and each pair of rows represents a matched parental tumor and derived GO. Color corresponds to sensitivity score, with dark green representing the potential best responders, and dark red the most resistant samples. The three selected candidates (dabrafenib, alectinib, ruxolitinib) are highlighted.

Using this approach, we focused on the top 3 compounds with higher scores for all 11 paired samples. Dabrafenib, a BRAF inhibitor was the top scoring compound in our cohort with a sensitivity score above 0.4 for all GO-tumor pairs. Alectinib, an ALK inhibitor, was next in the list, with scores of around 0.5 on all tumoral samples and GOs,

except for 3 GOs. Ruxolitinib, the JAK1/2 inhibitor was the last selected compound, with similar performance to alectinib. Three other compounds, VNLG/124, linifanib and motesanib also predicted strong effects in a subset of samples but were inconsistent across the tumoral cohort and discarded from subsequent experiments (Fig. 85).

5.6.2 ALK is expressed in GBM tumors and retained in matched GOs

To justify target relevance and the selection of compounds, we decided to evaluate the expression and protein status of ALK, the target of alectinib in our samples.

First, we compared our RNA-seq data with the average expression value of ALK in healthy brain samples from the GTEx portal (242) cohort. We observed a log₂ FC increase of 2 in both tumors and GOs (with no differences between them) compared to the healthy brain values (Fig. 86), hinting at elevated ALK expression in GBM, which is faithfully maintained in GOs.

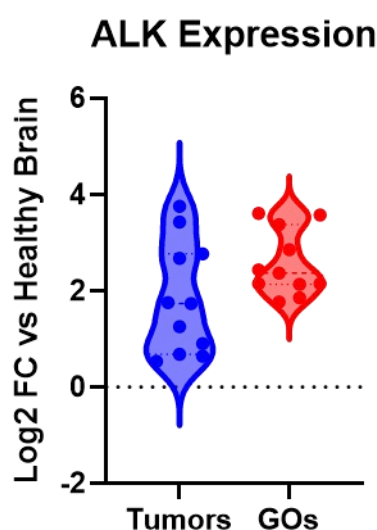


Figure 86: ALK expression is elevated in tumors and GOs compared to healthy tissue. Violin plots of log₂ FC of TPM+1 expression values of each paired tumor and derived GO as compared to the average ALK expression in healthy brain GTEx cohort. Blue depicts tumors, with GOs in red. N = 11 samples.

Moreover, ALK protein level presence was also confirmed in our GOs (Fig. 87), which together confirm target relevance and further support the *in silico* screening selection.

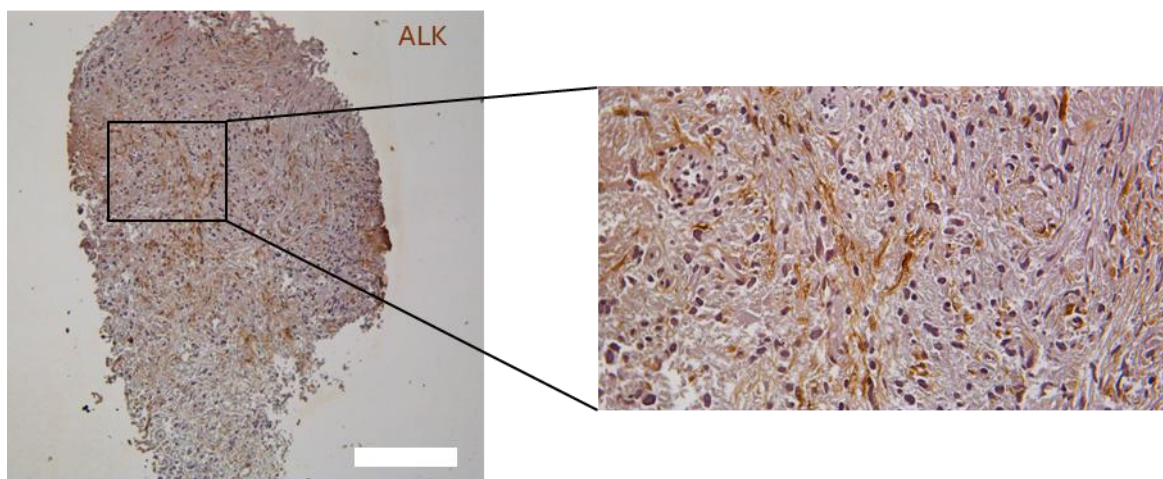


Figure 87: ALK is detected by IHC in GOs. Representative IHC staining for ALK (brown) in a two-week-old organoid, with a higher-magnification view of the central region (right). Scale bar: 200 μ m.

5.6.3 Dabrafenib, alectinib, and ruxolitinib reduce GO viability and increase apoptosis

To evaluate the *in vitro* effects of the top three ranking compounds, we established a new cohort of GOs that were subjected to treatment for 48 hours. Compound concentrations were selected again based on average IC₅₀ values from glioma cells available from the GDSC database (179).

Firstly, we evaluated the effects of these treatments on the histopathological characteristics of GOs. Qualitative assessment of H&E staining revealed that all three treatments produced marked reductions in GO cellularity, with a clear increase in necrotic areas (Fig. 88).

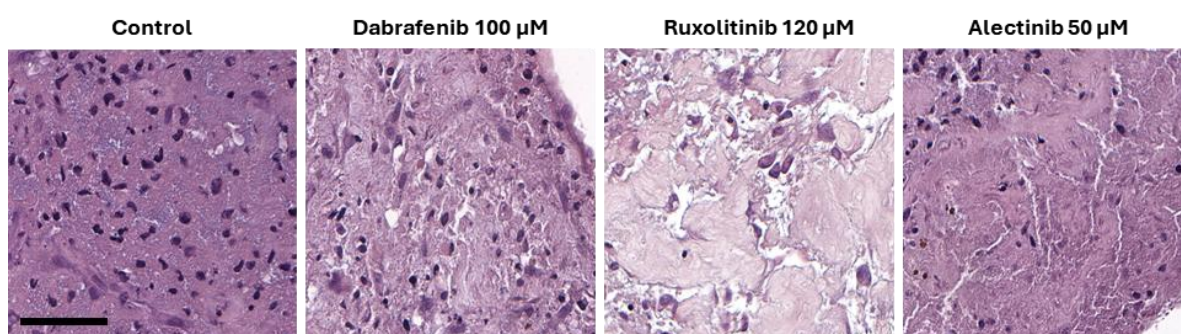


Figure 88: H&E reveals reduced cellularity and increased necrosis in treated GOs. Representative H&E images of GOs treated with dabrafenib, ruxolitinib and alectinib. Scale bar: 100 μ m.

We then evaluated metabolic activity via MTS assay. Here we observed that all three compounds significantly reduced metabolic activity. Alectinib and ruxolitinib showed the strongest effects, with 75 and 70% inhibition, respectively and *** $p < 0.001$ in comparison to the vehicle control. Dabrafenib showed a less potent but still significant metabolic activity inhibition (** $p < 0.01$), with GOs from one patient exhibiting a very low inhibition of only 30% as compared to the mean dabrafenib group of 50% (Fig. 89).

GOs metabolic inhibition after unbiased screening

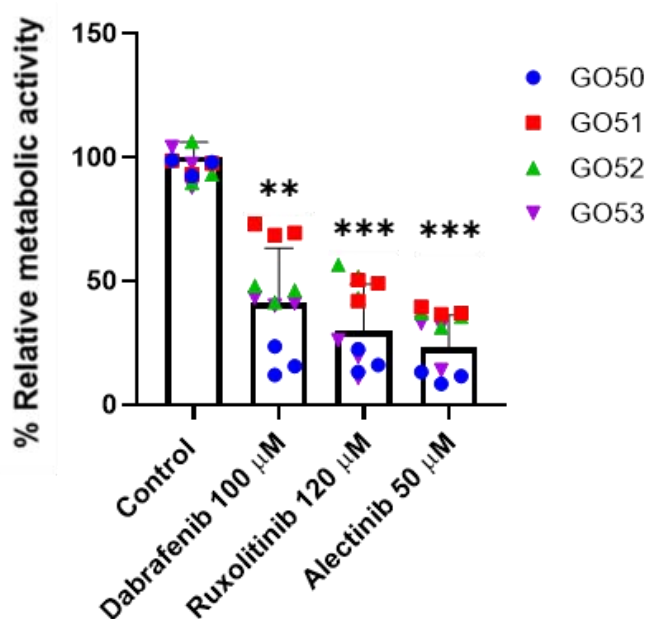


Figure 89: Dabrafenib, ruxolitinib and alectinib significantly inhibit metabolic activity in GOs. Bar graph of MTS results on GOwts after 48 hours of treatment with the indicated compounds. Bar height indicates mean relative metabolic activity as normalized to the control, with error bars for SD. Statistical comparisons were performed using ANOVA, ** $p < 0.01$; *** $p < 0.001$. GOs from $N = 4$ independent patients, indicated by color.

Extended treatment with these compounds showed a pronounced and progressive decrease in GO metabolic activity at 48, 96, and 120 hours, from close to 26-27% at 48 hours to around 5-6% remaining activity at the experimental endpoint in the case of alectinib and ruxolitinib; and from around 33% to 18% in the case of dabrafenib indicating strong and sustained antitumoral effects upon prolonged exposure (Fig. 90).

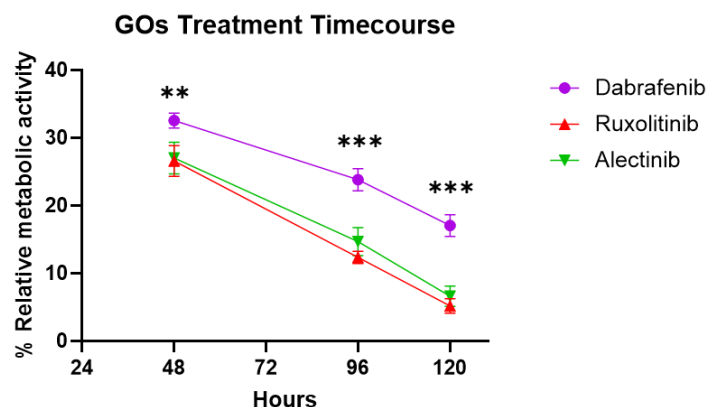


Figure 90: Extended treatment times further reduce metabolic activity of GOs. Line graph of MTS time course results on GOwts after 48, 96 and 120 hours with dabrafenib (purple), ruxolitinib (red) and alectinib (green). Points represent mean relative metabolic activity as normalized to the control, with error bars for SD. Statistical comparisons were performed using two-way ANOVA, ** $p < 0.01$; *** $p < 0.001$. GOs from $N = 3$ independent patients.

Following this, FC analysis confirmed a significant (** $p < 0.001$) reduction in cellular counts per mL in all treatments, accompanied by marked increases of about 10-fold for ruxolitinib and 8-fold for alectinib and dabrafenib in the late apoptosis / dead cells fraction. Necrotic fractions for ruxolitinib and alectinib also slightly increased, with the corresponding reduction in live cell fraction for all three compounds (Fig. 91).

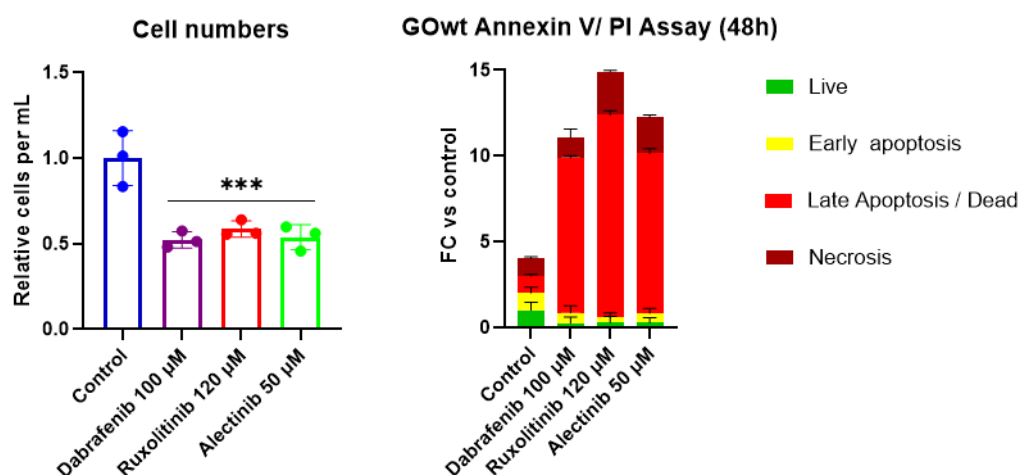


Figure 91: Dabrafenib, ruxolitinib and alectinib reduce recovered cell counts and increase late apoptotic/dead fraction in GOs. Left, bar graphs of relative number of cells per mL after 48 h treatment with the indicated compounds as determined by flow cytometry. Bar height indicates mean cell counts, with individual GOs shown. Right, segmented bar graph of fold change of number of cells in each apoptotic stage, each segment is color coded in the following manner: live cells, green; early apoptotic cells, yellow; late apoptotic/dead cells, red and necrotic cells as brown. Segment height indicates average fold change value. Error bars indicate SD. Statistical comparisons were performed using ANOVA, *** $p < 0.001$. GOs from $N = 3$ independent patients.

5.6.4 IC₅₀ estimation for dabrafenib, alectinib, and ruxolitinib in GOs

To further characterize GO responses to these compounds and enable comparison with other model systems, we estimated IC₅₀ values at 48 hours using MTS dose–response assays. IC₅₀ values were derived by nonlinear regression of normalized viability data.

Alectinib showed the lowest estimated IC₅₀ (25 μ M), followed by ruxolitinib (56 μ M) and dabrafenib (112 μ M) (Fig. 92).

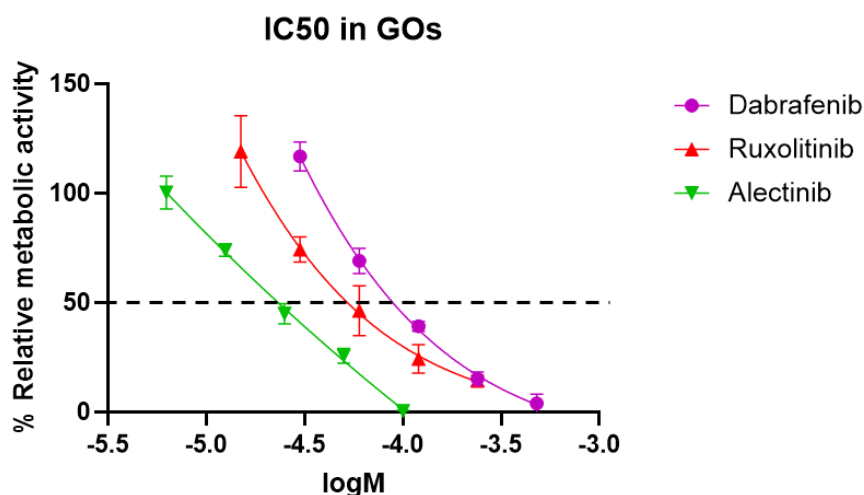


Figure 92: IC₅₀ determination in GOs: Line graph of MTS results on GOwts. Points represent mean relative metabolic activity as normalized to the control, with error bars for SD. Dashed line indicates 50% inhibition. GOs from N = 3 independent patients.

5.6.5 Dabrafenib, alectinib, and ruxolitinib inhibit cellular migration in GOs

We next assessed whether these compounds affected migration of GO-derived cells. To do so, dissociated cells from GOs were treated for 48 hours and subsequently subjected to a transwell migration assay (Fig. 93).

All three compounds significantly reduced transwell migration relative to vehicle control. Ruxolitinib and dabrafenib showed the strongest inhibition ($***p < 0.001$), while alectinib produced a smaller but significant reduction ($**p < 0.01$) (Fig. 94).

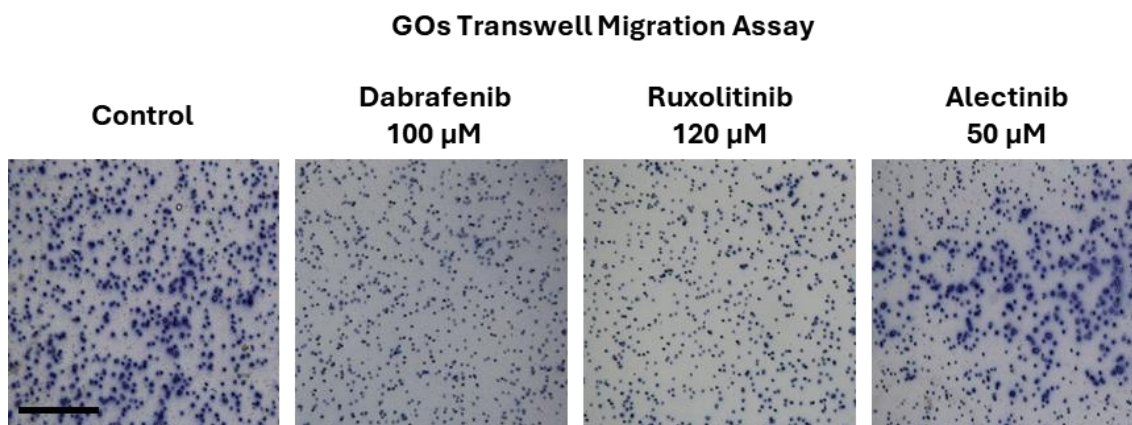


Figure 93: GOs transwell migration assay: Representative images of transwell center sections after 6 hours of incubation with dissociated GOs treated at the indicated concentration for 48 hours. Stained with 0.1% crystal violet. Scale bar: 200 μ m. GOs from N = 3 independent patients.

GOs transwell migration quantification

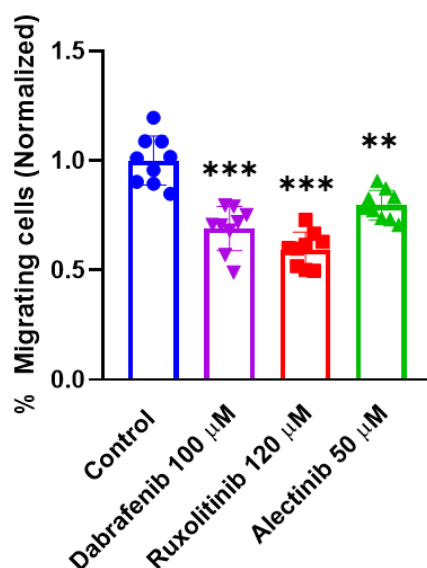


Figure 94: Dabrafenib, ruxolitinib and alectinib inhibit GO transwell migration. Bar graphs of normalized percentage of migrating cells after 6 hours of transwell incubation of dissociated GOs following 48 hours of treatment with dabrafenib (purple), ruxolitinib (red) and alectinib (green). Bars indicate mean values with error bars for SD. Statistical comparisons were performed using one-way ANOVA with appropriate multiple-comparisons correction; ** $p < 0.01$, *** $p < 0.001$. GOs from N = 3 independent patients.

5.6.6 Dabrafenib, alectinib, and ruxolitinib modulate key tumor-associated transcriptional programs in GOs

In order to offer some mechanistic insight into the effects of these compounds in GOs, we performed RNA-seq on GOs from 3 independent patients after 6 hours of treatment.

GSEA indicated significant modulation of pathways linked to proliferation, stress responses, and survival. Alectinib treatment was associated with downregulation of MYC targets, G2M checkpoint, and mitotic spindle genes, alongside positive enrichment of hypoxia response, TNF α signaling via NF- κ B, and apoptosis gene signatures (Fig. 95).

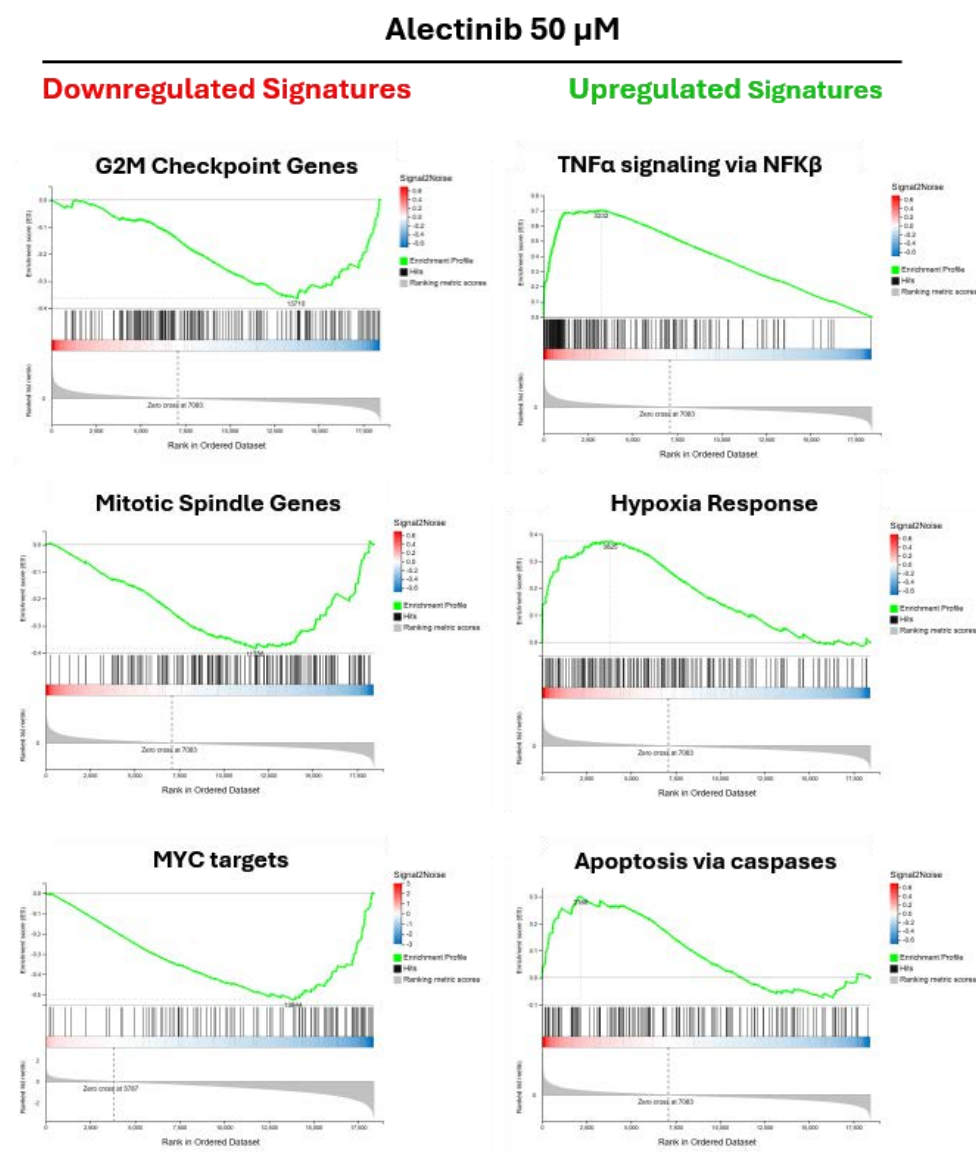


Figure 95: Alectinib treatment alters gene expression of GOs. GSEA plots of differentially downregulated (red, left) and upregulated (green, right) gene pathways from MSigDB hallmarks gene sets in GOs after 6 hours treatment with alectinib. Genes were ranked by signal-to-noise represented by red to blue color gradient. The green line shows the running enrichment score (ES) across the ranked gene list; the maximum deviation from zero corresponds to the peak ES (dashed line). Black vertical ticks indicate the positions of gene set hits within the ranked list. The lower gray panel shows the distribution of ranking metric scores across the ordered dataset.

RESULTS

Ruxolitinib showed negative enrichment of PI3K/AKT/mTOR signaling, DNA repair, and TGF- β 1 signaling, with positive enrichment of angiogenesis (Fig. 96).

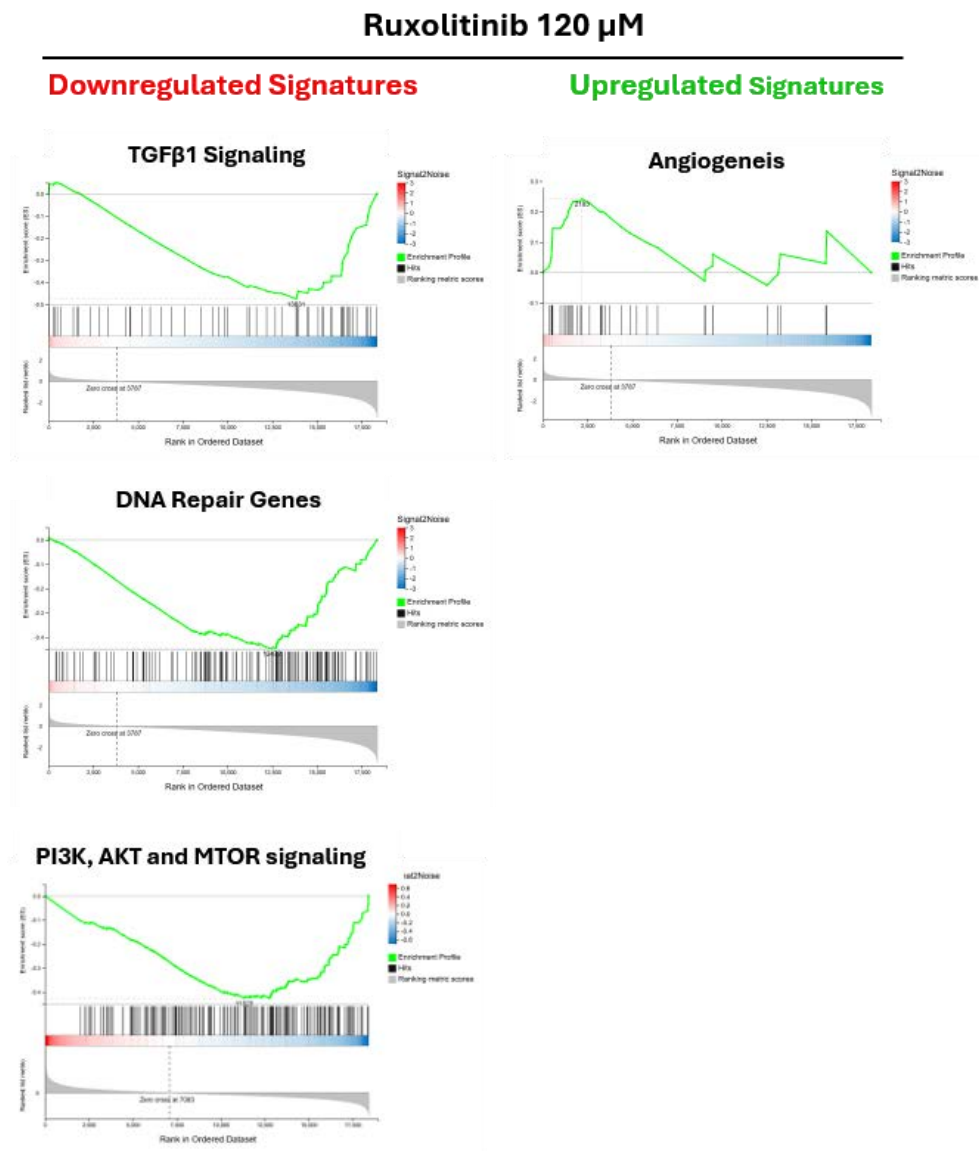


Figure 96: Ruxolitinib treatment alters gene expression of GOs. GSEA plots of differentially downregulated (red, left) and upregulated (green, right) gene pathways from MSigDB hallmarks gene sets in GOs after 6 hours treatment with ruxolitinib. Genes were ranked by signal-to-noise represented by red to blue color gradient. The green line shows the running enrichment score (ES) across the ranked gene list; the maximum deviation from zero corresponds to the peak ES (dashed line). Black vertical ticks indicate the positions of gene set hits within the ranked list. The lower gray panel shows the distribution of ranking metric scores across the ordered dataset.

Dabrafenib treatment was associated with upregulation of metabolic programs including oxidative phosphorylation and glycolysis, as well as hypoxia response, while showing downregulation of epithelial to mesenchymal transition (EMT), TGF- β 1 signaling, and G2M checkpoint signatures (Fig. 97).

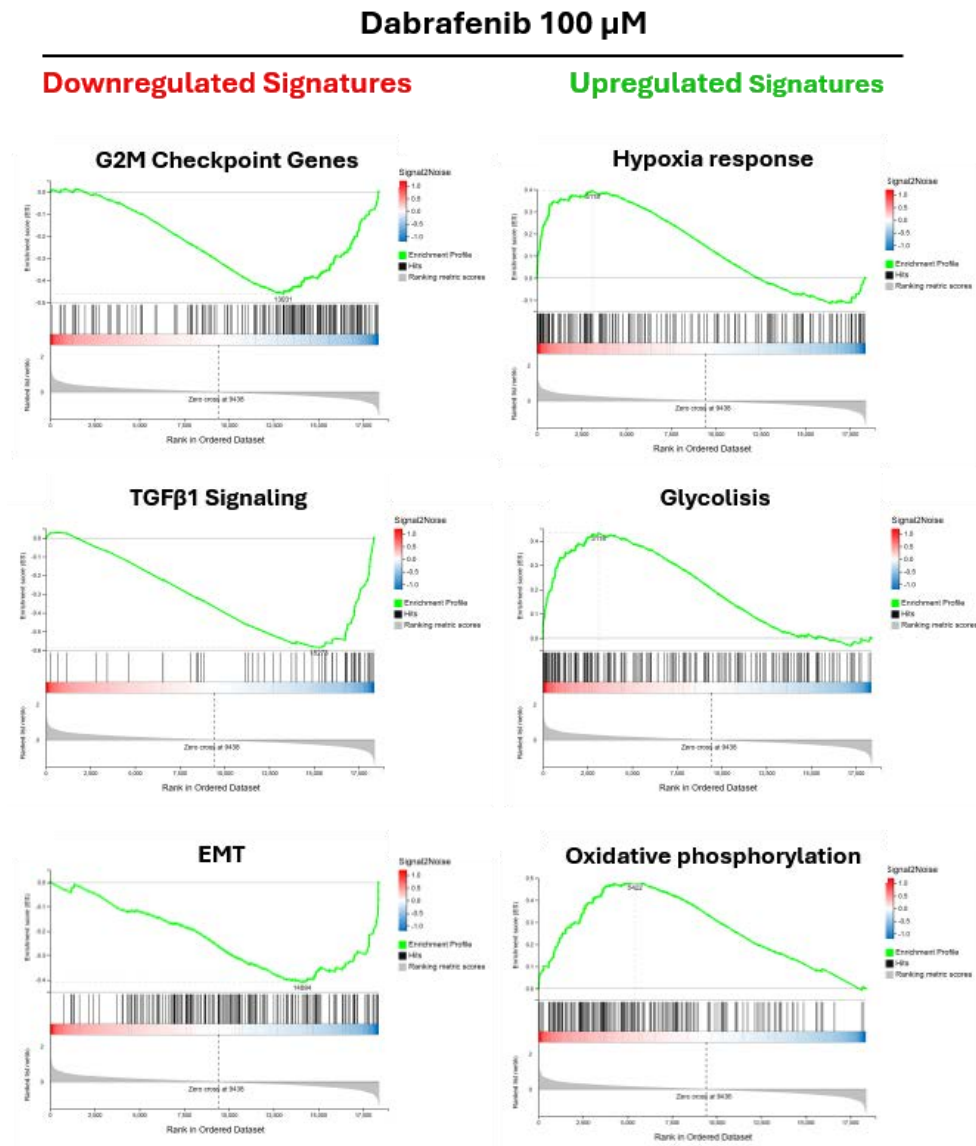


Figure 97: Dabrafenib treatment alters gene expression of GOs. GSEA plots of differentially downregulated (red, left) and upregulated (green, right) gene pathways from MSigDB hallmarks gene sets in GOs after 6 hours treatment with dabrafenib. Genes were ranked by signal-to-noise represented by red to blue color gradient. The green line shows the running enrichment score (ES) across the ranked gene list; the maximum deviation from zero corresponds to the peak ES (dashed line). Black vertical ticks indicate the positions of gene set hits within the ranked list. The lower gray panel shows the distribution of ranking metric scores across the ordered dataset.

5.7 Validation of GO results in glioma cell lines and GSCs

5.7.1 Dabrafenib, ruxolitinib, and alectinib reduce metabolic activity in glioma cell lines and GSCs

To understand whether our compounds could also present antitumoral effects in other *in vitro* glioma models, we evaluated metabolic activity in three classical glioma 2D cell lines, U87, U373 and LN229, together with the patient-derived GSC line GBM38, grown as 3D neurospheres.

MTS results showed that all three compounds reduced metabolic activity relative to vehicle control in each model, with alectinib producing the largest reduction in the 2D cell lines, whereas GBM38 spheres showed the greatest reduction following dabrafenib treatment (Fig. 98).

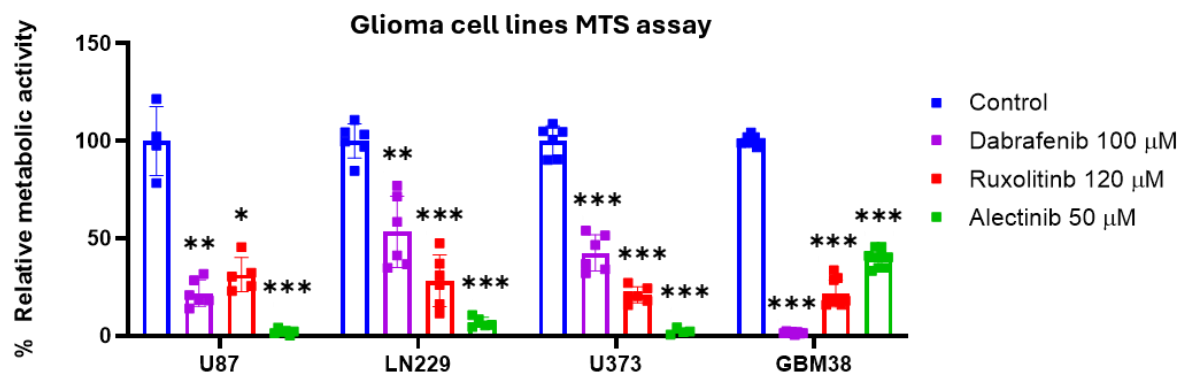


Figure 98: Dabrafenib, ruxolitinib and alectinib treatment reduces metabolic activity of glioma cell lines. Bar graph of MTS results on 2D glioma cell lines (U87, LN229 and U373) and 3D GSCs GBM38 after 48 hours of treatment with the indicated compounds. Bar height indicates mean relative metabolic activity as normalized to the control, with error bars for SD. Statistical comparisons were performed using one-way ANOVA, * $p < 0.05$; *** $p < 0.001$. $N = 2$ biological replicates with 3 technical replicates each.

5.7.2 IC50 estimation for dabrafenib, ruxolitinib, and alectinib in glioma cell lines and GBM38 GSCs

To enable direct comparison of drug sensitivity across models, we next estimated 48-hour IC50 values for dabrafenib, ruxolitinib, and alectinib in U87, LN229, U373, and GBM38 to enable comparison with GO responses.

IC50 values were derived by nonlinear regression of MTS dose–response curves (Fig. 99). For dabrafenib, estimated IC50 values were 13.4 µM (U87), 61.1 µM (LN229), 137

μM (U373), and $17.1 \mu\text{M}$ (GBM38). For ruxolitinib, IC_{50} values were $31.1 \mu\text{M}$ (U87), $21.8 \mu\text{M}$ (LN229), $27.5 \mu\text{M}$ (U373), and $43.2 \mu\text{M}$ (GBM38). For alectinib, IC_{50} values were $20.1 \mu\text{M}$ (U87), $22.5 \mu\text{M}$ (LN229), $22.1 \mu\text{M}$ (U373), and $247 \mu\text{M}$ (GBM38), indicating markedly reduced sensitivity to alectinib in the GBM38 GSC model relative to the 2D lines. (Table 12).

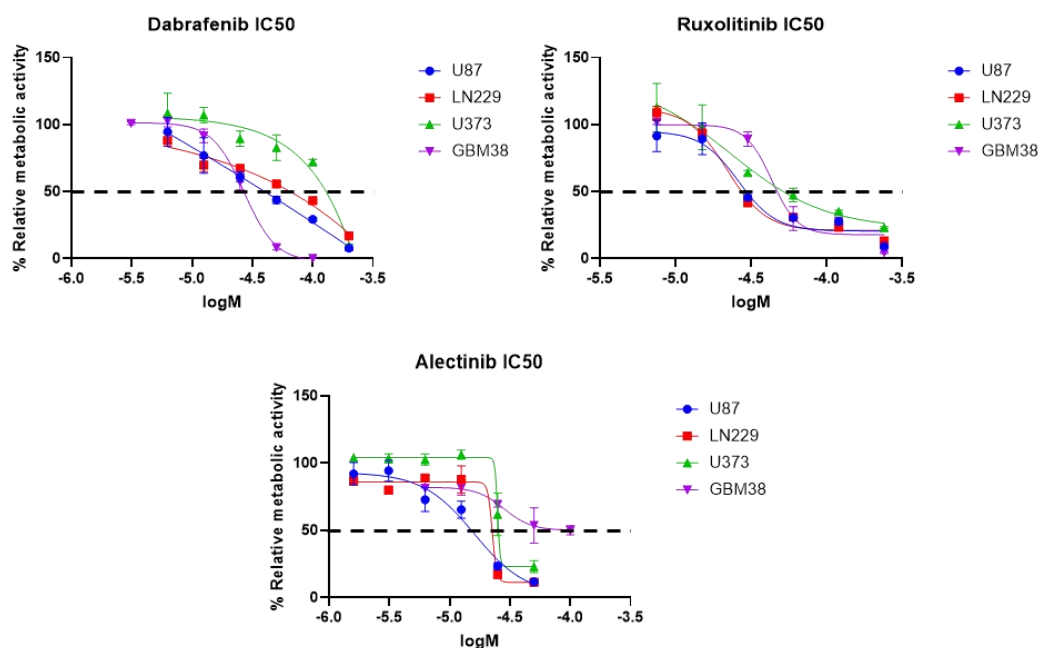


Figure 99: Dose–response curves used for IC_{50} estimation in glioma cell lines and GBM38 GSCs. Line graph of MTS results on glioma cell lines of dabrafenib (top), ruxolitinib (middle) and alectinib (bottom). Cell lines are color coded: U87 in blue, LN229 in red U373 in green and GBM38 in purple. Points represent mean relative metabolic activity as normalized to the control, with error bars for SD. Dashed line indicates 50% inhibition.

For dabrafenib, estimated IC_{50} values were $13.4 \mu\text{M}$ (U87), $61.1 \mu\text{M}$ (LN229), $137 \mu\text{M}$ (U373), and $17.1 \mu\text{M}$ (GBM38). For ruxolitinib, IC_{50} values were $31.1 \mu\text{M}$ (U87), $21.8 \mu\text{M}$ (LN229), $27.5 \mu\text{M}$ (U373), and $43.2 \mu\text{M}$ (GBM38). For alectinib, IC_{50} values were $20.1 \mu\text{M}$ (U87), $22.5 \mu\text{M}$ (LN229), $22.1 \mu\text{M}$ (U373), and $247 \mu\text{M}$ (GBM38), indicating markedly reduced sensitivity to alectinib in the GBM38 GSC model relative to the 2D lines. (Table 12).

RESULTS

Table 12: Estimated 48-hour IC₅₀ values for dabrafenib, ruxolitinib, and alectinib across glioma cell lines (U87, LN229, U373), GBM38 GSCs, and GOs.

Drug	Model				
	U87	LN229	U373	GBM38	GOs
Dabrafenib IC ₅₀ (μM)	13.4	61.1	137	17.08	112
Ruxolitinib IC ₅₀ (μM)	31.14	21.8	27.5	43.2	56.42
Alectinib IC ₅₀ (μM)	20.1	22.5	23.13	247.2	25.12

5.7.3 Dabrafenib, ruxolitinib and alectinib inhibit wound closure in U373 and LN229 cells

Finally, we assessed whether the selected compounds affected wound closure in 2D glioma models using a 24-hour wound healing assay in LN229 and U373 cells (Fig. 100).

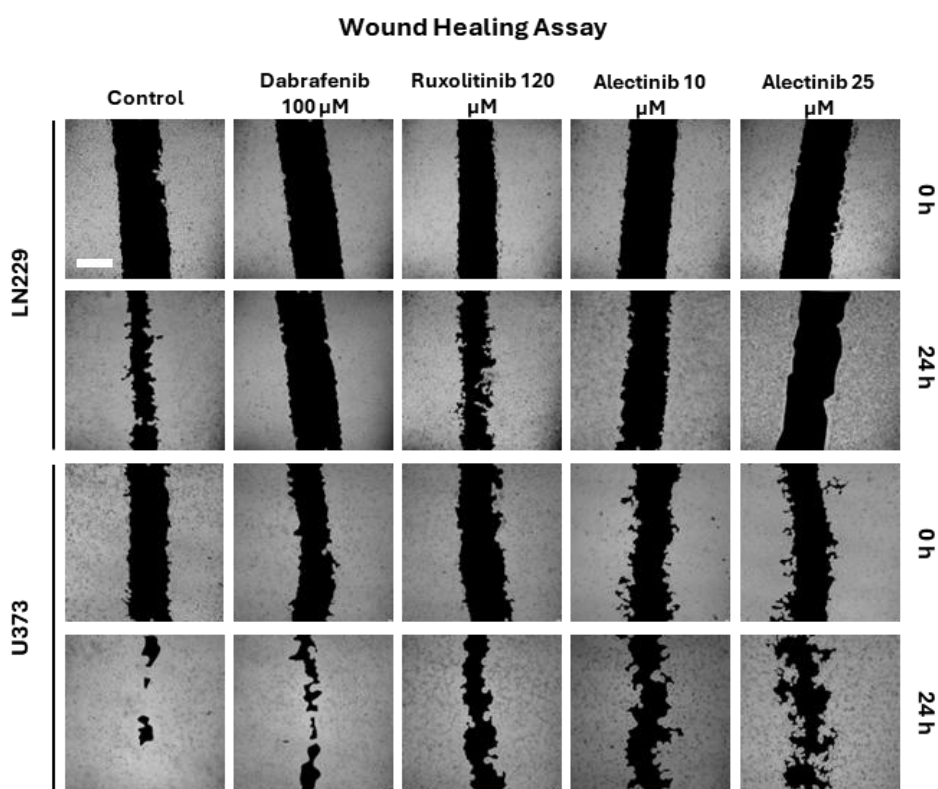


Figure 100: LN229 and U373 wound healing assay. Representative brightfield images from the wound healing assay at baseline and 24 hours post-treatment in LN229 (top) and U373 (bottom) cells. Scale bar: 500 μm.

GBM38 was excluded because it grows as non-adherent neurospheres, and U87 was excluded because it did not form stable confluent monolayers as it tended to aggregate at high confluence. Drug concentrations were matched to those used in prior assays; however, the alectinib dose was reduced (25 μM and 10 μM) due to marked cytotoxicity observed at 50 μM (Fig. Annex IC).

Across conditions, ruxolitinib and alectinib significantly reduced wound closure in both LN229 and U373 cells, whereas dabrafenib showed a weaker effect and did not reduce wound closure in LN229 (Fig. 101), suggesting that antimigratory effects on GOs are also extensible to 2D cultures.

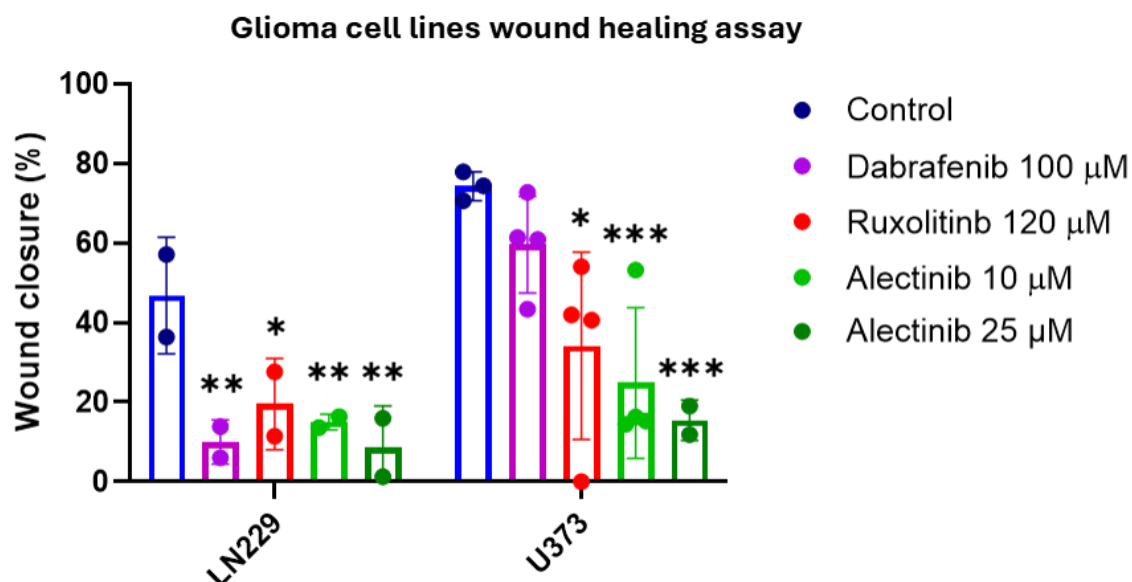


Figure 101: Ruxolitinib and alectinib inhibit wound closure in LN229 and U373 cell lines.
Bar graph of wound closure percentage versus baseline wound area 24 hours after monolayer injury in LN229 (left) and U373 (right) cells.

5.8 *In vivo* validation of GO drug screening

5.8.1 GO heterotopic xenograft model establishment

Next, to evaluate whether our *in silico* screening with GOs could identify compounds with antitumoral effectiveness in more complex *in vivo* systems, we decided to establish a novel GO heterotopic xenograft model, where GOs were surgically implanted subcutaneously into the flanks of athymic mice.

Implanted GOs showed progressive tumor growth, which maintained the histological characteristics of the original patient's tumor as assessed by H&E (Fig. 102), confirming the tumorigenic capabilities of GOs *in vivo*.

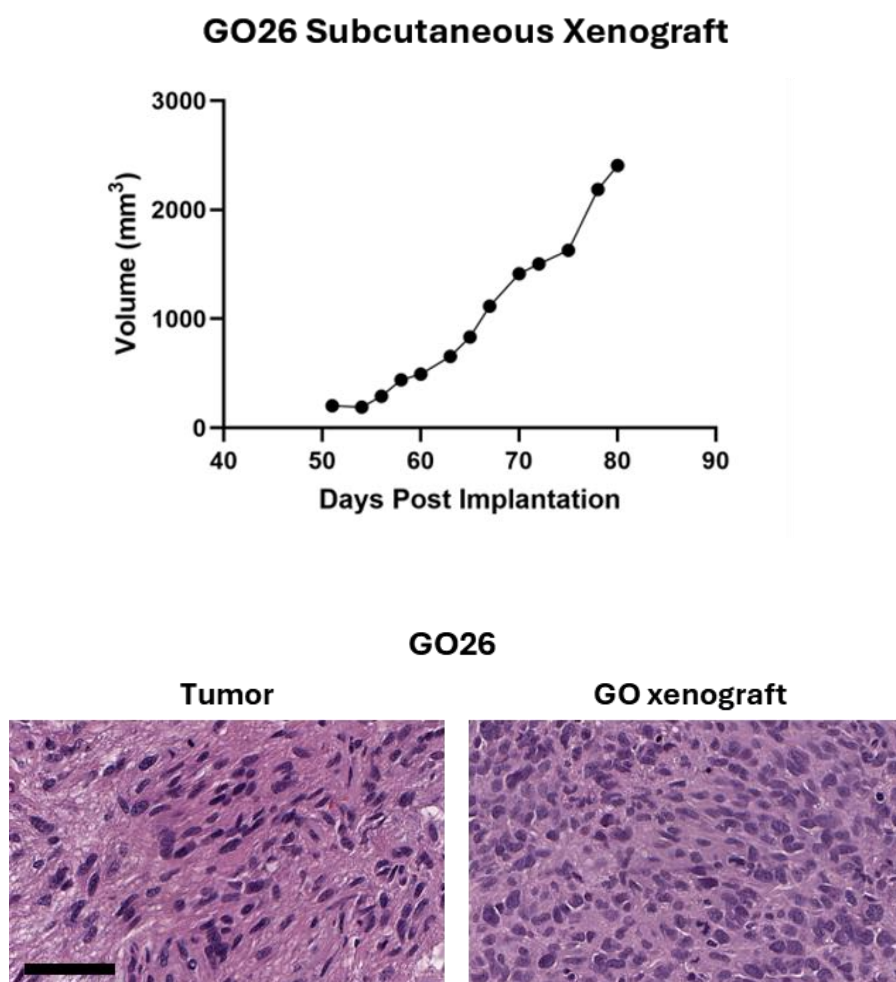


Figure 102: Heterotopic flank implantation of GOs produces tumors that replicate original parental characteristics. Top, line plot of tumor volume (mm³) in athymic mice after subcutaneous GO implantation. Each point indicates average volume across flanks at a different timepoint post-implantation. Bottom, representative H&E images of patient tumor and its corresponding GO xenograft. Scale bar: 200 μ m.

5.8.2 Alectinib and ruxolitinib significantly reduce tumoral growth in heterotopic models

After establishing the U87 and GO-derived heterotopic xenograft models, animals were randomized once tumors reached 200 mm³ and treated with dabrafenib, alectinib, or ruxolitinib, or vehicle control. Longitudinal caliper measurements showed that alectinib and ruxolitinib significantly reduced tumor growth compared with vehicle in both the U87 and GO xenograft models (**p < 0.01), whereas dabrafenib did not significantly alter tumor growth kinetics (Fig. 103).

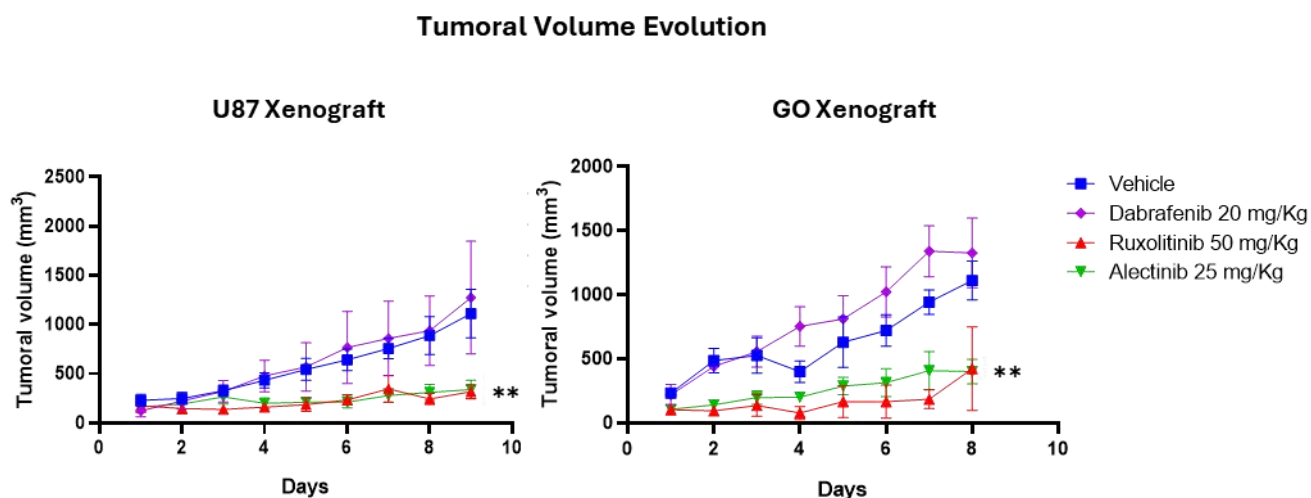


Figure 103: Alectinib and ruxolitinib treatment reduce heterotopic xenograft growth. Line graphs of tumoral volume evolution in U87 (left) and GO (right) heterotopic xenografts. Measured by caliper. Each colored line represents a treatment, with points indicating daily tumoral growth and SD. The baseline time was set when flank tumors reached 200 mm³ in size. Statistical comparisons were performed with two-way ANOVA. ** p < 0.01. N = 6 flanks per treatment.

Consistent with longitudinal growth curves, endpoint tumor volumes were significantly reduced in the alectinib and ruxolitinib treatment groups relative to vehicle (*p < 0.05), while dabrafenib showed no significant reduction and trended toward larger endpoint volumes (Fig. 104).

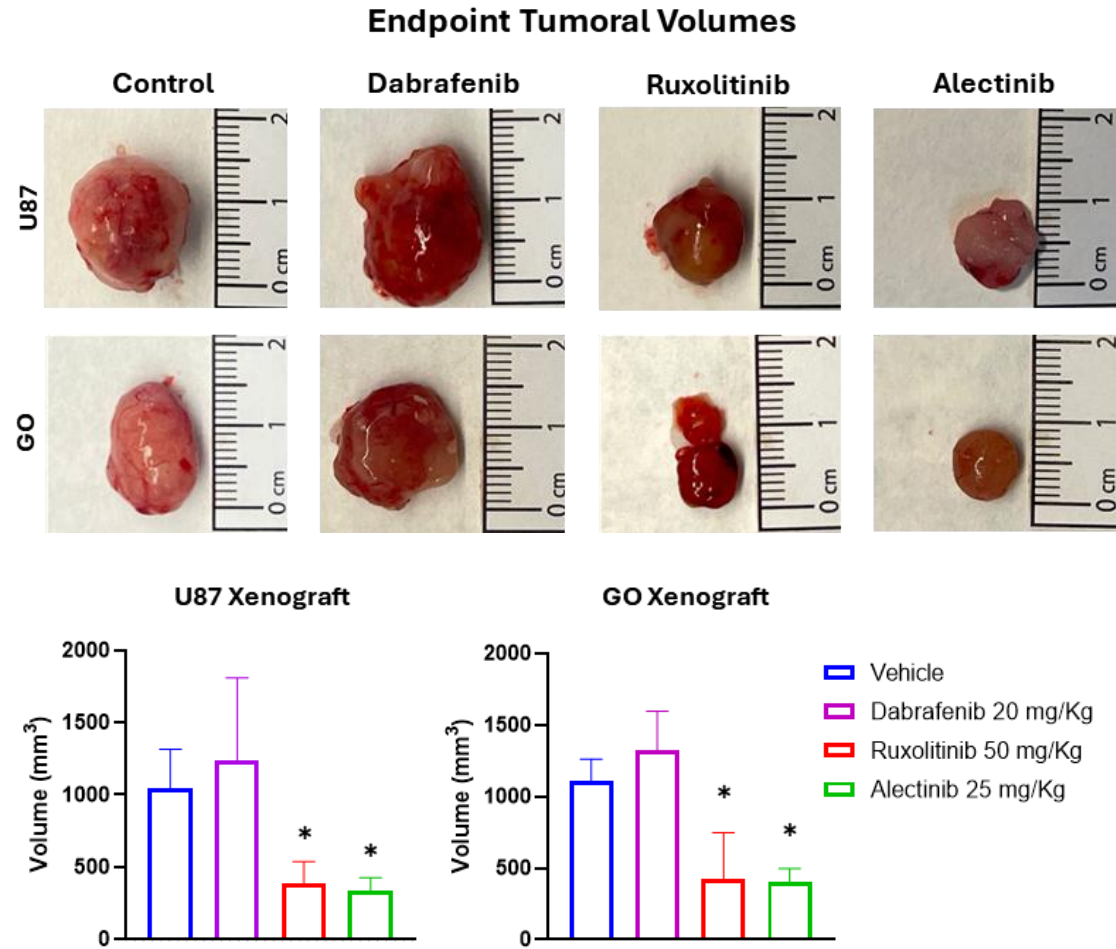
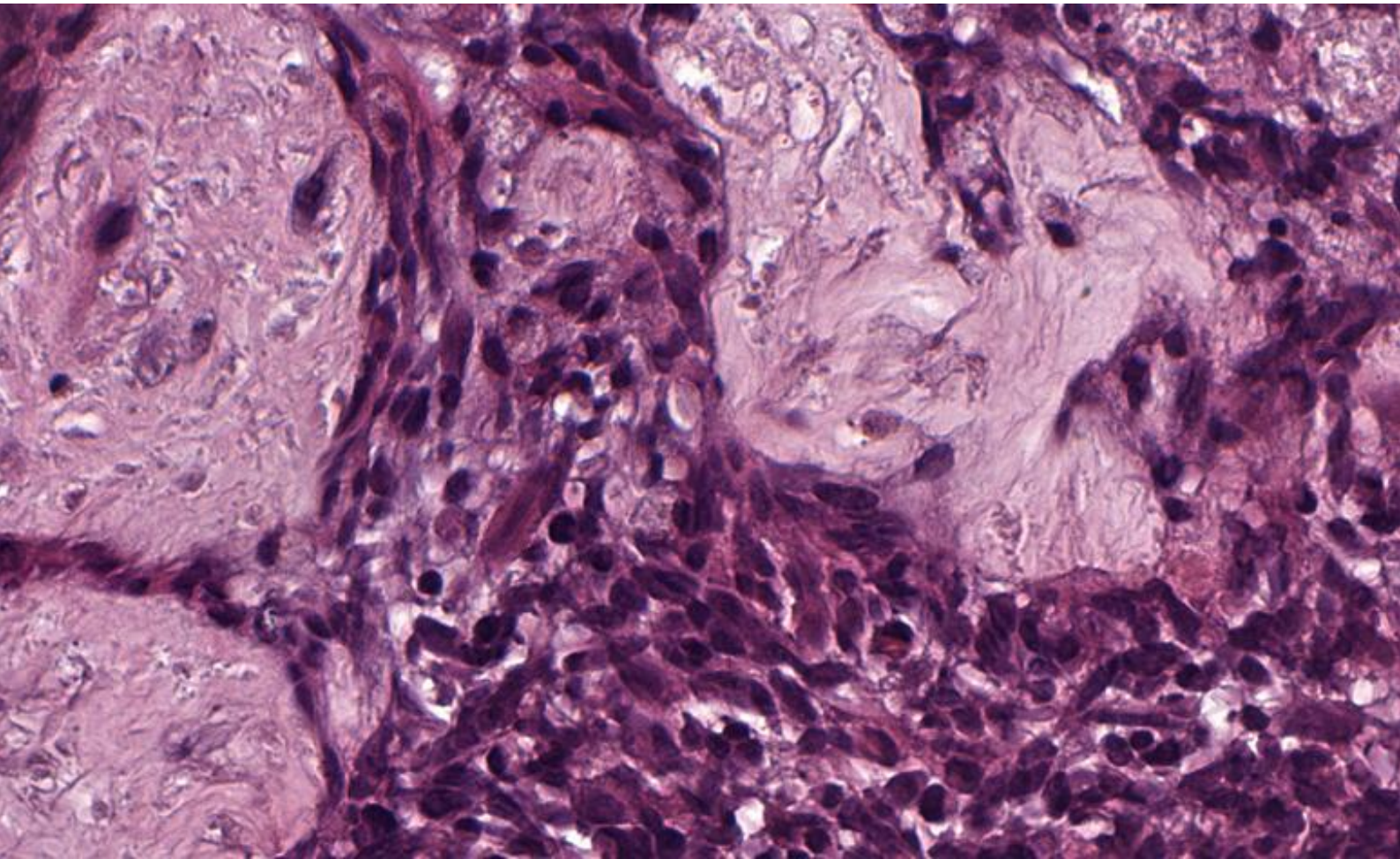


Figure 104: Alectinib and ruxolitinib treatment reduce tumoral volumes at endpoint. Top, representative images of U87 (top) and GO (bottom) derived xenografts at experimental endpoint. Ruler in centimeters to provide scale. Bottom, bar graphs of endpoint tumoral volume in U87 (left) and GO (right) heterotopic xenografts. Measured by caliper. Each colored bar represents a treatment. Statistical comparisons were performed with one-way ANOVA. * $p < 0.05$. $N = 6$ flanks per treatment.

6. DISCUSSION



“La naturaleza es la mejor maestra de la verdad”

- San Ambrosio

Adult diffuse gliomas constitute the majority of malignant primary brain tumors in adults. Despite major advances in molecular characterization, therapeutic outcomes under current SOC regimens remain unsatisfactory, particularly for GBM, the most common and lethal glioma subtype (11,13). A key barrier to effective therapy is the marked inter- and intratumoral heterogeneity of these tumors, which promotes rapid therapy resistance and complicates the identification of shared therapeutic vulnerabilities (101). In parallel, many candidate therapies that demonstrate activity in conventional preclinical systems fail to translate clinically, highlighting a persistent gap between experimental models and patient biology (172,185).

This translational bottleneck is partly explained by limitations of widely used glioma models. Established 2D cell lines and GSC cultures are scalable and experimentally tractable but often diverge from parental tumors in their genomic landscape and transcriptional programs while lacking key elements of the tumor microenvironment (194,258). On the other hand, animal models enable organism-level pharmacological studies and capture aspects of complex tumor–host interactions, but they are cost- and time-intensive, lower throughput, and frequently constrained by the immune background of the selected strain (259,260). Consequently, there is a need for model systems that preserve clinically relevant, patient-specific tumor features while remaining compatible with therapeutic testing within a practical timeframe (200,202).

In this context, the present thesis develops and evaluates a precision oncology framework centered on GOs established from both GBM and IDH-mutant glioma patients. This work integrates a comprehensive analysis of GO fidelity against parental tumors across histopathological, ultrastructural and molecular dimensions together with an assessment of their scalability through biobanking and xenografting and their integration into a transcriptomics-based drug prioritization strategy with *in vitro* and *in vivo* validation. Collectively, this integrated workflow is designed to address a central need in glioma research: bridging biologically faithful patient-derived models with actionable therapeutic testing to improve the translational relevance of preclinical findings.

For this, we have established a cohort of 61 glioma patients from 3 different hospitals over a period of 4 years, which amounts to an average of 1.3 patients per month. This amount aligns with the low incidence of gliomas, with around 6 cases per 100,000 individuals per year in Spain (3,4).

Using this cohort, GO establishment was successful in over 90% of cases, supporting robustness of the platform across routine surgical specimens and aligning with previously

DISCUSSION

reported organoid-based workflows. In particular, the comparable method developed by Jacob et al. (202,203) reported an almost identical 91.4% success rate in a comparable cohort of 53 patients. Notably, their IDH-mutant cohort only contained 3 patients with a 66% success rate, which complicates comparison to our more extensive cohort. They also evaluated recurrent tumors, which obtained a 77% success rate, samples which we did not collect in our cohort. Moreover, their method requires the use of a CO₂ and humidity-resistant orbital shaker, introducing additional equipment compared to our approach. Other authors utilizing the Jacob et al. approach like Abdullah et al. (261) report overall success rates of 91% in cohorts of 22 patients, with a success rate of 87 % for IDH-mutant tumors in much larger and representative cohorts, which better aligns to our observed results.

Consistent with other reports (262,263), tissue quality and amount was paramount to a successful GO generation, which probably was the leading cause of a lower success rate in IDH-mutant tumors, as these tumoral samples presented less densely cellularized tumoral cores and smaller tumoral volumes.

In practical terms, our GO method is particularly compelling compared to other patient-derived *in vitro* models, like traditional 2D established cell lines and GSC cultures which have reported significantly lower establishment rates of 56% (264) and 70% (265), respectively. Therefore, the higher success rate achieved with GOs may increase the likelihood of generating patient-matched models for downstream characterization and therapeutic testing in a broader fraction of cases. This is particularly interesting in the context of IDH-mutant gliomas, where cell line establishment ratios are even lower, thus difficulting the study of these patients (266).

Since mutations in the *IDH* genes are among the most clinically relevant alterations in diffuse gliomas, with major implications for diagnosis, prognosis, and therapeutic stratification (25,26), their preservation is an essential requirement for any model intended for precision-oncology applications. However, maintaining *IDH* mutations *in vitro* has historically been challenging: in traditional culture systems, the mutant population may fail to expand or can be lost during early passaging, resulting in cultures that no longer retain this defining alteration (267,268). Mechanistically, this limitation has been linked by some authors to an early activation of senescence-associated programs in IDH-mutant glioma cells upon *in vitro* culture, which can markedly restrict proliferative capacity and contribute to the loss of mutant clones over time (269).

In this context, our GOMuts preserved the clinically relevant *IDH1* alteration at multiple levels. We confirmed conservation of the *IDH1* G395A mutation at the genetic level,

together with expression of the mutant IDH1 R132H protein and maintenance of its biochemical activity, as demonstrated by production of the oncometabolite D-2HG. Moreover, the mutation remained detectable after eight weeks in culture, supporting the suitability of these GOs for longer-term studies of IDH-mutant gliomas. Other groups developing patient-derived IDH-mutant GOs have similarly reported retention of IDH1 R132H and associated metabolic activity, consistent with our observations (261,270,271). For GO cryopreservation and biobank generation, we followed the protocol established by Jacob et al. (202,203). After recovery, GOs retained the histopathological features of both the parental tissue and fresh cultures; however, unlike the original report, they did not recover proliferative capacity. Similar observations have been described by other groups, which reported preservation of histological and molecular features after thawing, although without evaluating organoid size or proliferation markers in detail (261,272). Likewise, Golebiewska et al. reported the biobanking of GOs, but did not provide post-thaw functional characterization (201,273). Together, these findings indicate that although cryopreserved GOs may preserve key features of the original tumor, efficient recovery of proliferative GOs remains a major unresolved limitation of the model. This constraint is particularly relevant for longitudinal studies and for workflows that rely on biobanking; however, it does not affect the use of GOs for prospective patient-specific drug screening, which can be performed during the primary culture window without the need for cryostorage.

Several factors may contribute to the limited success of GO biobanking. GO size is likely to be one of the main determinants, as larger 3D structures may show diffusion-limited penetration and incomplete incorporation of cryoprotective agents, resulting in heterogeneous protection and increased core damage during freezing and thawing. In addition, the combined stress associated with sectioning, cryopreservation, and thawing may induce adaptive stress responses that favor the acquisition of a senescent-like state, thereby impairing subsequent proliferation (274). These limitations may be mitigated through further optimization of cryopreservation procedures and through the development of preservation media specifically tailored to larger GO structures (275). As an alternative strategy to avoid prolonged *in vitro* maintenance and conventional cryopreservation, Golebiewska et al. proposed the generation of a living biobank by orthotopic implantation of GOs, which was shown to preserve the main characteristics of the original tumors (201). We have also confirmed that GOs are able to successfully be maintained as heterotopic xenografts, aligning with her observations. Nonetheless, a limitation of long-term GO maintenance via *in vivo* passaging is the continued

DISCUSSION

requirement for animal use, raising ethical considerations and reducing the extent to which organoid platforms can function as animal-replacement approaches (276).

In accordance with the capacity of tumoral organoids to better replicate the TME, we proceeded to characterize our GOs. We confirmed the conservation of key tumoral histopathological characteristics in accordance to other reports of GOs (201,203,215). Moreover, these characteristics remained unaltered after long-term culture up to 10 weeks, which positions GOs as more clinically relevant platforms to perform long term experiments than *ex vivo* organotypic slice cultures, which have been reported to conserve these histological characteristics for only 2 to 4 weeks when derived from GBM (277,278).

To our knowledge, our work provides the first TEM analysis of a purely patient-derived GO model, which represents a unique opportunity to understand tissular ultrastructure and cell to cell interactions. Using TEM, we observed preservation of key pathological features compatible with the parental tumors, including necrosis and edema like tissue alterations, together with a diverse cellular landscape in which neuronal- and glial-like cells and tumor cells coexist in close proximity and form physical contacts with conserved intracellular organelles. This cell type diversity, together with the conservation of vascular features and stemness elements, was also confirmed. This heterogeneous composition, including partial preservation of tumor microenvironment elements, further supports the use of GOs over traditional 2D culture systems, where non-malignant components are typically lost and clonal expansion of rapidly proliferating tumor cells under culture selection pressures can yield a biologically simplified representation of context-specific interactions (175,279).

Linkous et al. showed similar conservation of GBM neuroanatomical and ultrastructural characteristics in their assembloid GBM model. This team also reported on the presence of a network of tumor microtubes which maintained GBM growth (215), which has been previously recognized as a major driver of invasion, proliferation, and therapy resistance (280). Our TEM images also identified tubular structures loaded with mitochondrial consistent with this microtube network. In addition, Sun et al. recently presented a hybrid iPSC and GBM cell co-culture which described the presence of tumor-neuronal synapses, aligning with our report of conserved cellular interactions in GOs (281).

Molecular profiling further supported the biological similarity between GOs and their parental tumors. At the transcriptomic level, GOs largely preserved tumor-associated expression programs across multiple feature sets, including signatures linked to cellular composition as well as stromal- and immune-related markers. This indicates that, despite

culture outside the native brain environment, GOs retain clinically relevant molecular states that reflect the source tissue.

Subtype analysis provided an additional layer of validation. For most patients, GOs were assigned to the same transcriptional subtype as the corresponding parental tumor, consistent with preservation of dominant regulatory programs. However, a subset of GOs deviated from their parental assignment and shifted toward classical and/or mesenchymal-like states. Several complementary explanations could account for this divergence. First, bulk RNA-seq captures an average signal that is sensitive to regional sampling; because GBM exhibits strong intratumoral transcriptional heterogeneity, tissue fragments used for sequencing may differ in the relative abundance of subtype-associated programs, leading to apparent discordance even when both samples remain biologically representative (103,282). Second, the directionality of the observed subtype evolutions is itself informative: transitions toward classical and mesenchymal signatures are consistent with reported state changes during disease progression and treatment-associated evolution, which favours mesenchymal-like expression states (99,100), a process which may be accelerated by the distinct *in vitro* culture conditions which may impose selective pressures on GOs, promoting the outgrowth of more aggressive mesenchymal-like phenotypes (208). Consistent with these culture promoted changes, GSEA analysis of matched GO/tumor pairs identified significantly differing expression of metabolism related pathways.

Since conventional glioma therapy has remained stagnant for decades, alternative options like immunotherapies have been explored, with limited success so far. Two of the most common approaches are the use of ICIs and CAR-T cells. Clinically used ICIs primarily target T-cell inhibitory pathways such as PD-1/PD-L1 and CTLA-4/B7 (283). While these therapies have transformed treatment for multiple cancers such as hematologic malignancies (284), melanoma (285) and NSCLC (286), they have not yet demonstrated consistent survival benefit in glioma clinical trials (287–289), consistent with an immune landscape characterized by limited effector T-cell infiltration and prominent myeloid-driven immunosuppression. On the other hand, CAR-T cells have also shown limited durable clinical benefit, likely reflecting tumoral antigen heterogeneity, adaptive resistance by masking, and immunosuppressive microenvironmental constraints (290,291).

To improve the outcome of these immune-based therapies it is therefore fundamental to better understand the TME (292,293).

DISCUSSION

However, immune-focused glioma research has historically been constrained by model limitations. Conventional *in vitro* cultures typically lack endogenous immune populations, while reductionist co-culture systems rely on manually introduced immune subsets and therefore capture only selected interactions rather than the full cellular and spatial complexity of the tumor immune ecosystem (172,260). GOs provide a compelling approach because they can retain, at least transiently, components of the tumor-associated microenvironment present in the surgical specimen, thereby preserving aspects of patient-specific cellular context and local cellular interactions without requiring complex microfluidic or OOC engineering (294,295).

Immune profiling in our GOs demonstrated a clear imbalance between myeloid and lymphoid compartments. Myeloid populations were consistently detected by transcriptomic signatures and IF, including expression of canonical markers such as CD68 and IBA1. Further assessment of microglial/macrophage-associated activation markers suggested heterogeneous activation states, with evidence of both pro-inflammatory-like and immunosuppressive-like programs coexisting within the GO microenvironment. In contrast, CD4⁺ and CD8⁺ T cells were sparse and often difficult to detect in tissue sections. Notably, immune gene signatures in GOs closely resembled those of the matched parental tumors, supporting retention of key immune-related transcriptional features. These observations align with unbiased analyses of publicly available scRNA-seq datasets and with the broader literature describing adult gliomas as typically myeloid-dominated and relatively deficient in lymphocytes-immunologically cold tumors, whose composition correlates to patient outcomes (296,297). However, there was a key limitation to our immune system characterization, GOs were generated from densely cellular tumor cores; infiltrative margins (where lymphoid cells are relatively richer and more active) (298,299) were not sampled. Rather than excluding lymphoid-based strategies, this context may help explain why approaches that rely on robust endogenous T-cell infiltration and activation frequently underperform in glioma, and it highlights the tumor-associated myeloid compartment as a major candidate for therapeutic modulation, an area that is currently under preclinical and phase I clinical investigation in many cancers (300–302).

In our GOs, expression of T-cell associated checkpoint pathways was relatively low, whereas myeloid related checkpoints and innate immune regulatory programs were comparatively more prominent, mirroring the overall myeloid-dominant immune composition. Together, these findings explain why ICI therapy is not ideal in the context of gliomas and support the view that focusing on the myeloid compartment for immunomodulatory strategies, including myeloid-associated checkpoint targets, may be

particularly relevant to explore in these tumors. In fact, recent preclinical studies in glioma focus on leveraging the macrophage-associated CD47 immune checkpoint (303,304). Moreover, some authors have suggested the use of macrophage-derived CAR-M cells instead of CAR-Ts, as these cells can increase antitumoral effects and generate immune memory in *in vivo* models (305). In this regard, GOs are well positioned as a preclinical platform to prioritize and mechanistically evaluate such approaches based on their close immune profile matches to parental tumors and the prevalence of microglial populations.

Regarding conventional CAR-T approaches, patient-derived organoids systems like GOs can contribute at the preclinical stage by enabling antigen-dependent killing assays in a 3D tumor context and by capturing intratumoral heterogeneity that can drive immune escape. For example, Jacob et al. (203) used EGFRvIII-directed CAR-T cells in their patient-derived glioma organoid model and observed CAR-T expansion and apoptosis preferentially in organoids with higher EGFRvIII expression, alongside with persistence of EGFRvIII tumor cells consistent with selection and escape dynamics.

While these GO systems have shown higher fidelity in immune system conservation than conventional models, they are still inherently limited by their *in vitro* context, which still lacks structural and signaling elements present in full organisms. To address these limitations, xenotransplantation of organoids presents a compelling strategy (306).

Several groups have reported successful orthotopic implantation of patient-derived GOs into the mouse brain, resulting in tumors that recapitulate key features of the parental disease. For example, Golebiewska et al. (201) and Jacob et al. (203) describe orthotopic approaches in athymic nude mice that support tumor formation and enable downstream therapeutic testing within the CNS niche.

In contrast, we adopted a heterotopic subcutaneous implantation strategy for several reasons. First, this approach is technically simpler, more reproducible, and faster to establish than orthotopic implantation. Second, it enables longitudinal assessment of tumor volume as well as treatment response without specialized equipment, intracranial surgery, or the use of reporter genes. Third, because our primary aim was to present GOs as an alternative platform to conventional xenograft models, a more complex orthotopic system was not essential for these experiments. The main disadvantage of this approach is that it does not model the brain stromal component and its immune regulation or BBB constraints. The lack of immune regulation is shared with the previously mentioned studies, as xenografting human cells requires immunodeficient hosts, which further limits the evaluation of immune-dependent mechanisms; this limitation can be only partially addressed by the use of humanized mouse models. Owing

DISCUSSION

to their remarkable capacity to mimic individual tumoral contexts, GOs have been demonstrated to be invaluable tools for preclinical compound screening, as they can very closely mimic the patient's response to therapies *ex vivo*.

Some clinical case studies show the remarkable benefits of organoid-based drug screening approaches in oncological treatment selection. He et al. showcased a complex unresponsive cholangiocarcinoma case where patient-derived organoids were utilized to adjust clinical regimen, causing tumoral conversion and no subsequent recurrence (307). Perhaps even more striking, Chen et al. established an organoid biobank from 125 breast cancer patients, which closely mirrored response to conventional therapies. Moreover, for unresponsive or recurrent patients, organoids were used to guide tumoral rechallenge, proving very successful in this extensive cohort (308).

In glioma, organoid-based functional testing has not yet been widely implemented to retrospectively guide therapy selection at the individual patient level, but several studies indicate that patient-derived GOs can reproduce key aspects of clinically observed treatment response, particularly the limited efficacy of SOC regimens. Chen et al. (309) showed that glioma patients and their derived organoids were resistant to the SOC TMZ, but responded positively to a combined vemurafenib and trametinib combined regimen. Similarly, Jacob et al. (203) showed that their GBM organoids were largely refractory to TMZ and to high-dose irradiation (10 Gy), while Majc et al. (310) observed no significant changes in organoid viability or invasive behavior after TMZ exposure, consistent with the well-established clinical refractoriness of many gliomas to TMZ monotherapy.

In this thesis, we first used an *in silico* guided screening approach focused on clinically used agents, including TMZ and other compounds frequently evaluated in glioma such as irinotecan and vincristine, in matched parental tumors and derived GOs. Predicted sensitivity scores were highly concordant between tumors and GOs, supporting a high degree of response commonality and further reinforcing the molecular fidelity of GOs in a pharmacological context. Across these screened compounds, all agents except gefitinib were classified as non-responsive, and this was confirmed experimentally by the absence of measurable antitumoral effects in GOs. Notably, TMZ induced hallmarks consistent with DNA damage in GOs without a corresponding reduction in viability, mirroring observations reported by Majc et al. (310) and aligning with the concept that DNA damage induction alone is insufficient to translate into effective tumor control in resistant glioma contexts (311). In contrast, gefitinib produced a marked reduction in GO viability, consistent with its *in silico* results.

Gefitinib is a first-generation, reversible EGFR tyrosine kinase inhibitor that reduces EGFR autophosphorylation and can attenuate downstream signaling through core GBM pathways, including PI3K. Preclinical studies support biological activity of EGFR inhibition particularly in tumors with strong EGFR pathway dependence, including those with high EGFR expression and EGFRvIII mutations (312,313). However, heterogeneous target engagement, pathway redundancy, and adaptive resistance mechanisms have limited consistent clinical benefit (314). In this context, GOs may potentially serve as a pragmatic platform to functionally assess whether EGFR inhibition produces a meaningful antitumor effect in a specific patient, thereby informing therapeutic choices.

After establishing that GOs recapitulated *in silico* predicted sensitivity patterns observed in the corresponding parental tumors, we next leveraged this integrated workflow to nominate candidate therapies with potential activity in our cohort. Unbiased screening again showed strong concordance between predicted responses in tumors and matched GOs, reinforcing that the organoid transcriptomic state is sufficiently aligned to support comparative prediction. At the same time, the resulting sensitivity compound rankings differed across patients, as expected given the pronounced interpatient molecular heterogeneity that characterizes diffuse gliomas (83,233). Within this patient-specific landscape, three compounds consistently emerged with the top predicted sensitivity scores across the cohort, suggesting potential activity that is comparatively robust in all patients. These three compounds, alectinib, ruxolitinib, and dabrafenib were therefore selected for subsequent experimental validation.

Alectinib is a second-generation tyrosine kinase inhibitor targeting ALK, impeding its associated downstream proliferative and cell survival activity. It is effective against tumors with overexpression of ALK or ALK rearrangements, like those usually presented in NSCLC (315). Very relevant for its use in neuro-oncology is its very extensive BBB penetration capability (316). In the context of gliomas, some authors have reported high levels of ALK expression, which is suggested to promote glioma growth by favouring proliferation and neovascularization (317,318). These reports are aligned to the observed ALK expression values in our cohort compared to the average ALK expression in healthy brain and explain the *in silico* results. Preclinical research shows that alectinib can be effective against glioma cell lines, even those with acquired TMZ resistance (319). Though no glioma trial has utilized alectinib, it has shown encouraging results in neuroblastoma patients (320) and is currently being investigated in phase I/II studies for children and adolescents with treatment resistant CNS tumors (321).

DISCUSSION

Ruxolitinib is a JAK1/JAK2 inhibitor designed to suppress signaling through the JAK–STAT pathway (322). In gliomas, this axis is frequently engaged by inflammatory cytokines like the IL-6 family ligands, which activate JAKs and downstream STAT3, promoting transcriptional programs associated with proliferation/survival, invasion, angiogenesis, and stem-like phenotypes (323). In parallel, STAT3 signaling contributes to the characteristic immunosuppressive microenvironment of gliomas by supporting tumor-associated myeloid suppressive states and limiting effective T-cell activity (324). Inhibiting this pathway can therefore have a plethora of combined effects in gliomas. A recent clinical trial has demonstrated the safety of ruxolitinib in glioma patients, in combination with RT and TMZ, which proves the feasibility of incorporating it into clinical practice (325), though efficacy still remains unknown. Ruxolitinib has been shown to be effective in myeloproliferative tumors (326), with active clinical trials in pancreatic (327), breast (328) and NSCLC (329) as well.

Dabrafenib is a selective inhibitor of mutant BRAF, designed to suppress oncogenic signaling through the MAPK pathway (330). In clinical practice, dabrafenib is usually combined with MEK co-inhibition using trametinib, providing deeper MAPK pathway suppression and mitigating pathway reactivation mechanisms (331,332). In gliomas, the therapeutic relevance of dabrafenib as monotherapy is highly context-dependent, with the strongest activity observed in tumors harboring the activating BRAF V600E mutation (333). Such cases represent around 4% of all adult diffuse gliomas (334), limiting broad applicability outside molecularly selected cohorts. Several clinical trials have incorporated dabrafenib in both adult and pediatric *BRAF* mutant gliomas, usually in combination with trametinib. These trials have reported objective response rates that are substantially higher than those typically achieved with conventional chemotherapy (335–337). Together, these data support BRAF/MEK inhibition as an effective targeted alternative for appropriately stratified glioma patients.

In this thesis, we did not assess the *BRAF* status on our glioma cohort, which limits mechanistic interpretation of dabrafenib's prioritization by the *in silico* screening framework. Therefore, rather than inferring the presence of BRAF V600E, this recurrent hit is more conservatively interpreted as a signal of broad MAPK pathway activation or dependency captured by the transcriptomic state used for prioritization.

In vitro validation of these three compounds aligned with the *in silico* prioritization, as all exhibited marked antitumoral activity in our GOs and significantly impaired migratory behavior. Importantly, these effects were also reproduced in conventional glioma model systems, including established glioma cell lines and GSC cultures, supporting that the

observed responses are not restricted to a single experimental platform and may reflect broader antitumor activity across diverse glioma contexts.

In vivo validation provided an additional translational layer to the integrated screening pipeline. In both U87 and GO-derived heterotopic xenografts, alectinib and ruxolitinib produced a marked inhibition of tumor growth kinetics and reduced endpoint tumor size, supporting that the antitumoral effects observed *in vitro* can be maintained under organism-level constraints. The concordance between *in vitro* and *in vivo* efficacy for these two compounds strengthens the argument that the GO platform can nominate therapeutically relevant candidates and that the selected hits are not limited to short-term culture artifacts.

In contrast, dabrafenib did not produce a measurable antitumoral effect *in vivo*, despite exhibiting activity *in vitro*, indicating a divergence in response. This discordance can be interpreted through several mechanisms. First, the antitumoral effect observed *in vitro* may reflect partial MAPK pathway vulnerability that is insufficient to constrain tumor growth *in vivo*, where additional microenvironmental signals, growth factor availability, and clonal competition can buffer pathway inhibition. Second, pharmacologic factors may have limited effective target engagement *in vivo* even in a heterotopic setting where BBB constraints are minimized. Finally, MAPK pathway feedback and reactivation mechanisms are well described for RAF inhibition *in vivo* which do not play a major role in primary *in vitro* cultures due to the shorter experimental timelines *in vitro*.

In this thesis, we have developed and validated an integrated precision-oncology framework centered on patient-derived GOs that aims to bridge biologically faithful modeling with actionable therapeutic prioritization. Across a multi-institutional cohort, GOs could be established at high efficiency and recapitulated key tumoral features across histopathology, ultrastructure, cellular composition, and transcriptomic programs, including preservation of clinically defining alterations such as IDH1 R132H when present. We demonstrated that predicted *in silico* sensitivities were highly concordant between parental tumors and matched GOs and that GOs reproduced clinically familiar resistance patterns to SOC agents. Unbiased screening then identified candidate therapies with subsequent *in vitro* and *in vivo* validation.

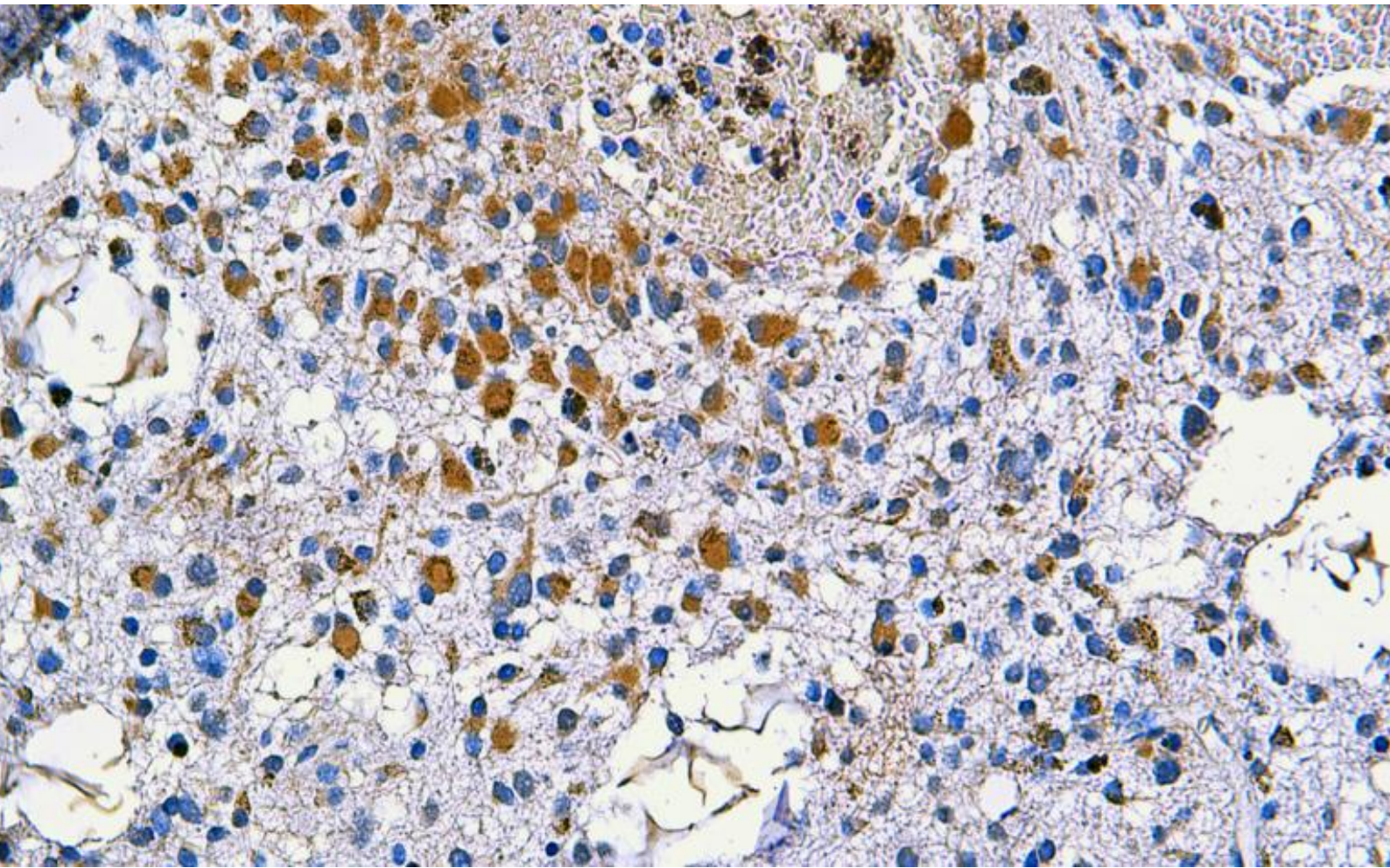
Several limitations define the boundaries of interpretation. Organoid systems remain *ex vivo* models that incompletely reproduce the full CNS niche, including BBB constraints and systemic immune dynamics, and some microenvironmental components may be transient or variably retained. In addition, *in vivo* results highlighted that *in vitro* antitumoral activity does not guarantee therapeutic efficacy, emphasizing the need for

DISCUSSION

target confirmation, on-target pathway modulation, and pharmacologic considerations when translating candidates across model layers. These limitations do not diminish the utility of GOs; rather, they motivate a layered modeling strategy in which GOs provide patient-matched functional evidence that is subsequently refined and validated in appropriate *in vivo* systems.

Overall, this work supports GOs as a practical and biologically relevant component of precision-oncology pipelines, enabling functional prioritization of therapies under patient-specific constraints. Future efforts should focus on optimizing biobanking and orthotopic engraftment, validating therapies in human immune competent *in vivo* systems, and prospectively testing GO prioritization in clinical workflows. Collectively, the framework presented here provides a foundation for more reliable preclinical to clinical translation in adult diffuse gliomas and nominates alectinib and ruxolitinib for further clinical assessment.

7. CONCLUSIONS



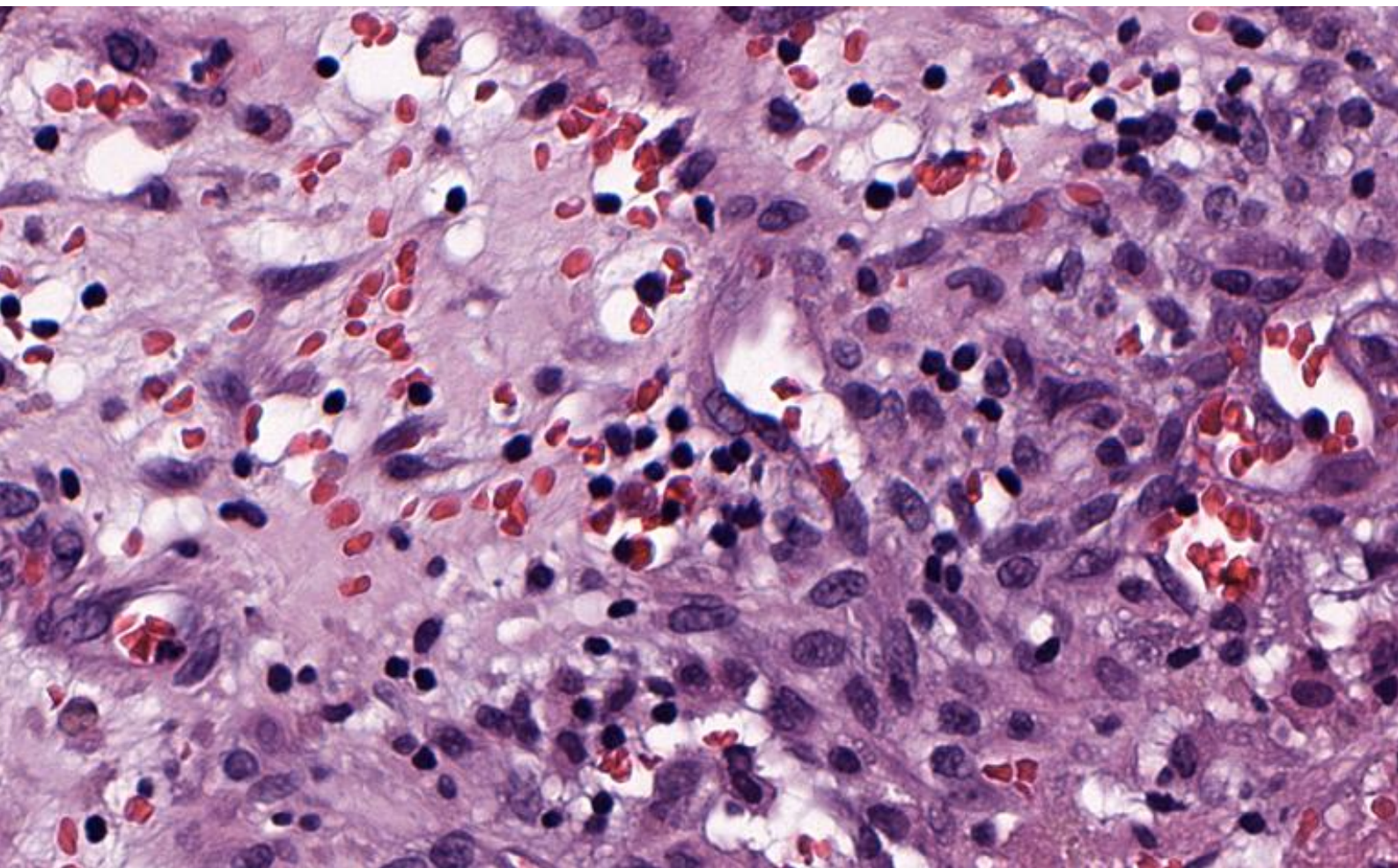
“No temáis, pues el mismo Espíritu que levantó a Jesús de entre los muertos habita ahora en vosotros”

- San Pablo.

Collectively, this thesis establishes a precision oncology workflow that integrates high-fidelity patient-derived GOs with computational drug prioritization and preclinical validation, providing a scalable framework with potential relevance for precision medicine approaches in currently incurable gliomas. From this general conclusion, we can draw the following specific conclusions:

1. GOs were successfully established from both IDH-wildtype GBM and IDH-mutant gliomas.
2. GOs recapitulate key parental features, including histopathological hallmarks, ultrastructural architecture, cellular composition, a myeloid-dominant immune landscape, and major molecular/transcriptional patterns.
3. GOMuts preserve the clinically relevant IDH1 R132H mutation, presenting themselves as a promising platform to study IDH-mutant gliomas *in vitro*.
4. GO biobanking maintained histopathological identity but substantially impaired post-thaw proliferative recovery *in vitro*, defining an important practical limitation for downstream assays.
5. Subcutaneous xenografts of GOs produced heterotopic tumors which recapitulate core parental tumor features, supporting preservation of tumorigenic capacity.
6. *In silico* screening in GOs and parental tumors yielded comparable sensitivity scores and gave high sensitivity scores to alectinib, ruxolitinib, and dabrafenib which were selected for functional testing.
7. *In vitro* testing in GOs and glioma cells confirmed antitumoral activity of the prioritized compounds and demonstrated modulation of tumor-relevant programs/pathways.
8. *In vivo* testing in heterotopic xenografts showed that alectinib and ruxolitinib significantly reduced tumor growth and endpoint tumor burden, supporting their prioritization for further translational evaluation.

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“Si he podido ver más lejos es porque estaba a hombros de gigantes”

- Isaac Newton.

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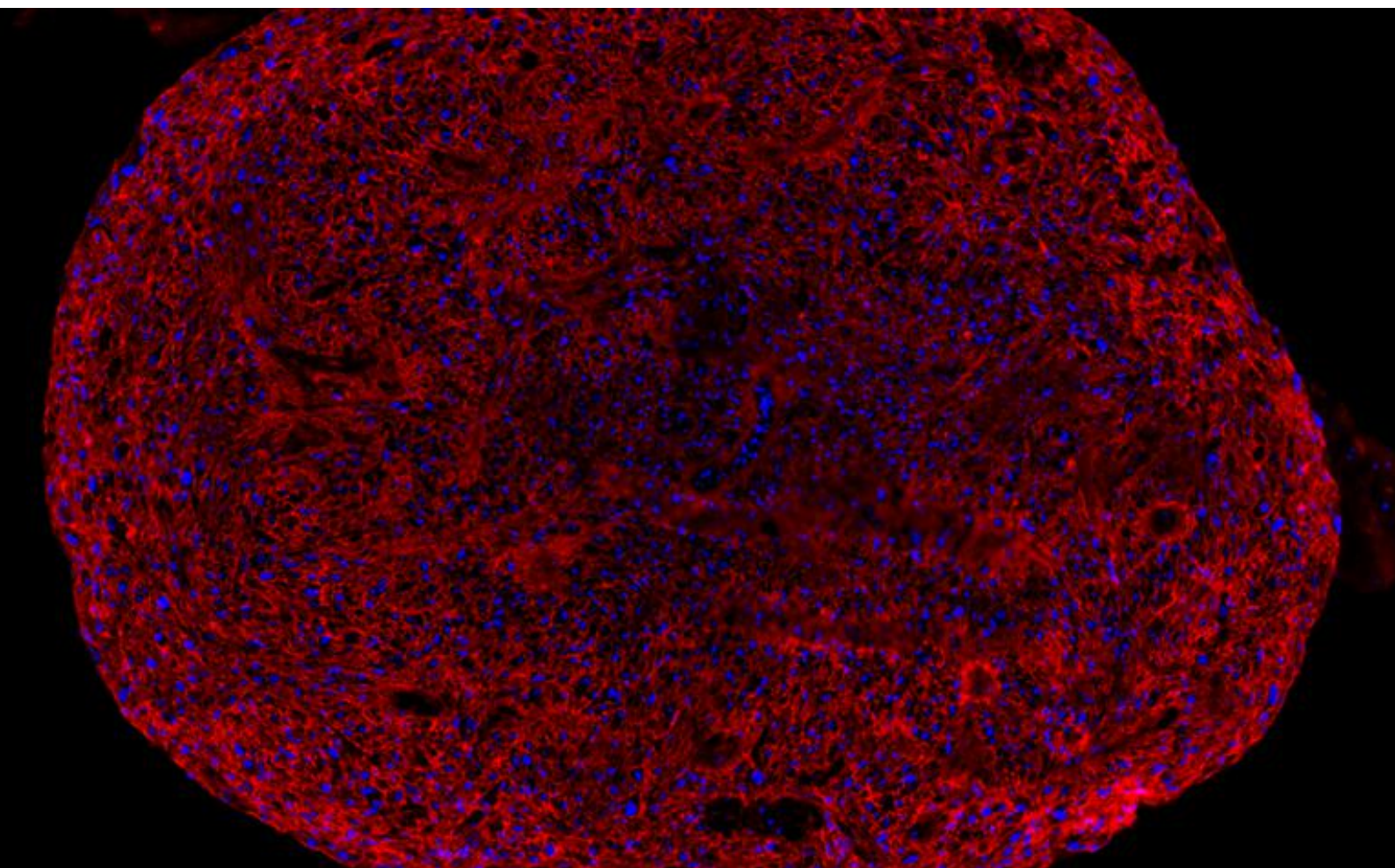
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9. ANNEXES



Annex I: Additional Figures

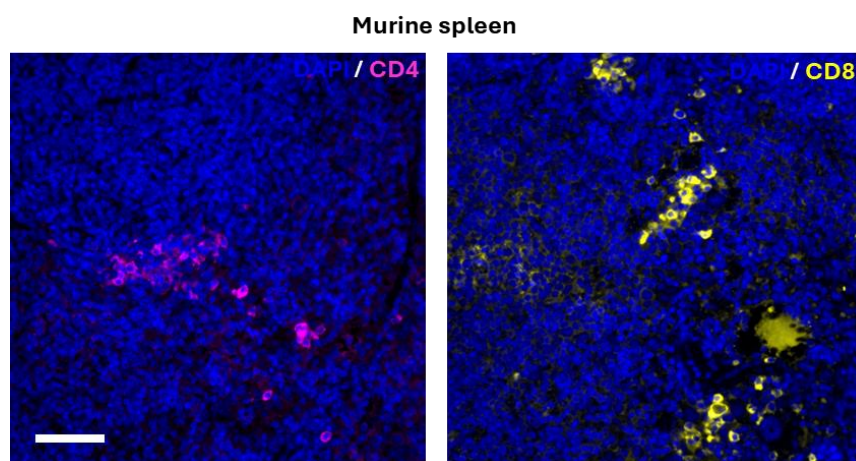


Figure Annex IA: CD4 and CD8 antibodies validation in murine spleen. Representative IF images for CD4 (magenta) and CD8 (yellow) in murine spleens. Counterstained with DAPI in blue. Scale bar: 100 μ m.

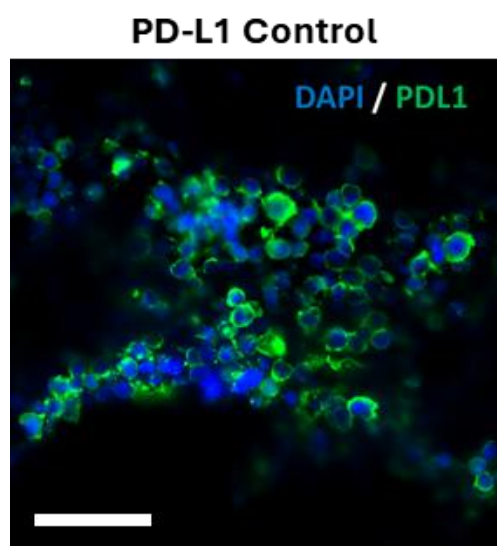


Figure Annex IB: PD-L1 antibody reactivity validation in positive sample. Representative IF image of PD-L1 in green counterstained with DAPI in blue in a PD-L1 positive sample. Scale bar: 100 μ m.

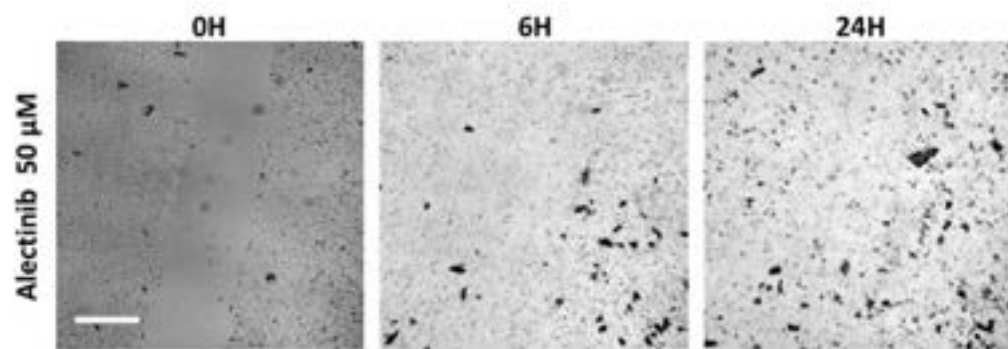


Figure Annex IC: Alectinib 50 μ M induces high cellular toxicity and impedes monitoring of wound healing assay. Representative brightfield images from alectinib treated cell monolayers at the time of injury, 6 h and 24 h. Scale bar is 500 μ m..

Annex II: Written informed consent provided to patients

HOJA DE INFORMACION AL PACIENTE Y CONSENTIMIENTO INFORMADO ADULTOS

TÍTULO DEL ESTUDIO: Predicción de respuesta a inmunoterapia en pacientes con GBM basado en su perfil inmune, e identificación de biomarcadores en biopsia líquida.

INVESTIGADOR PRINCIPAL:

Nombre: Angel Ayuso Sacido

Cargo: Director Científico en Vithas Hospital, director gerente en Fundación Vithas y director del laboratorio de tumores cerebrales Fundación Vithas-Universidad Francisco de Vitoria.

Afiliación: Fundación Vithas

Dirección: Arturo Soria 107, 28043 Madrid

PROMOTOR: Fundación Vithas

Nº DE VERSIÓN Y FECHA: V02, 27 de Junio de 2023

1. INTRODUCCIÓN

Nos dirigimos a Ud. para informarle sobre un proyecto de investigación, aprobado por el Comité Ético de Investigación con medicamentos (CEIm/CEIC) de HM Hospitales, en el que se le invita a participar.

Nuestra intención es que Ud. reciba la información correcta y suficiente para que pueda evaluar y decidir, si quiere o no participar en este estudio.

Para ello le ruego lea esta hoja informativa con atención, pudiendo consultar con las personas que considere oportuno, y nosotros le aclararemos las dudas que le puedan surgir.

2. PARTICIPACIÓN VOLUNTARIA

Debe saber que su participación en este estudio es voluntaria, y que puede decidir no participar, sin que por ello se altere la relación con su médico ni produzca perjuicio alguno en su tratamiento.

3. DESCRIPCIÓN GENERAL DEL ESTUDIO

Los gliomas malignos son los tumores cerebrales más agresivos en adultos. En la última década parece que hay un nuevo enfoque que ha funcionado con éxito en otros tumores, como por ejemplo en melanoma metastásico y/o en cáncer de pulmón de células no pequeñas. Éstos nuevos tratamientos intenta ayudar al propio sistema inmune a luchar contra el tumor. En Glioblastoma (GBM), un tipo de tumor cerebral, los ensayos clínicos que prueban este enfoque han fallado. Sin embargo, casos aislados de GBM han mostrado una importante respuesta positiva, lo que sugiere que algunos pacientes podrían beneficiarse de esta terapia. Por lo tanto, es importante encontrar biomarcadores que nos permitan identificar a aquellos pacientes que podrían beneficiarse de dicha terapia.

En el presente proyecto, nuestro objetivo es identificar biomarcadores que nos permitan saber que pacientes diagnosticados con GBM pudieran beneficiarse de la inmunoterapia.

Para conseguir nuestro objetivo, analizaremos grandes cohortes públicas y gratuitas de datos de pacientes con GBM para identificar los subgrupos de pacientes que potencialmente podrían beneficiarse de esta terapia. Después, evaluaremos los resultados obtenidos, en modelos creados en el laboratorio a partir de muestras de tejido tumoral procedente de pacientes con GBM, y modelos de ratón, con moléculas dirigidas a las células inmunes en el entorno del tumor, ya utilizadas en ensayos clínicos para GBM. Además, analizaremos la sangre de los modelos animales tratados con esta terapia donde podremos estudiar la progresión de estos biomarcadores a lo largo de la enfermedad. Para posteriormente, identificarlos en muestras de sangre de pacientes.

Para este proyecto se recogerán muestras biológicas de tejido de tumor cerebral y de sangre periférica. Dado que Ud. va a someterse a un procedimiento de cirugía, en caso de que usted lo permita, pretendemos guardar parte de esta muestra para emplearla en dicho proyecto de investigación, cuya finalidad es la siguiente:

1. Crear modelos de laboratorio, a partir de muestras de tejido tumoral, para estudiar los tumores cerebrales
2. Evaluar la actividad de nuevas terapias dirigidas al sistema inmune
3. Buscar nuevos biomarcadores que nos permitan identificar aquellos pacientes que se pueden beneficiar de dichas terapias.

Su participación en este estudio conlleva utilización de muestras biológicas con fines de investigación, para lo que se observará la Ley 14/2007 de investigación biomédica y el Real Decreto 1716/2011, normativas que garantizan el respeto a los derechos que le asisten. Al firmar este documento, revisado y evaluado favorablemente por el Comité de Ética de la Investigación con medicamentos que ha aprobado este estudio, usted acepta que se utilicen sus muestras para las finalidades del presente estudio.

4. BENEFICIOS Y RIESGOS DERIVADOS DE SU PARTICIPACIÓN EN EL ESTUDIO.

Los inconvenientes y riesgos.

El inconveniente deriva de la toma de la muestra de biopsia líquida (extracción de sangre por punción venosa). La obtención de muestras de sangre por punción venosa puede provocar dolor, sangrado, hematoma o inflamación de la zona de inserción de la aguja.

Salvo lo indicado anteriormente, el estudio no supone riesgo adicional para Ud.

Los beneficios.

Ud. no se beneficiará directamente del estudio. Sin embargo, los resultados derivados del estudio podrían ser de gran utilidad en la elaboración de nuevos tratamientos contra tumores cerebrales en el futuro y, por lo tanto, de un enorme beneficio para la sociedad.

5. COMPENSACIÓN ECONÓMICA

Usted no recibirá compensación económica alguna por su participación en este proyecto de investigación. Su participación en el proyecto de investigación no le supondrá ningún gasto extra con respecto a lo que le supondría si no participara en el mismo.

6. CONFIDENCIALIDAD Y PROTECCION DE DATOS

Los datos personales que se recojan serán obtenidos, tratados y almacenados cumpliendo en todo momento el deber de secreto, de acuerdo con la legislación vigente en materia de protección de datos de carácter personal, Reglamento (UE) 2016/679 del Parlamento Europeo y del Consejo de 27 de abril de 2016 de Protección de Datos (RGPD). Asimismo, aunque los resultados obtenidos de la investigación realizada con sus datos se publiquen en revistas científicas, su identidad no será facilitada.

De acuerdo a lo que establece la legislación mencionada, sus datos personales serán tratados con la finalidad de participar en el Proyecto de investigación indicado en el documento objeto de firma, siendo su causa de legitimación el cumplimiento de las obligaciones legales en relación con el interés general en llevar a cabo investigaciones científicas; y la mejora y garantía de los niveles de calidad y seguridad.

El promotor es responsable del tratamiento de sus datos y se compromete a cumplir con la normativa de protección de datos en vigor. Los datos clínicos serán tratados con la única y exclusiva finalidad de llevar a cabo la

investigación biomédica que usted haya autorizado en el marco del proyecto antes descrito. Los datos recogidos para el estudio estarán identificados mediante un código, de manera que no se incluya información que pueda identificarle, y sólo los investigadores /colaboradores podrán relacionar dichos datos con usted y con su historia clínica. Por lo tanto, su identidad no será revelada a ninguna otra persona salvo a las autoridades sanitarias o los Comités de Ética de la Investigación.

En cualquier momento podrá ejercer los derechos de acceso, rectificación, supresión, portabilidad, limitación y oposición al tratamiento de datos, dirigiéndose al investigador principal del estudio. Le recordamos que los datos no se pueden eliminar, aunque deje de participar en el estudio para garantizar la validez de la investigación. Así mismo tiene derecho a dirigirse a la Agencia de Protección de Datos si no quedara satisfecho.

En caso de que se quieran utilizar los datos obtenidos en el presente estudio para investigaciones futuras (con objetivos diferentes a los del presente estudio) se solicitará, en ese momento el consentimiento del paciente o representante legal del paciente, utilizando una hoja informativa independiente, previamente aprobada por el Comité Ético de Investigación (CEIm).

7. FINALIDAD DEL DOCUMENTO

Este documento tiene como objeto solicitar su autorización para la realización de pruebas genéticas en sus muestras biológicas.

En cumplimiento de la normativa vigente (Artículo 47 de la Ley 14/2007 de Investigación Biomédica) Ud. da su consentimiento informado para la realización del estudio genético indicado, y declara entender y aceptar los siguientes aspectos:

La naturaleza del estudio consiste en:

- Desarrollo de nuevas técnicas de diagnóstico y seguimiento de tumores basados en el análisis de moléculas analizadas a partir de tejido tumoral y un simple análisis de sangre.
- Identificar marcadores y perfiles de sensibilidad a fármacos relacionados con el tratamiento de tumores cerebrales a partir de biopsias de tejido y un análisis de sangre.

Dicho análisis será realizado en el laboratorio de Investigación dirigido por el Dr. Ángel Ayuso y situado en los laboratorios de la Facultad de Ciencias Experimentales, , localizado en el campus de Universidad Francisco de Vitoria. Las muestras biológicas serán entregadas al Dr. Angel Ayuso Sacido, en el caso de las muestras de tejido, en el interior de un frasco de plástico estéril, en suero

salino y con hielo para mantenerlas a 4°C; en el caso de las muestras de sangre periférica, en tubos separadores de suero de 10 ml. El Dr. Angel Ayuso-Sacido custodiará las muestras en las instalaciones de la Facultad de Ciencias Experimentales de la UFV hasta su uso y/o destrucción, de acuerdo a la normativa para el tratamiento y destrucción de muestras humanas. Las muestras de sangre periférica se utilizarán para la extracción de suero y se mantendrán hasta la finalización del proyecto. Las muestras sobrantes del estudio serán destruidas de acuerdo con la normativa para la eliminación de residuos biológicos/ o bien, si usted está de acuerdo, la posible muestra sobrante se guardará para futuros estudios de investigación, para lo cual se le facilitará un consentimiento específico.

Únicamente el Investigador Principal, personal sanitario debidamente autorizado por HM hospitales, Vithas HOSPITALES, Hospital La Fe de Valencia, Hospital Gregorio Marañón, Hospitales Quirón, Hospital Puerta de Hierro, Hospital de Móstoles y Hospital de Alcorcón y los facultativos solicitantes podrán acceder a los datos personales y a los resultados de las pruebas genéticas.

Los resultados de estas determinaciones no tendrán influencia en el manejo clínico de su enfermedad, ni tendrán implicaciones para miembros de su familia, por lo tanto, no se suministrará consejo genético una vez obtenidos y evaluados los resultados del análisis.

CONSENTIMIENTO INFORMADO

Título del estudio:

Predicción de respuesta a inmunoterapia en pacientes con GBM basado en su perfil inmune, e identificación de biomarcadores en biopsia líquida.

Centro del estudio: Universidad Francisco de Vitoria, con la colaboración de Vithas hospitales, HM hospitales, Hospital La Fe de Valencia, Hospital Gregorio Marañón, Hospitales Quirón, Hospital Puerta de Hierro, Hospital de Móstoles y Hospital de Alcorcón

Yo, <<nombre y apellidos del participante>>

- ☐ He leído la hoja de información que se me ha entregado sobre el estudio.
- ☐ He podido hacer preguntas sobre el estudio.
- ☐ He recibido suficiente información sobre el estudio.
- ☐ He hablado con <<nombre del investigador>>
- ☐ Comprendo que mi participación es voluntaria.
- ☐ Comprendo que puedo retirarme del estudio:
- Cuando quiera.

- Sin tener que dar explicaciones.
- Sin que esto repercuta en mis cuidados médicos.

Recibiré una copia firmada y fechada de este documento de consentimiento informado Presto libremente mi conformidad para participar en el estudio.

Firma del participante

Firma del investigador

Fecha: ____/____/____

Fecha:

____/____/____

(Nombre, firma y fecha de puño y letra por el paciente)

Firma del representante legal,

Firma del investigador

Fecha: ____/____/____

Fecha: ____/____/____

(Nombre, firma y fecha de puño y letra por el paciente)

(Nombre, firma y fecha de puño y letra por el paciente).

Annex III: Papers authored by the PhD candidate during his thesis period



Caspase-Mediated Regulation and Cellular Heterogeneity of the cGAS/STING Pathway in Kaposi's Sarcoma-Associated Herpesvirus Infection

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Tate Tabtieng and Rachel C. Lent contributed equally to this work. Author order is based on the fact that Tate Tabtieng initiated the project and Rachel Lent completed it.

ABSTRACT As a result of the ongoing virus-host arms race, viruses have evolved numerous immune subversion strategies, many of which are aimed at suppressing the production of type I interferons (IFNs). Apoptotic caspases have recently emerged as important regulators of type I IFN signaling both in noninfectious contexts and during viral infection. Despite being widely considered antiviral factors since they can trigger cell death, several apoptotic caspases promote viral replication by suppressing innate immune response. Indeed, we previously discovered that the AIDS-associated oncogenic gammaherpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV) exploits caspase activity to suppress the antiviral type I IFN response and promote viral replication. However, the mechanism of this novel viral immune evasion strategy is poorly understood, particularly with regard to how caspases antagonize IFN signaling during KSHV infection. Here, we show that caspase activity inhibits the DNA sensor cGAS during KSHV lytic replication to block type I IFN induction. Furthermore, we used single-cell RNA sequencing to reveal that the potent antiviral state conferred by caspase inhibition is mediated by an exceptionally small percentage of IFN- β -producing cells, thus uncovering further complexity of IFN regulation during viral infection. Collectively, these results provide insight into multiple levels of cellular type I IFN regulation that viruses co-opt for immune evasion. Unraveling these mechanisms can inform targeted therapeutic strategies for viral infections and reveal cellular mechanisms of regulating interferon signaling in the context of cancer and chronic inflammatory diseases.

IMPORTANCE Type I interferons are key factors that dictate the outcome of infectious and inflammatory diseases. Thus, intricate cellular regulatory mechanisms are in place to control IFN responses. While viruses encode their own immune-regulatory proteins, they can also usurp existing cellular interferon regulatory functions. We found that caspase activity during lytic infection with the AIDS-associated oncogenic gammaherpesvirus Kaposi's sarcoma-associated herpesvirus inhibits the DNA sensor cGAS to block the antiviral type I IFN response. Moreover, single-cell RNA sequencing analyses unexpectedly revealed that an exceptionally small subset of infected cells (<5%) produce IFN, yet this is sufficient to confer a potent antiviral state. These findings reveal new aspects of type I IFN regulation and highlight caspases as a druggable target to modulate cGAS activity.

KEYWORDS Kaposi's sarcoma-associated herpesvirus, cGAS, caspases, heterogeneity, interferons

Editor Blossom Damania, University of North Carolina, Chapel Hill

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The authors declare no conflict of interest.

Received 30 August 2022

Accepted 19 September 2022

Published 18 October 2022

Type I interferon (IFN) cytokines are potently antiviral and play an essential role in controlling infections and starting systemic immune responses. Type I IFNs are generally turned on during viral infection after sensing of viral nucleic acids by cellular pattern recognition receptors, such as the cytosolic DNA sensor cGAS and the RNA sensor RIG-I (1). These sensors signal through adaptor proteins (STING and MAVS, respectively) and the kinases TBK1 and IKK ϵ to activate the transcription factor IRF3 and trigger induction of type I IFN cytokines (1). Type I IFNs in turn stimulate expression of interferon-stimulated genes (ISGs) in an autocrine and paracrine manner to limit viral replication and infection, as well as alerting the immune system to the infection (2). As ISGs create a strong antiviral response, all viruses have evolved one or more strategies to block IFN induction (3). The AIDS-associated oncogenic gammaherpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV), which is the etiological agent of one of the leading causes of cancer death in sub-Saharan Africa, Kaposi's sarcoma, is a prime example of this. KSHV induces minimal type I IFN responses when reactivating from latency (4, 5), demonstrating that it can strongly block this response.

While many virus-encoded factors directly target the type I IFN response, viruses like KSHV have also evolved strategies to usurp cellular pathways to counteract immune responses. We recently established that KSHV exploits the activity of the apoptotic caspases to suppress the type I IFN response and promote viral replication (4). Indeed, while KSHV lytic reactivation normally does not induce a strong type I IFN response, lytically infected cells express and secrete high levels of IFN- β if caspases are inhibited (4). Although apoptotic caspases are canonically thought to be antiviral by facilitating cell death of infected cells, multiple recent studies have demonstrated caspase-dependent inhibition of innate immune signaling during apoptosis and viral infection (4, 6–8). However, it remained unclear from our previous studies how caspases enact this IFN-regulatory function during KSHV infection. In particular, while caspase-3 and -7 can directly cleave cGAS, MAVS, and IRF3 to block type I IFN signaling (8), our previous findings suggest that in the case of KSHV infections, caspase-8 is the major caspase suppressing the type I IFN response (4). Moreover, caspase-8 appears to act independently of caspase-3/7 activation and of cell death to regulate IFNs in KSHV-infected cells (4). As different caspases have different proteolytic targets, these results suggest that the mechanism we found in KSHV-infected cells may also be different from what has previously been reported.

To decipher how caspases disrupt IFN signaling during KSHV infection, we used RNA interference (RNAi)-mediated depletion and pharmacological inhibition to identify which IFN signaling components are targeted by caspase activity. This revealed that caspase activity specifically inhibits cGAS to impede IFN signaling during KSHV lytic infection. We also performed single-cell RNA sequencing (scRNA-Seq) to examine the heterogeneity of immune responses and viral gene expression during KSHV lytic infection. This uncovered considerable heterogeneity of the KSHV lytic cycle. Moreover, it surprisingly revealed that a small percentage of infected cells produce IFN and suggested that differences in cellular gene expression may account for this heterogeneity.

RESULTS

Caspase activity blocks cGAS/STING signaling during KSHV lytic infection. We previously showed that the type I IFN response that occurs upon caspase inhibition in KSHV-infected cells is dependent on the kinase TBK1 (4). TBK1 can be activated by several pathogen sensors, including sensors of viral nucleic acids (1), and both the DNA- and the RNA-sensing pathways can be activated during KSHV infection (5, 9–15). To discern which pathogen-sensing pathway is inhibited by caspase activity during KSHV infection, we knocked down key proteins for DNA and RNA sensing: the DNA sensors cGAS and IFI16 and their signaling adaptor STING, and the RNA sensor RIG-I and its signaling adaptor MAVS (Fig. 1A to C; also, see Fig. S1 in the supplemental material). For these experiments, we used iSLK.219 cells, which are latently infected with recombinant rKSHV.219. rKSHV.219 expresses green fluorescent protein (GFP) constitutively and red

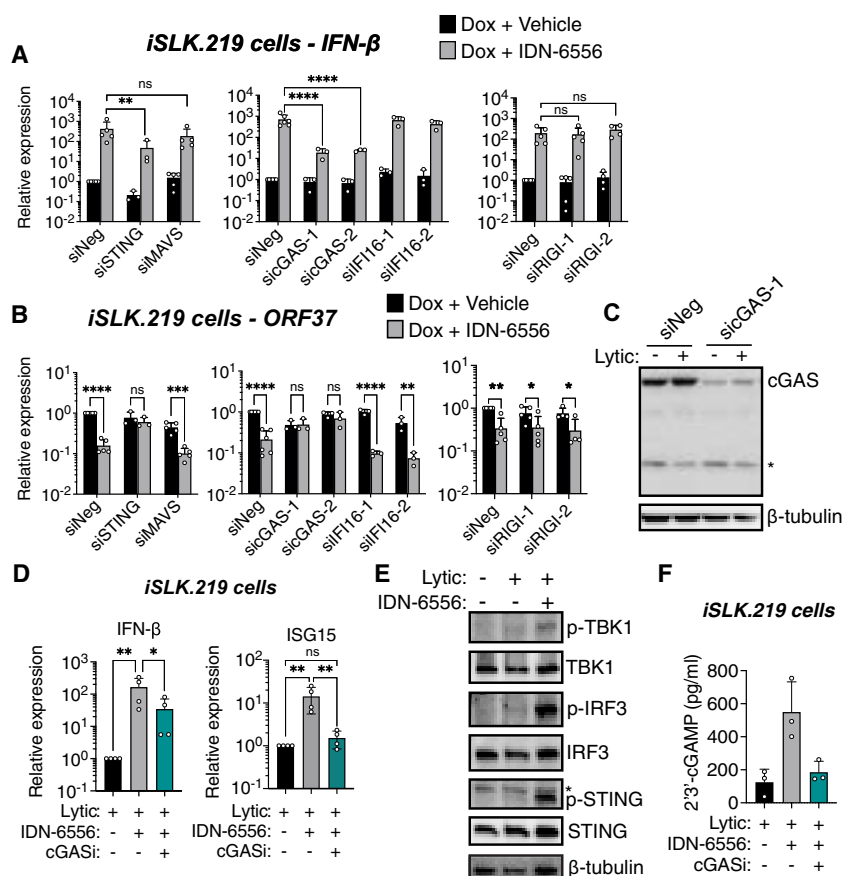


FIG 1 Caspase activity prevents cGAS activation during KSHV lytic replication to block IFN- β induction. (A to C) iSLK.219 cells were transfected with a negative-control siRNA or siRNAs targeting the indicated proteins. For cGAS and RIG-I, the transfection was carried out twice, 2 days prior to and on the day of lytic cycle induction; for STING, MAVS, and IFI16, one transfection was carried out 2 days prior to induction. The cells were then lytically reactivated with doxycycline (1 μ g/mL) and treated with either dimethyl sulfoxide (DMSO, vehicle) or IDN-6556 (10 μ M) as indicated. (A and B) Total RNA was extracted at day 5 postreactivation, and the levels of IFN- β (A) and ORF37 (B) mRNA were measured by RT-qPCR and normalized to levels of 18S rRNA ($n \geq 3$). (C) Cell lysates were harvested at day 4 postreactivation and subjected to Western blotting for cGAS and β -tubulin as a loading control. The asterisk indicates a nonspecific band. Blots are representative of 3 replicates. (D to F) iSLK.219 cells were lytically reactivated with doxycycline (1 μ g/mL) and treated with either DMSO (vehicle) or IDN-6556 (10 μ M) and the cGAS inhibitor RU.521 (cGASi; 24.1 μ M), where indicated. (D) Total RNA was extracted from iSLK.219 cells 3 days after reactivation. Levels of IFN- β mRNA were measured by RT-qPCR and normalized to 18S rRNA ($n = 3$). (E) Cell lysates were harvested at day 4 postreactivation and subjected to Western blotting for p-TBK1, TBK1, p-IRF3, IRF3, p-STING, STING, and β -tubulin (as a loading control) as indicated. The asterisk indicates a nonspecific band. Blots are representative of 3 replicates. (F) Levels of 2',3'-cGAMP in lysate collected from iSLK.219 cells at day 4 postreactivation were measured by ELISA ($n = 4$). ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (Tukey's multiple-comparison test after two-way ANOVA).

fluorescent protein (RFP) under the control of a KSHV lytic promoter (16). These cells also express an exogenous copy of the KSHV master lytic regulator replication and transcription activator (RTA) under the control of a doxycycline-inducible promoter, so that reactivation of the viral lytic replication cycle can be induced by doxycycline treatment (16). IFN- β induction upon caspase inhibition was severely reduced by STING and cGAS depletion, suggesting that caspase activity targets STING-dependent type I IFN signaling during KSHV replication (Fig. 1A). Moreover, upon STING and cGAS knockdown, caspase inhibitors no longer caused a reduction in KSHV replication, which we previously showed is a result of type I IFN induction (4) (Fig. 1B). In contrast, IFI16, RIG-I, and MAVS knockdowns had minimal effects on IFN- β induction and viral gene expression in the presence of caspase inhibitors (Fig. 1A and B). These results suggest that cGAS/STING-mediated

DNA sensing is disabled by caspase activity. To corroborate this conclusion, we also tested pharmacological inhibition of cGAS enzymatic activity with RU.521. When bound to DNA, cGAS synthesizes the cyclic dinucleotide 2',3'-cyclic GAMP (2',3'-cGAMP), which in turn binds and activates STING (17). Consistent with our knockdown results, cGAS inhibition reduced induction of IFN- β and of the interferon-stimulated gene ISG15 upon caspase inhibition (Fig. 1D). Collectively, these findings suggest that caspase activity prevents activation or signal transduction of the cGAS/STING DNA-sensing pathway during KSHV lytic replication.

Caspases inhibit cGAS activity without reducing cGAS protein levels. To determine how caspases interfere with cGAS/STING signaling, we tested whether there was differential activation of various effectors in the cGAS/STING pathway in the presence versus absence of caspase activity. We observed increased phosphorylation of the downstream proteins STING, TBK1, and IRF3, which indicates that they are activated upon caspase inhibition (Fig. 1E). These results suggest that activation of the entire pathway is blocked by caspases. Indeed, we also found that 2',3'-cGAMP levels were increased upon caspase inhibition and were reduced by treatment with a cGAS inhibitor (Fig. 1F). Taken together with our results from the knockdown experiments, these findings identify the target of caspases as a component or regulator of the cGAS/STING pathway that acts to promote activation or activity of cGAS.

We considered the possibility that the target was cGAS itself. One report suggests that caspase-3 and -7 can cleave cGAS during apoptosis and other viral infections (8). However, we have found that the activity of these caspases is dispensable for IFN regulation during KSHV lytic infection, and that caspase-8 is likely the key regulator (4). Also, we do not detect an overall reduction in cGAS levels nor any cGAS cleavage fragments during lytic reactivation (Fig. 1C). Ectopic coexpression of caspase-8 and cGAS also did not reveal caspase cleavage fragments, even though caspase-8 overexpression also leads to activation of caspase-3 and -7 (data not shown). It is thus likely that during KSHV lytic infection, caspases do not cleave cGAS directly but instead target a regulatory factor that modulates its activity.

Caspase activity also regulates cGAS in KSHV-infected primary effusion lymphoma B cells. To determine if caspases also suppress the cGAS/STING pathway in a cell type that is physiologically relevant for KSHV infection and tumorigenesis, we examined the activity of caspases in BC3 cells, a KSHV-infected B cell line derived from a primary effusion lymphoma (PEL) patient (18). Similarly to iSLK.219 and BCBL1 cells (4), caspase-8 was also activated upon lytic reactivation of BC3 cells (Fig. 2A). Moreover, caspase inhibition increased expression of both IFN- β and ISGs (ISG15 and IFIT1) (Fig. 2B), as well as IRF3 phosphorylation (Fig. 2C). Consistent with what we observed in iSLK.219 cells, caspase inhibition potentiated 2',3'-cGAMP production in lytically reactivated BC3 cells (Fig. 2D). Moreover, inhibition of cGAS with RU.521 reduced the induction of IFN- β (Fig. 2E). These results strengthen our conclusion that caspases have a major role in inhibiting cGAS activity or activation to block IFN induction during KSHV lytic infection in multiple cell types. These include cell types that are relevant to KSHV infection in humans and KSHV-associated diseases.

scRNA-Seq highlights heterogeneity of KSHV reactivation and confirms the proviral role of caspase activity. The results so far provide a bulk analysis of the IFN pathway during KSHV infection. However, not all iSLK.219 cells in the population re-enter the lytic cycle upon doxycycline addition (16), suggesting that there could be significant cell-to-cell variability in responses to infection. To understand both lytic reactivation and IFN signaling on a single-cell basis, we carried out scRNA-Seq on uninfected cells, latently infected cells, lytically infected cells, and lytically infected cells treated with IDN-6556 (Fig. 3A; Table S1). Uninfected cells are SLK cells containing the inducible KSHV RTA transgene but no rKSHV.219 (iSLK.RTA) (16). We also included lytically infected cells treated with IDN-6556 and anti-IFN antibodies to block autocrine and paracrine signals (Fig. 3A; Fig. S2A; Table S1). Analysis using the Seurat package (19, 20) unbiasedly identified 13 clusters with different gene expression across the 4 samples (Fig. 3B). Analysis of the mapped reads showed that, as expected, there was a

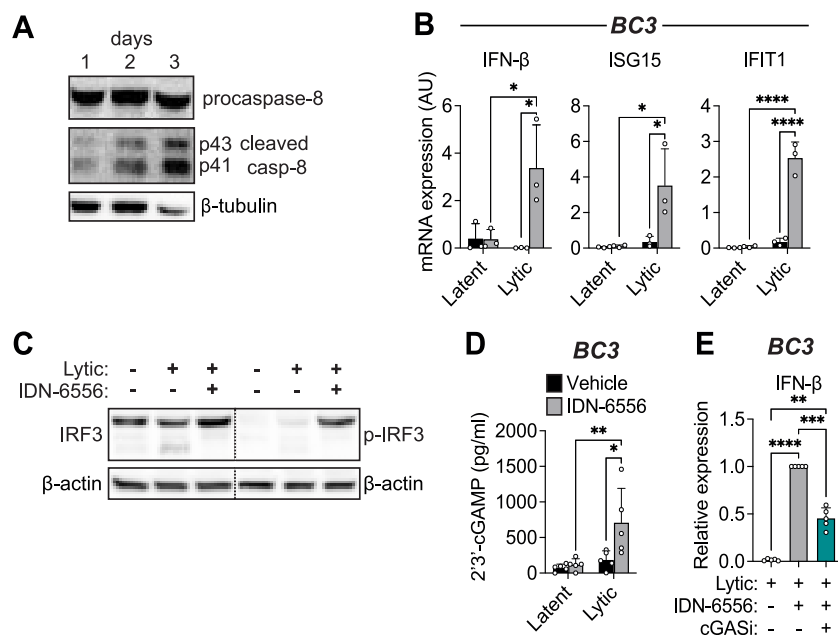


FIG 2 Caspases also inhibit cGAS activity in KSHV-infected B cells. BC3 cells were lytically reactivated with 12-O-Tetradecanoylphorbol-13-acetate (TPA) (20 ng/mL) for 1 to 3 days as indicated (A) or for 2 days (B to E) and treated with DMSO (vehicle), the pan-caspase inhibitor IDN-6556 (10 μ M), and/or the cGAS inhibitor RU.521 (cGASi, 24.1 μ M) as indicated. (A) Cell lysates were collected from latent and lytically reactivated BC3 cells at the indicated time points and subjected to Western blot analysis for activated (cleaved) caspase-8, and β -tubulin as a loading control. Blots are representative of 3 replicates. (B) Total RNA was extracted from BC3 cells. Levels of the indicated mRNAs were measured by RT-qPCR and normalized to 18S rRNA ($n = 3$). (C) Cell lysates were collected from lytically reactivated BC3 cells and subjected to Western blot analysis for activated p-IRF3, total IRF3, and β -actin as a loading control. The lower band for total IRF3 is likely a caspase-3 product as described by Aresté et al. (52). Blots are representative of 3 replicates. (D) Levels of 2',3'-cGAMP in lysate collected from BC3 were measured by ELISA ($n = 5$). (E) Total RNA was extracted from BC3 cells. Levels of IFN- β mRNA were measured by RT-qPCR and normalized to 18S rRNA ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (Tukey's multiple-comparison test after two-way ANOVA).

higher percentage of KSHV reads in cells that were lytically reactivated (Fig. 3C and D). Certain clusters were clearly enriched in KSHV transcripts (Fig. 3C and E; Fig. S2B) and these corresponded to clusters that appeared only in the lytic samples (Fig. 3B and C). Analysis of the cell cycle status indicated that most of the cells were in G_1 , but some of the clusters were enriched in cells in S or G_2 /M phase. This tended to correlate with lower viral gene expression, presumably indicative of lower reactivation (Fig. S2C).

We further analyzed the type of genes that were seen in the different clusters in the lytic infection+vehicle sample. We saw that cells from multiple clusters had on average 5 to 30% of reads mapping to KSHV genes, while more than 75% of the reads mapped to KSHV genes in cells from cluster 12 (Fig. 3E and F; Fig. S2B). Because our scRNA-Seq is directed at the 3' end of RNAs and many mRNAs in KSHV have the same 3' end (21, 22), we were not able to conclusively distinguish expression of all the viral genes. Therefore, in the heat maps, we list together all the open reading frames (ORFs) that each 3' end corresponds to. Nonetheless, analysis of viral genes and of the rKSHV.219-encoded GFP, RFP, and puromycin *N*-acetyltransferase (PAC) reporters agrees with the general picture obtained from percentage reads. Most clusters show some lytic gene expression, but only a few demonstrate substantial expression of lytic genes (Fig. 3E; Fig. S2B). Interestingly, we noted that some of the clusters that expressed lytic genes did not express high levels of RFP (cluster 0, for example) (Fig. 3E). Because KSHV lytic genes are expressed in several waves, termed early lytic, delayed early lytic, and late lytic genes (23–25), we roughly categorized the clusters based on the pattern of lytic-gene expression, as well as the cell cycle stage and the expression of IFNs. We classified clusters as “uninfected,” “latent,” “latent (dividing),” “lytic (early),” “lytic (intermediate),”

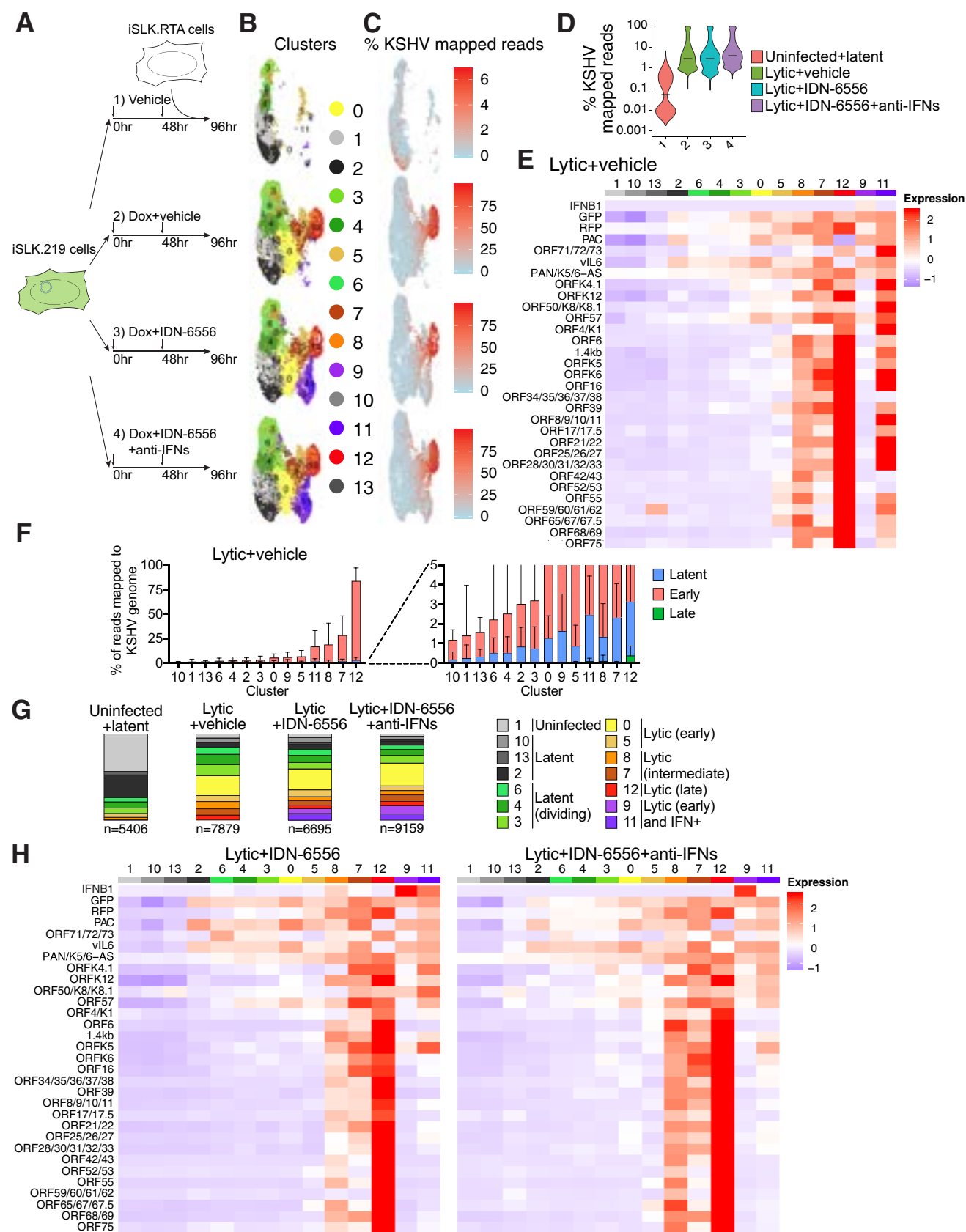


FIG 3 Single-cell RNA-Seq analysis reveals subsets of lytically reactivating cells. (A) Diagram of the scRNA-Seq experiment using 4 samples. Sample 1 was a mixture of uninfected iSLK.RTA and latently infected iSLK.219 cells treated with DMSO (vehicle). Samples 2 to 4 were iSLK.219 treated with (Continued on next page)

"lytic (late)," "lytic (early)" and "IFN⁺" (Fig. 3E, G, and H and 4A to C). The levels of most viral transcripts increased monotonically in cells that had progressed further through the lytic cycle, except for ORF57, K4.1, and vIL-6, whose levels appeared to decrease in the late lytic cell cluster (cluster 12) (Fig. 3E).

Figure 3G and Fig. S3B show how the different clusters expanded and contracted in each sample. Interestingly, addition of the caspase inhibitors and the consequent type I IFN induction did not reduce the overall percentage of cells expressing lytic genes (Fig. 3G; Table S2), but they reduced the number of cells that we classified as lytic (intermediate) and lytic (late) (particularly clusters 7, 8, and 12) (Fig. 3G). Moreover, caspase inhibitor treatment reduced the levels of most viral genes in many clusters, most notably the "lytic" clusters 5, 8, and 12 (Fig. 3E and H; Fig. S2B and S3). The effect of caspase inhibitors on the size of these clusters and the reduction in lytic gene expression was largely rescued by blocking type I IFN signaling with anti-IFN antibodies (Fig. 3G and H; Fig. S2B and S4). These results confirm that caspase activity is required for reactivation and/or progression through the lytic cycle and that inhibition of the lytic cycle upon caspase inhibition is due to the paracrine and autocrine effects of type I IFN secretion. These data thus recapitulate our bulk-level findings on the role of caspases during KSHV replication (4). The scRNA-Seq results also confirm the wide variability in stage of reactivation of iSLK.219 cells and reveal that the RFP signal in iSLK.219 cells may underestimate the number of cells expressing lytic genes.

Only a small subset of infected cells express IFNs to confer a potent antiviral state. An outstanding question from our bulk analysis was the identity of the cells that produce IFN- β in the caspase inhibitor-treated samples. We had hypothesized that lytically reactivating cells, rather than latent ones, produce IFNs. Indeed, our scRNA-Seq data showed that type I IFN expression is limited to cells that express viral lytic genes (Fig. 3H). However, the scRNA-Seq also surprisingly revealed that only 3.8% of the cells in the caspase inhibitor-treated samples expressed IFNs, particularly IFN- β (Fig. 4B). The IFN- β ⁺ cells were concentrated in clusters 9 and 11, which appeared only in the +IDN-6556 samples (Fig. 4A and B). We did not detect expression of IFN- α s, but we saw that cells in clusters 9 and 11 also expressed the type III IFN IFN- λ 1 (Fig. 4C; Fig. S3A). The IFN coming from this small fraction of cells was sufficient to elicit a strong IFN response in the entire population of cells, as indicated by the IFN-dependent increase in expression of ISGs after IDN-6556 treatment across almost all clusters (Fig. 4D and E; Fig. S5B). The only exception was cluster 12, which contains cells expressing late lytic gene. These cells likely undergo host shutoff and generally express reduced levels of host genes (26). To ensure that the variability in IFN- β induction is not an artifact of a limit of detection by scRNA-Seq, we constructed iSLK.219- and BC3-based cell lines that expressed the TagBFP or tdTomato proteins under the control of the IFN- β promoter. When the lytic cycle was induced in these cells concomitantly with caspase inhibitor treatment, we found that ~1 to 2% of the cells became TagBFP⁺ (Fig. 4E) or tdTomato⁺, in line with our scRNA-Seq results (Fig. 4F). In addition, we found that caspase inhibitor treatment of latent BC3 cell lines did not increase the percentage of tdTomato⁺ cells (Fig. 4F), consistent with our previous results that lytic induction is also required for the type I IFN induction (Fig. 2B and D) (4). Overall, these data show that an exceedingly small fraction of cells induce IFNs even under caspase inhibition,

FIG 3 Legend (Continued)

doxycycline (Dox) to reactivate lytic replication and treated with DMSO (vehicle), the caspase inhibitor IDN-6556, or IDN-6556 and a cocktail of anti-IFN antibodies to block paracrine IFN signaling. All samples were collected 4 days after the start of treatments. (B to H) Analysis of data from the scRNA-Seq experiment presented in panel A. (B) UMAP diagram of the clusters unbiasedly defined by Seurat in the 4 samples. (C) UMAP diagram showing what percentage of reads from each cell comes from the KSHV genome. Legends on the right define what percentage the colors represent. (D) Violin plot of the distribution of cells in each sample based on percentage of KSHV reads. Sample 1 has a bimodal distribution because it is a mix of uninfected and latent cells. (E) Heat map of average expression of KSHV genes in each cluster in the lytic infection+vehicle sample. The legend on the right defines what expression level the colors represent (arbitrary units). A corresponding heat map with gene expression in each individual cell can be found in Fig. S2B. (F) Plot of percentage reads per cell that map to each of three types of KSHV genes (latent, early, and late) in each cluster in the lytic infection+vehicle sample. Clusters are sorted based on the total average percentage of KSHV reads. (G) Proportion of cells that map to each cluster in the four samples. (H) Heat map of average expression of KSHV genes in each cluster in the lytic infection+IDN-6556 and lytic infection+IDN-6556+anti-IFN samples. The legend on the right defines what expression level the colors represent (arbitrary units). Corresponding heat maps with gene expression in each individual cell can be found in Fig. S2B.

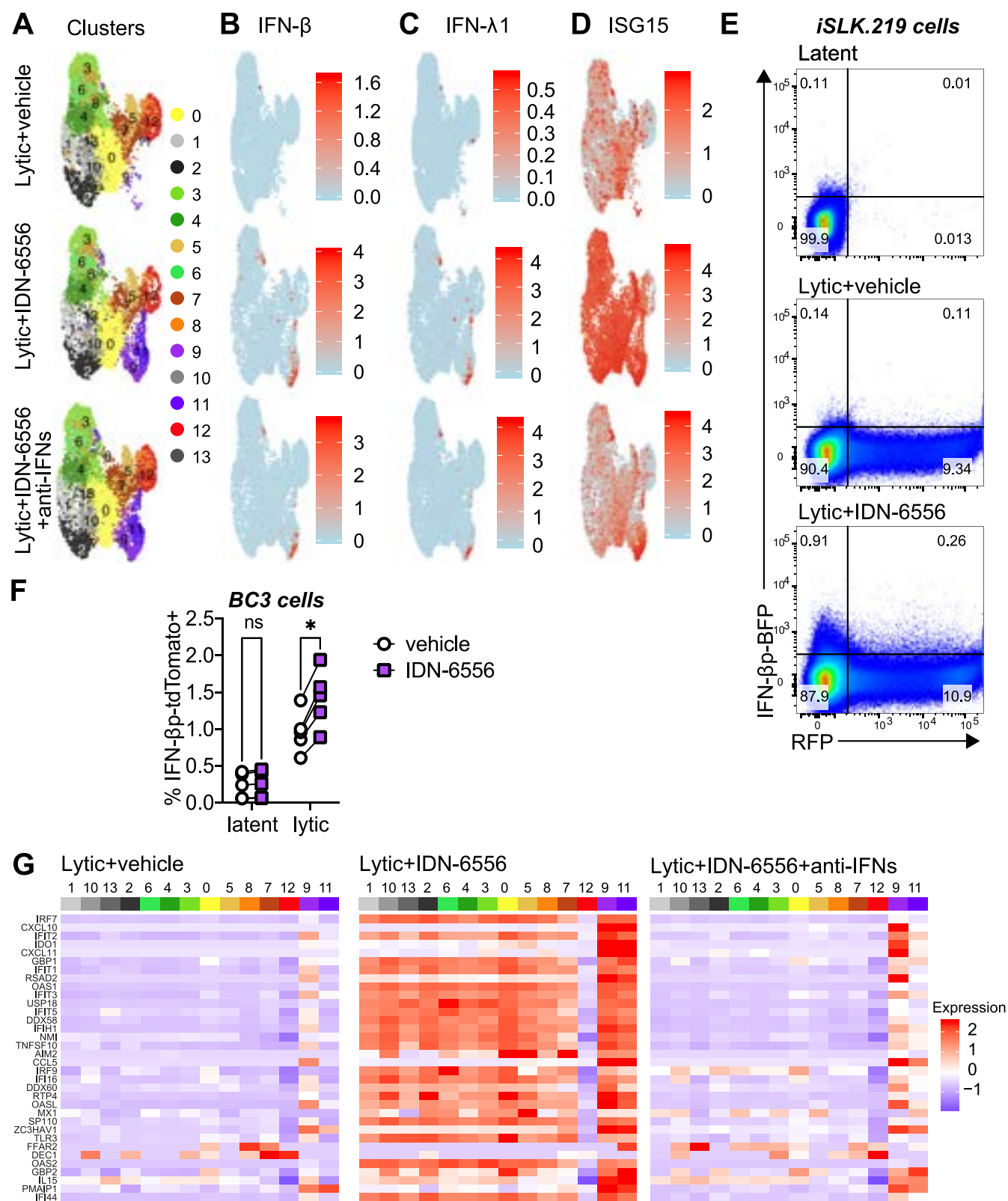


FIG 4 Only a small fraction of KSHV lytic cells express IFN- β . (A to D) Analysis of data from the scRNA-Seq experiment presented in Fig. 3A. (A) UMAP diagram of the clusters unbiasedly defined by Seurat in the 3 lytic samples (as in Fig. 3B). (B to D) UMAP diagram of the level of expression of IFN- β (B), IFN- λ 1 (C), or ISG15 (D) in each cell for the 3 lytic samples. The legends on the right define what expression level the colors represent (arbitrary units). (E) *iSLK.219* expressing TagBFP from a human IFN- β promoter were treated with doxycycline to reactivate the lytic cycle and IDN-6556, where indicated. Flow cytometry was carried out on the cells 4 days after doxycycline addition. The RFP axis reflects the RFP signal in lytic cells, and the BFP (blue fluorescent

(Continued on next page)

yet they can elicit a strong antiviral response in the entire cell population. Moreover, this heterogeneous induction of type I IFN occurs in both cell types we tested.

The heterogeneity of type I IFN induction is not easily explained by differential viral gene expression. We considered the possibility that only a subset of KSHV-infected cells make IFN- β due to differential expression of viral genes. For example, IFN- β^+ cells could lack expression of KSHV-encoded anti-IFN factors. Consistent with this idea, cells in the IFN- β^+ clusters (clusters 9 and 11) expressed only early lytic genes and low levels of the RFP lytic marker (Fig. 3H and 5A; Fig. S2B and S5C). However, their viral gene expression pattern was similar to that of cells from other clusters that do not express IFNs, especially cluster 0 (Fig. 3H and 5A; Fig. S2B and S5C). Moreover, the viral gene expression pattern is also similar between IFN- β^- and IFN- β^+ cells within clusters 9 and 11 (Fig. 5A; Fig. S5C). One notable exception was the K5 mRNA, which was differentially expressed depending on IFN status. However, expression of K5 was higher in the IFN- β^+ cells, rather than lacking in these cells. These observations suggest that lack of expression of viral anti-IFN regulators does not explain the restricted IFN- β induction. Another possibility is that some cells express higher basal levels of genes in the type I IFN induction pathway and are thus more sensitive to IFN-inducing cues. This model was proposed in the case of other viral infections by Zhao et al. (27). Analyzing these genes is complicated by the fact that many of them are themselves ISGs. Indeed, their expression was increased by IFN signaling in all clusters (except cluster 12) in the caspase inhibitor-treated samples and was reduced back to basal levels by anti-IFN antibody treatment (Fig. 5B; Fig. S5B). Nonetheless, there was a clear enrichment of transcripts for the NF- κ B transcription factor family members (NFKB1, NFKB2, RELA, and RELB) in IFN- β^+ clusters 9 and 11 (Fig. 5B; Fig. S5B). NF- κ B is also needed for IFN- β transcription downstream of pathogen sensors, together with IRF3 (1). Indeed, levels and phosphorylation of NF- κ B were increased in lytic cells treated with IDN-6556, showing that caspase activity also modulates NF- κ B activation (Fig. 5C). It is thus possible that high NF- κ B expression is needed to induce type I IFN in these cells, perhaps as a feed-forward mechanism to ensure commitment to IFN production.

DISCUSSION

This study provides mechanistic insights into multiple levels of type I IFN regulation during KSHV infection. We found that caspases regulate cGAS activity during KSHV lytic infection, likely indirectly, either through a cGAS regulator/cofactor or by modulating access of cGAS to its stimulating DNA (Fig. 1 and 2). Moreover, we found that although caspase inhibition results in a strong type I IFN induction, type I and III IFNs are turned on in only a very small percentage of cells. IFN expression appears to correlate with strong induction of NF- κ B transcripts, rather than differences in viral gene levels.

The cGAS/STING pathway has emerged in recent years as a crucial component of the innate immune response to DNA viruses and various other pathogens (28). This is highlighted by the multitude of strategies employed by pathogens (29), including herpesviruses (30), to disrupt the cGAS/STING pathway. While this immunosuppression is often driven by virus-encoded proteins, we have uncovered an intriguing mechanism in KSHV infection, which involves the hijacking of the host cell caspases to subvert immune activation and viral clearance (4). Caspase-3 and -7 have been reported to directly cleave cGAS during apoptosis and other viral infection (8). However, our previous results suggest that caspase-8 activity mediates type I IFN suppression in KSHV-infected cells without triggering apoptosis, whereas caspase-3/7 activity is dispensable

FIG 4 Legend (Continued)

protein) axis reflects activity of the IFN- β promoter. Percentage of cells mapping to each quadrant are listed. Flow cytometry plots are representative of 3 repeats. (F) BC3 expressing tdTomato from a human IFN- β promoter were treated with TPA to reactivate the lytic cycle and the caspase inhibitor IDN-6556, where indicated. Flow cytometry was carried out on the cells 2 days after TPA addition. The percentage of cells that were tdTomato positive is plotted for 5 biological replicates. ns, not significant ($P > 0.05$); *, $P < 0.05$ (Šidák's multiple-comparison test after two-way ANOVA). (G) Analysis of data from the scRNA-Seq experiment presented in Fig. 3A. Heat maps of average expression of ISGs in each cluster for the three lytic-infection samples. The legend on the right defines what expression level the colors represent (arbitrary units). Corresponding heat maps with gene expression in each individual cell can be found in Fig. S5B.

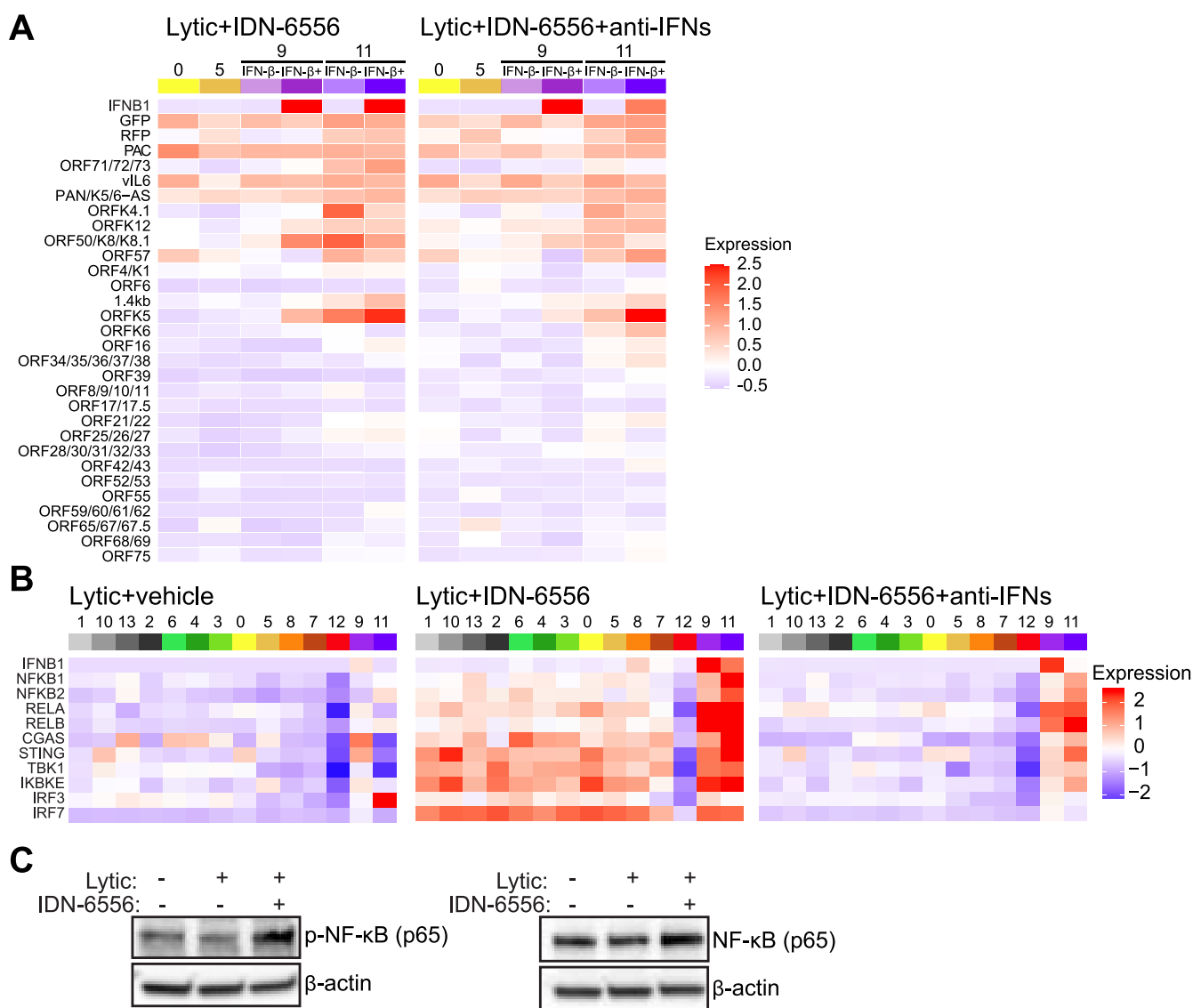


FIG 5 IFN- β -producing cells express high levels of NF- κ B transcripts. (A and B) Analysis of data from the scRNA-Seq experiment presented in Fig. 3A. Heat maps of average expression of KSHV genes in clusters 0, 5, 9, and 11, with 9 and 11 divided based on IFN- β status (A) and of average expression of select genes from the type I IFN induction pathway in all clusters (B) for the indicated samples. The legends on the right define what expression level the colors represent (arbitrary units). Corresponding heat maps with gene expression in each individual cell can be found in Fig. S5B and C. (C) iSLK.219 were treated with doxycycline to reactivate the lytic cycle and with IDN-6556 where indicated. Protein lysates were collected at day 4 and analyzed by Western blotting for NF- κ B, phospho-NF- κ B (p-NF- κ B), and β -tubulin as a loading control. Blots are representative of 3 replicates.

(4). Moreover, we have been consistently unable to detect cleavage fragments or downregulation of cGAS in infected cells. Together, our previous report and the current study suggest an unexpected type I IFN regulatory function of caspase-8 activity in targeting the cGAS/STING pathway that is separate from its apoptotic activity. Perhaps caspases like caspase-8 cleave regulators of the cGAS/STING pathway. Alternatively, they may affect access of the DNA to cGAS. The latter is hard to test, because we do not know what the cGAS stimulating DNA is during KSHV reactivation. We have previously reported that viral DNA replication is not required for IFN induction (4). Moreover, we have found that depletion of mitochondrial DNA, a common stimulus of cGAS, reduced reactivation of the lytic cycle, confounding analysis of IFN induction (data not shown). Identifying potential relevant caspase targets will be important to determine how they regulate cGAS activity. Nonetheless, caspases are an attractive therapeutic target that could be exploited to potentiate cGAS/STING-mediated immune responses for viral clearance. This could have far-reaching implications for

other pathologies, as regulation of the cGAS/STING pathway is fundamental in various inflammatory diseases and in tumor immunity (31, 32) and thus is a current focus of intensive drug discovery research efforts.

The notable cellular heterogeneity of type I IFN production reveals an additional layer of complexity and regulation of innate immune responses during KSHV infection. We detect an exceptionally small fraction of IFN- β ⁺ cells upon caspase inhibitor treatment (<5%) (Fig. 4B). Remarkably, this small group of IFN- β ⁺ cells still induces a robust IFN response in the whole population (Fig. 4D and F). On a practical level, the high heterogeneity in IFN- β expression complicates our analysis of caspase regulation of cGAS. It is possible that the upstream portions of the pathway (i.e., cGAS) are also active only in the IFN- β ⁺ cells and that cell sorting may be needed to detect differences in the levels of the direct target(s) of caspases in IFN regulation. Alternatively, it is possible that only a subset of the cells in which the IFN induction pathway is activated end up expressing IFN- β . In support of this idea, we were able to easily detect phosphorylation of many of the components of the IFN induction pathway using bulk analysis (Fig. 1E, 2C, and 5C).

Single-cell RNA-Seq studies in herpesviruses have started to come out in the last few years, but many have not analyzed IFN responses, focusing instead on latency and reactivation (33–38). However, several studies in multiple viruses have examined IFN induction and have also shown that only a small number of cells respond to infection in a population during infection with a number of other viruses (27, 35, 39–45). Interestingly, studies that investigated infections in innate immune cells like macrophages and dendritic cells or fibroblasts saw higher percentages of responding cells (10 to 30%) (27, 39, 40). In contrast, iSLK.219 cells are of epithelial origin (46). Epithelial cells are often the first exposed to invading pathogens and mount responses that alert and prime surrounding cells to counteract viral spread. Therefore, it is possible that epithelial cell responses are even more tightly regulated to prevent inadvertent activation of the pathway. Consistent with this idea, influenza A virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection of epithelial cells also elicit responses in a small minority of cells (41–43). Moreover, while we did not perform scRNA-Seq on BC3 cells, flow cytometry analysis of BC3 cells encoding an IFN reporter also indicated that IFN- β is induced in less than 2% of these cells (Fig. 4F). Taken together, our findings suggest differences in IFN responsiveness depending on the cell type as well as the virus infecting the cells.

It is unclear what the source of interferon heterogeneity is in KSHV-infected cells. Viral gene expression, particularly expression of viral immune regulators, is often considered the reason for limited type I IFN responses (43). A previous study in herpes simplex virus 1 reports that type I IFN signaling occurs in only a small percentage of cells that are all abortively infected (35). This is also a potential explanation for our results, since we found that the cells that make IFNs express only a subset of viral genes, mostly early genes (Fig. 3H and 5A). However, this pattern of gene expression is not unique to the small percentage of IFN- β ⁺ cells. Multiple clusters of cells do not express high levels of late lytic genes. Indeed, the pattern of viral gene expression is generally similar in clusters 0, 5 (IFN- β negative), 9, and 11 (IFN- β positive) (Fig. 5A; Fig. S5C). Moreover, when we compared IFN- β -positive and -negative cells within the same clusters (clusters 9 and 11), we found that they also have a similar viral gene expression pattern (Fig. 5A; Fig. S5C). We also do not know whether this gene expression pattern represents abortive infection or simply an early stage of reactivation that facilitates IFN induction, since we know that iSLK.219 reactivation is heterogeneous. This also means that we cannot distinguish a situation whereby progression to the late stage of the lytic cycle prevents IFN- β induction from one where IFN- β induction prevents progression through the lytic cycle. It thus appears that abortive or early-stage infection alone does not explain the restricted pattern of IFN gene expression in our experiments.

Our results also do not support the idea that some cells express IFN because they are missing expression of a viral negative regulator of type I IFN, although we cannot

fully exclude this model because we did not reliably detect all viral genes. However, we did find that the KSHV gene K5 displayed much higher expression in the IFN- β -positive cells (Fig. 5A; Fig. S5C). This was surprising to us, as we would not expect viral genes to positively regulate IFN induction. There is no known role of K5 in regulating type I IFN signaling, so it is unclear why its expression correlates with IFN- β expression at present. Future studies will hopefully elucidate this correlation.

Currently, our results point to the possibility that inherent differences in the cells govern the heterogeneity of the interferon response. Stochasticity in gene expression, which is common among cytokines, was previously proposed as a driver of type I IFN expression heterogeneity. Zhao et al. suggested that some of this stochasticity may be epigenetically encoded (27), reducing the number of cells that are able to express type I IFN, perhaps to prevent overactivation and inflammation. Paracrine IFN signaling is also thought to influence and modulate heterogeneity (44). This cell-cell communication may fine tune immune signal amplification in a cell density-dependent manner. Indeed, we observed 3.8% IFN- β ⁺ cells in the IDN-6556-treated sample but only 1.6% IFN- β ⁺ cells in the sample treated with IDN-6556 and anti-IFN antibodies that block IFN signaling. Moreover, while cells in cluster 9 retained high average IFN- β expression even when IFN signaling was blocked, cluster 11 cells had lower IFN- β without IFN signaling (Fig. 5A; Fig. S5A and C), suggesting two different modes of IFN induction in these groups of cells. This reinforces the notion that self-amplification of the type I IFN response ensures that robust responses develop only in the appropriate contexts for infection control, thus avoiding tissue damage from unnecessary inflammation.

MATERIALS AND METHODS

Cell lines, constructs, reagents, and treatments. All cells were cultured at 37°C and 5% CO₂. iSLK.219, iSLK.219-IFNBp-BFP, iSLK.RTA (16), and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS) (HyClone). BC3 cells were grown in RPMI supplemented with 20% FBS, 2 mM GlutaMAX supplement, and 50 μ M β -mercaptoethanol (Gibco/Thermo Fisher). iSLK.219 cells were reactivated with 1 μ g/mL of doxycycline (Thermo Fisher). Reactivation was confirmed visually for all iSLK.219 samples by examining the appearance of RFP-positive cells (RFP expression is driven by the promoter for polyadenylated nuclear (PAN) RNA, an early lytic gene). BC3 cells were reactivated with 20 ng/mL of 12-O-Tetradecanoylphorbol-13-acetate (TPA) for 48 h. iSLK.219-IFNBp-BFP cells were generated by transducing iSLK.219 cells with lentiviruses containing pJP1-IFNBp-tagBFP and BC3-IFNBp-tdTomato by transducing BC3 cells with pLJM1_2xIFNBp-tdTomato. All pLJM and pJP1 plasmids are based on pLJM1-GFP, a gift from David Sabatini (Addgene plasmid number 19319; <http://n2t.net/addgene:19319>; RRID, Addgene_19319) (47). For pJP1-IFNBp-tagBFP, the cytomegalovirus (CMV) promoter of pLJM1 was replaced with a human IFN- β promoter fragment subcloned from a luciferase reporter construct (kind gift from Michaela Gack) and the GFP sequence was replaced with a TagBFP sequence. In addition, the antibiotic resistance gene was changed from a puromycin resistance to a zeocin resistance gene. For pLJM1_2xIFNBp-tdTomato, the CMV promoter and multiple cloning sites were replaced with two copies of a human IFN- β promoter 303-bp fragment cloned from IFN-Beta_pGL3, a gift from Nicolas Manel (Addgene plasmid number 102597; <http://n2t.net/addgene:102597>; RRID, Addgene_102597) (48). To add the 54-bp 5' untranslated region of human IFN- β , sequences were added to the primers used to assemble the construct. Lentiviral packaging was carried out using packaging plasmids pMDLg/pRRE (Addgene plasmid number 12251; <http://n2t.net/addgene:12251>; RRID, Addgene_12251), pRSV-Rev (Addgene plasmid number 12253; <http://n2t.net/addgene:12253>; RRID, Addgene_12253), and pMD2.G (Addgene plasmid number 12259; <http://n2t.net/addgene:12259>; RRID, Addgene_12259), kind gifts from Didier Trono (49). Where indicated, cells were treated with vehicle (dimethyl sulfoxide [DMSO]; Sigma-Aldrich), 10 μ M IDN-6556 (MedChem Express or Selleck Chemicals), 24.1 μ M RU.521 (InvivoGen), and 1:50, 1:100, or 1:500 dilutions of a mixture of neutralizing antibodies against type I IFNs (PBL Assay Science, catalog number 39000-1). The concentrations of all drug treatments were chosen based on common concentrations used in previously published studies.

siRNA knockdown. iSLK.219 cells were transfected while in suspension (reverse transfection) with 10 nM concentrations of small interfering RNAs (siRNAs; purchased from Thermo Fisher) against STING (HSS139156), MAVS (HSS148537), cGAS (siRNA 1, HSS132955; siRNA 2, HSS132956), IFI16 (siRNA 1, HSS105205; siRNA 2, HSS105206), RIG-I (siRNA 1, s24144; siRNA 2, s223615), or a nontargeting siRNA (12935300) at a density of 16,700 cells/cm² using the Lipofectamine RNAiMAX transfection reagent (Life Technologies/Thermo Fisher) per the manufacturer's protocol. Six hours later, the medium was replaced. The lytic cycle was induced 2 days later. For cGAS and RIG-I knockdowns, the reverse transfection process was done twice, once 2 days prior to induction and a second time on the day of lytic-cycle induction. All siRNAs were obtained from Life Technologies/Thermo Fisher.

RT-qPCR. For RNA analysis, iSLK.219 cells were seeded at a density of 33,000 cells per well in a 24-well plate, and the lytic cycle was induced by the addition of doxycycline (1 μ g/mL). RNA samples were collected at the indicated time points in RNA lysis buffer (Zymo Research). BC3 cells were seeded at 500,000 cells per mL and induced using TPA for 48 h. One milliliter of the cells was collected, and the

cell pellet was lysed in RNA lysis buffer (Zymo Research). Total RNA was extracted using the Quick-RNA MiniPrep kit (Zymo Research) by following the manufacturer's protocol. For mRNA measurements, cDNA was prepared using an iScript cDNA synthesis kit (Bio-Rad) per the manufacturer's protocol. In all cases, 18S rRNA levels were used as an internal standard to calculate relative mRNA levels. Real-time quantitative PCR (RT-qPCR) was performed using iTaq Universal SYBR green Supermix (Bio-Rad) in a CFX Connect real-time PCR detection system (Bio-Rad). No-template and no-RT controls were included in each replicate. CFX Manager software was used to analyze the data. Primers are listed in Table S3.

Protein analysis. iSLK.219 cells were seeded at a density of 167,000 cells per well in a 6-well plate and treated with doxycycline (1 μ g/mL). Lysates were collected at the indicated time points. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) or an NP-40-only buffer for caspase blots and phospho-protein blots (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with 50 μ g/mL phenylmethylsulfonyl fluoride (PMSF; G-Biosciences) and cComplete protease cocktail inhibitor (Roche). Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The Cell Signaling Technologies antibody anti-cGAS (D1D3G) (no. 15102) was used on PVDF membranes at a 1:1,000 dilution in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST). The following Cell Signaling Technologies antibodies were used with PVDF at a 1:1,000 dilution in 5% bovine serum albumin (BSA) in TBST: anti-caspase-8 (no. 9746), anti-cleaved caspase-8 (no. 9496), anti-IRF3 (D83B9) (no. 4302), anti-phospho-IRF3 Ser396 (4D4G) (no. 4947), anti-TBK1 (D1B4) (no. 3504), anti-phospho-TBK1 Ser172 (D52C2) (no. 5483), anti-STING (D2P2F) (no. 13647), anti-phospho-STING Ser366 (D7C3S) (no. 19781), anti-RIG-I (D14G6) (no. 3743), anti-NF- κ B p65/RelA (D14E12) (no. 8242S), anti-phospho-NF- κ B p65 Ser536/RelA (93H1) (no. 3033), and anti- β -tubulin (9F3) (no. 2128). Anti-IFI16 (1:1,000; ab169788; Abcam) and anti- β -actin (1:500; ab8229; Abcam) were diluted in 5% BSA in TBST and used with PVDF membranes blocked in 5% BSA in TBST. Anti-MAVS (1:1,000 dilution; sc-166583, Santa Cruz Biotechnology) was diluted in 0.5% milk in phosphate-buffered saline with 0.1% Tween 20 (PBST) and used with PVDF membranes blocked in 5% milk in PBST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (both 1:5,000 in blocking buffer) were purchased from Southern Biotechnology. HRP-conjugated donkey anti-goat IgG (1:5,000 in 0.5% milk in PBST) was purchased from Santa Cruz Biotechnology. All membranes were exposed using Pierce ECL Western blotting substrate (Thermo Fisher) and imaged with a Syngene G:Box Chemi XT4 gel documentation system.

cGAMP ELISA. Cell lysates were harvested 4 days after reactivation in RIPA buffer and subjected to enzyme-linked immunosorbent assay (ELISA) analysis for 2',3'-cGAMP (Cayman Chemical) according to the manufacturers' protocols. Each biological replicate consisted of three technical replicates per condition.

Flow cytometry analysis. 10^6 IFN- β p-TagBFP cells were treated with DMSO, IDN-6556 (10 μ M), doxycycline (1 mg/mL) or doxycycline and IDN-6556 for 4 days to induce KSHV reactivation. BC3-IFN- β p-TdTomato cells (10^6) were treated with DMSO, IDN-6556 (10 μ M), TPA (20 ng/mL), or TPA and IDN-6556 for 48 h to induce KSHV reactivation. Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature and washed in PBS before being analyzed on a BD Biosciences LSR II flow cytometer in the Tufts Laser Cytometry Core facility. Five hundred thousand events were collected per sample. TagBFP, RFP, and TdTomato expression was gated based on the corresponding latently infected and vehicle (DMSO)-treated cells.

Single-cell RNA sequencing. iSLK.219 and iSLK.RTA cells were seeded onto T25 flasks at a density of 16,700 cells/cm² and induced with doxycycline in the presence or absence of IDN-6556 (10 μ M) and a mixture of neutralizing antibodies against type I IFNs at a 1:500 dilution (39000-1; PBL Assay Science). The medium was replaced at day 2 postreactivation with fresh doxycycline, IDN-6556, and type I IFN-neutralizing antibodies. Cells were harvested at day 4 postreactivation, washed twice with PBS, and strained to create single-cell suspensions at a concentration of 1,000,000/mL. Single-cell samples were generated according to 10X Genomics' sample preparation protocol for cultured cell lines (CG00054) and gel-beads-in-emulsion (GEM) generation protocol (CG000183). Briefly, cells were trypsinized and washed twice with Dulbecco's phosphate-buffered saline (DPBS; Life Technologies). Cells were resuspended in DPBS and passed through a 30- μ m strainer to remove clumps. Cells were counted and diluted to a concentration of 1,000,000 cells/mL. Cells were mixed with a reverse transcription master mix [including a primer with a poly(dT) anchor] and loaded onto a Chromium B microfluidic chip. Gel beads and partitioning oil were loaded onto the chip, and the chip was run through the chromium controller device to generate the GEMs. GEMs were incubated in a thermal cycler for 1 cycle to generate cDNA. The emulsion was broken, and cDNA was isolated with magnetic beads, amplified for 12 cycles, and then cleaned up with SPRIselect reagent.

Libraries were prepared according to the 10X Genomics library construction protocol (CG000183). Briefly, cDNA was fragmented and then end repaired and A tailed with the enzymes in the provided 10X Genomics kit (version 3; PN-1000092). cDNA was size selected using SPRIselect, and adaptor oligonucleotides were ligated onto the cDNA and further cleaned up with SPRIselect. Paired-end sequencing with a 75-cycle read length was performed on an Illumina NextSeq 550 instrument at Tufts Genomics Facility. Raw sequencing data (.bcl files) were processed using the 10X Genomics CellRanger (3.0.0) software. Fastq files were generated for the reads using the mkfastq function as described on the 10X Genomics website (<https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/mkfastq>). Also using CellRanger, the count function was used to align the reads to a reference genome, including the human genome (GRCh38), the KSHV genome (GQ994935.1), and the additional sequence of the RFP/GFP/PAC insertion cassette (GenBank number [MZ617352](https://www.ncbi.nlm.nih.gov/nuclot/MZ617352)). This third reference was added because the GQ994935.1 reference genome corresponds to the KSHV BAC16 genome, whereas our cells contain the rKSHV.219 virus (16, 50). To our knowledge, a full reference for rKSHV.219 is not available. We thus amplified the missing region and sequenced it using Sanger sequencing.

The matrix table of expression for each gene in each single cell from CellRanger was used as an input for integration and clustering analyses using the R-based Seurat package (version 3.1.1.9021; R version 3.6.1) (19, 20). Cell cycle analysis was done as described by Tirosh et al. (51). The matrices were converted to Seurat objects and then preprocessed by performing quality control analyses. First, the four objects from the different samples were integrated into one object using the Seurat functions `FindIntegrationAnchors()` and `IntegrateData()`. Dying cells with mitochondrial contamination or possible cell doublets were filtered out by eliminating cells that had >20% mitochondrial RNA counts and cells that have unique feature (RNA) counts of >7,500. Feature expression measurements for each cell were normalized by the total expression, multiplied by a scale factor (10,000), and log-transformed using the Seurat function `NormalizeData()`. The expression was scaled so that the mean expression across all cells was 0 and the variance was 1, using the Seurat function `ScaleData()`. For linear dimensional reduction, principal-component analysis (PCA) was performed using the Seurat function `RunPCA()`. Only the PCs that account for the most variation were included, to overcome technical noise when clustering cells. These were chosen by visualizing each PC with the Seurat functions `JackStrawPlot()` and `ElbowPlot()` and determining that variance was negligible after 30 PCs. Therefore, UMAP (uniform manifold approximation and projection) cluster plots were created using dimensions 1 to 30.

Statistical analysis. All statistical analysis was performed using GraphPad Prism version 8.4.2 or later (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). Statistical significance was determined by Student's *t* test or analysis of variance (ANOVA) followed by a *post hoc* multiple-comparison test (Dunnett's, Šidák's or Tukey's) when multiple comparisons were required. All data are plotted as means and standard deviations.

Data availability. Raw and processed data are available on NCBI GEO, record number [GSE190558](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE190558). Scripts used for analysis are available on GitHub (https://github.com/mgaglia81/ISLK.219_scRNAseq).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 1 MB.

FIG S2, PDF file, 1.1 MB.

FIG S3, PDF file, 0.6 MB.

FIG S4, PDF file, 0.6 MB.

FIG S5, PDF file, 1.2 MB.

TABLE S1, PDF file, 0.04 MB.

TABLE S2, PDF file, 0.1 MB.

TABLE S3, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank members of the Gaglia laboratory for suggestions and feedback on the project and the manuscript. We thank the Tufts Laser Cytometry and Genomics core staff for technical and conceptual assistance. We thank M. Gack, D. Trono, D. Sabatini, and N. Manel for sharing reagents.

This work was supported by American Cancer Society grant 131320-RSG-17-189-01-MPC and NIH R01 R01CA268976 to M.M.G., T.T. was supported by the Tufts Collaborative Cancer Biology Award. R.C.L. was supported by NIH training grant T32 GM007310.

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OPEN ACCESS

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SPECIALTY SECTION

This article was submitted to
Neuro-Oncology and
Neurosurgical Oncology,
a section of the journal
Frontiers in Oncology

RECEIVED 15 June 2022

ACCEPTED 25 July 2022

PUBLISHED 18 August 2022

CITATION

Erices JI, Niechi I, Uribe-Ojeda A,
Toro MLÁ, García-Romero N,
Carrión-Navarro J, Monago-Sánchez Á,
Ayuso-Sacido Á, Martin RS and
Quezada-Monrás C (2022) The low
affinity A2B adenosine receptor
enhances migratory and invasive
capacity *in vitro* and angiogenesis *in vivo*
of glioblastoma stem-like cells.
Front. Oncol. 12:969993.
doi: 10.3389/fonc.2022.969993

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The low affinity A2B adenosine receptor enhances migratory and invasive capacity *in vitro* and angiogenesis *in vivo* of glioblastoma stem-like cells

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Glioblastoma (GBM) is the most common and deadly malignant brain tumor, with a median survival of 15 to 17 months for a patient. GBM contains a cellular subpopulation known as GBM stem-like cells (GSCs) that persist in hypoxic niches and are capable of infiltrating into healthy brain tissue. For this reason, GSCs are considered one of the main culprits for GBM recurrence. A hypoxic microenvironment increases extracellular adenosine levels, activating the low affinity A2B adenosine receptor (A_{2B}AR). Adenosine, through A_{2B}AR, is capable of modulating invasiveness. However, its role in the invasion/migration of hypoxic-GSCs is still unknown. This study aims to understand the importance of A_{2B}AR in modulating the migratory/invasive capacity of GSCs under hypoxia. Data analysis from The Cancer Genome Atlas (TCGA) program correlates A_{2B}AR expression with high-grade glioma and hypoxic necrotic areas. U87MG and primary culture-derived GSCs under hypoxic conditions (0.5% O₂) increased A_{2B}AR mRNA and protein levels. As expected, the migratory and invasive capacity of GSCs increased under hypoxia, which was counteracted by blocking A_{2B}AR, through the downregulation of MMP9 activity and epithelial–mesenchymal transition marker expression. Finally, in a xenograft mouse model, we demonstrate that treatment with MRS1754 did not affect the tumor volume but could decrease blood vessel formation and VEGF expression. Our results suggest that extracellular adenosine, through the activation of A_{2B}AR, enhances the migratory and invasive capacity of GSCs *in vitro* under hypoxic conditions. Targeting A_{2B}AR can be an effective therapy for GBM recurrence.

KEYWORDS

glioblastoma stem-like cells, hypoxia, adenosine, invasion, angiogenesis

Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor, accounting for around 54% of all gliomas (1). Current GBM tri-modal treatment includes total surgical resection, radiotherapy, and chemotherapy. However, the survival median is <15 months and only 2% of patients survive for 24 months (2, 3). The poor treatment outcome is mainly caused by the high invasiveness of GBM toward healthy tissue, the difficulty of total removal of malignant cells, and limiting the effects of localized radiotherapy (4). During the past few years, a subpopulation called GBM Stem-like Cells (GSCs) has been identified as being responsible for GBM aggressiveness (5). These cells have stem cell properties (e.g., self-renewal and multi-lineage differentiation), but, unlike neural stem cells, GSCs can initiate the formation and progression of a malignant tumor (6). Different studies have associated GSCs and their high infiltrative capability with tumor relapse, and for this reason, GSCs are becoming more relevant as a target for new treatments for improved patient outcome (6). Tumoral tissue has poorly organized blood vessels, which are unable to correctly provide oxygen, causing hypoxia, which sustains stemness, survival, growth, proliferation, and GSC invasion (7). Additionally, the hypoxic niche of GSCs is composed of endothelial cells (ECs), known as perivascular hypoxia (8). It has been reported that ECs are essential in maintaining the stemness characteristics of GSCs. In fact, studies using co-culture models demonstrated that ECs can induce the expression of genes involved in the stemness phenotype, such as Sox2, Olig2, and Bmi1 (9, 10). This has also been identified in biopsies from patients diagnosed with GBM, in which it has been reported that CD133+ GSCs reside closely with ECs, indicating the importance of this cellular interaction (11). This hypoxic microenvironment promotes the release of ATP and AMP into the extracellular space, which are hydrolyzed by membrane ectonucleotidases, enhancing adenosine production and allowing cells to persist in the hypoxic niche, thereby promoting stemness-related chemoresistance and invasiveness (12, 13). Previously, we and others have reported that aberrant levels of extracellular adenosine produced by GSCs activate the A₃ adenosine receptor (A₃AR) and promote tumorigenic characteristics (14, 15). Interestingly, the A_{2B} adenosine receptor (A_{2B}AR) has the lowest affinity for adenosine in comparison to other adenosine receptors, suggesting a relevant role under hypoxic conditions where adenosine is aberrantly increased (A_{2B}AR Ki = 15,000 nM vs A_{2A}AR Ki = 310 nM, A₃AR Ki = 290 nM, and A₁AR Ki = 100 nM) (16, 17). Additionally, hypoxia induces A_{2B}AR expression in different cancer cells, activating signaling pathways involved in cancer progression (18, 19). Indeed, A_{2B}AR downregulation dramatically decreases tumor

metastasis in breast cancer, melanoma, and bladder urothelial carcinoma, suggesting an important role of A_{2B}AR in cell invasiveness (20). Despite this, the relationship between A_{2B}AR and GSC invasion capacity under hypoxic conditions is unknown. Here, we demonstrate that A_{2B}AR is highly expressed in high-grade glioma as well as in patient-derived GSCs under hypoxia. Pharmacological blockage of A_{2B}AR reduced GSC migration/invasion, expression of EMT markers and MMP-9 activity *in vitro*. In a xenograft model, A_{2B}AR blockage GSCs decreased blood vessel formation and angiogenesis markers without affecting tumor volume mass. Our results suggest that extracellular adenosine through the activation of A_{2B}AR enhances the migratory and invasive capacity of GSCs under hypoxic conditions *in vitro* and modulates angiogenesis *in vivo*. Targeting A_{2B}AR can be an effective therapy for GBM recurrence.

Material and methods

Cell culture

The human U87MG GBM (ATCC[®], HTB-14TM) cell line was grown in differentiation DMEM-F12 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) in standard culture conditions (37°C and 5% CO₂).

GBM stem-like cells culture and hypoxia and *in vitro* treatment

For generating GSC from the U87MG cell line (ATCC[®] HTB-14TM), the cells were grown in neurobasal medium (Gibco, Waltham, MA, USA) supplemented with EGF (20 ng/ml; Peprotech[®], Rocky Hill, NJ, USA), bFGF (20 ng/ml; Peprotech[®]), 1× B27 (GibcoTM), 1× Glutamax (Gibco) and penicillin/streptomycin (100 U/ml, Gibco) at 37°C. After 5 days of culture, GSCs were plated to conduct different tests and treatments. Further, GSC primary cultures (GBM27 and GBM38) were obtained from resected human GBM cultured in M21 medium as described in (21). The work was carried out following the rules of the Declaration of Helsinki. All patients participating in the study gave their informed consent, and protocols were approved by institutional ethical committees. For expansion, GSCs were washed with PBS 1× and then treated for 10 min at 37°C with StemPro[®] Accutase[®] (ThermoFisher, Waltham, MA, USA). Subsequently, the cells were maintained in M21 medium for generating new GSC. Normoxia (21% O₂) and hypoxia (0.5% O₂) conditions were generated using a gas mixing chamber (5% CO₂ and 95% N₂ mixture) for 24 h (18). In *in vitro*

studies, MRS1754 (50 nM; Tocris[®]) was used as a selective A_{2B}AR antagonist.

GlioVis analysis

Gene expression and survival data for patients diagnosed with GBM were obtained from records collected by the Cancer Genome Atlas (TCGA) and the Ivy Glioblastoma Atlas, and these were analyzed using the GlioVis web service (Version 0.20) (<http://gliovis.bioinfo.cnio.es/>, accessed 10 May 2021) (22). Only adult patients whose tumors had mRNA expression obtained by RNA-seq for our genes of interest were included. Pairwise comparisons were performed using the t-test (with Bonferroni correction). The p-values of the pairwise comparisons are indicated on the graphs as ***p < 0.001.

RNA extraction and qRT-PCR cell culture

U87MG and GSCs were maintained under standard culture conditions (37°C, 5% CO₂) for 4 days. Then, total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) and reverse transcription was performed with 1 µg of RNA using the M-MLV Reverse Transcriptase (Thermo Fisher Scientific) following the instructions of the manufacturer. Then, qPCR was performed using the 2^{-ΔΔCT} and β-actin as normalizer genes using HOT FIREPol[®] EvaGreen[®] qPCR Mix Plus ROX (SolisbioScience) following the instructions of the manufacturer. The qPCR reactions were performed with 250 nM of each primer (Supplementary Table 1).

Western blot

Total proteins extracts (40 µg) obtained from U87MG GSCs and PC GSCs were fractionated by SDS-PAGE, transferred to 0.22 µm PVDF membranes (General Electric, GE[®], Boston, MA, USA) and blocked with 1× PBS/0.05% Tween/1% BSA or 5% non-fat milk for 1 h. Then, membranes were incubated overnight with primary antibodies at 4°C followed by a secondary antibody-HRP conjugate during 1 h. Western blots were revealed using the SuperSignal[™] West Dura Extended Duration Substrate kit (Thermo Fisher Scientific) and images were quantified by densitometry analysis (ImageJ, NIH). Primary antibodies targeting the following proteins were used: A_{2B}AR (Abcam, cat#ab222901; 1:1,000); Mmp9 (Cell Signaling, cat#13667, 1:1,000); β-actin (Santa Cruz, cat#sc-47778, 1:5,000).

Cell adhesion assay

Pre-treated GSCs were seeded in a 96-well plate (2 × 10⁴ cells/well) and incubated at 37°C for 2 h in neurobasal medium plus 1% FBS to trigger cell adhesion (23). Non-adherent GSCs were carefully removed by inverting the culture plate, and adherent GSCs were fixed (PFA 3.7% for 10 min) and stained (0.5% crystal violet/20% methanol for 10 min). GSCs were washed with 1× PBS and incubated with 10% acetic acid (v/v) for 5 min. Cell adhesion was analyzed by measuring the optical density of the eluted crystal violet stain at 570 nm in a microplate reader (Synergy 2 Biotek).

Migration and invasion assay

GSCs were plated (75,000 cells/chamber) on the top side of a polycarbonate Transwell chamber (6.5 mm, 8.0 µm pore Polycarbonate membrane; Corning) for migration assay or in a 300 µg/ml matrigel-coated Transwell chamber (Corning) for invasion assay. Cells were seeded in serum-free neurobasal medium for U87 or M21 for primary cultures. As a chemoattractant medium, 10% FBS DMEM-F12 was used in the bottom chamber. Cells were incubated at 37°C for either 6 h or 12 h for migration or invasion assays, respectively. The cells were stained for 15 min with 1% crystal violet/20% methanol, after they were washed with water, and the sediment in the top chamber was carefully removed with cotton swabs. Cells were counted using ×10 objectives in five different fields on the underside of the insert. The mean number of cells was normalized to 1 using the control conditions and then plotted.

Gelatinase activity

A Biovision Gelatinase Fluorogenic Activity Assay kit (Millipore Sigma, Burlington, MA, USA) was used to assess the activity of gelatinase in cells. This assay uses a triple-helical collagen-like peptide that is highly selective for MMP2 and MMP9. This approach relies on the fluorescence resonance energy transfer (FRET) phenomenon. The fluorescence of the FRET peptide is quenched while it is intact and released only when the peptide is cleaved. Accordingly, this peptide is used as an indicator of MMP activity. The manufacturer protocol was followed. Finally, fluorescence was measured *via* a microplate reader (Synergy 2 Biotek) with excitation and emission wavelength of 320 and 405 nm, respectively.

Heterotopic xenograft assay and MRS1754 treatment

Resuspended in culture media mixed with cold liquid Matrigel (BD) were 1.5×10^6 GBM38 cells, and they were subcutaneously injected into athymic nude mice ($N =$ five mice per group). Tumor volumes were measured every two days with a caliper and treatment with MRS1754 started when the tumors reached $>25 \text{ mm}^3$, administering 160 ng per kg every 48 h. Mice were euthanized 20 days after the beginning of treatment, before the tumor burden became symptomatic.

RT-qPCR from tumor tissue samples

For qRT-PCR, total RNA from tumor samples was isolated by phenol–chloroform extraction. One microgram of isolated RNA was used for cDNA synthesis (High-Capacity cDNA Reverse Transcription Kit; Applied BioSystems). QRT-PCR reactions were performed in an optical 96-well plate equipped with a Bio-Rad CFX Connected Real-Time PCR Detection System, using SYBR Green. Gene expression levels were quantified using the primers listed in [Supplementary Table 1](#), while the β -actin housekeeping gene was used to normalize the data.

Immunofluorescence staining and quantification

Tumor pieces were fixed with 4% PFA, embedded in paraffin and cut into $3 \mu\text{m}$ sections. Tumor sections were immersed in a 10% blocking solution of the specific serum and then incubated (overnight, 4°C) in solutions containing the following primary antibodies: goat anti-mouse CD105 (R&D Systems, ref. AF1320), mouse anti-human vimentin (Santa Cruz Biotechnology, ref. sc-6260) and rabbit anti-VEGF (Abcam, ref. ab52917). Then, Alexa Fluor-conjugated secondary antibodies were used for 1 h (donkey anti-goat 568 and goat anti-mouse 660, LifeTechnologies, ref. A-11057 and A-21055, respectively; and donkey anti-rabbit 647, Abcam ref. ab150075), and the nuclei were counterstained with DAPI. Coverslips were mounted using Fluor-SaveTM reagent (Calcbiochem, ref. 345789). Five random areas of each tumor were imaged using a Leica Microsystems THUNDER 3D Assay Imager epifluorescence microscope. Immunofluorescence images were quantified with the open-source software Fiji version 2.3.051. The background was subtracted from CD105 and VEGFA raw intensity images, then the integrated intensity density of each image was multiplied by the number of pixels in the image and divided by the number of cells that were stained by DAPI.

Statistical analysis

GraphPad Prism[®] 8.01 software was used to perform the statistical analysis. Values are shown as mean $\pm$ S.D., where n indicates the number of independent experiments. A Student's t -test was applied for unpaired data. P - and P -adjusted values ≤ 0.05 were considered statistically significant. For *in vivo* analysis, data were assessed for normality with either the Shapiro–Wilk or Kolmogorov–Smirnov tests depending on the number of samples. Group comparison was performed with an unpaired, two-tailed T -test for data that passed the normality test or by the Mann–Whitney U -test for data that were not deemed normal. For both cases, p -values are represented as follows: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; differences among groups were not considered statistically significant when $p > 0.05$.

Results

The expression of A_{2B} adenosine receptor is associated with high glioma grade and lower patient survival

A_{2B}AR influence in glioma-patient survival was studied using data from Gliovis (including Oligodendroglioma, Oligoastrocytoma, Astrocytoma, and GBM). We performed Kaplan–Meier curves to explore correlations between A_{2B}AR expression and patient survival ([Figure 1A](#)). Patients with high A_{2B}AR levels have shorter overall survival than those who have low A_{2B}AR levels (Median Survival for Low A_{2B}AR 87.50 months vs High A_{2B}AR 32.50 months vs Low High (p -value = 0.001). Finally, A_{2B}AR levels increase with glioma tumor grade, with overexpression in GBM tissue ([Figure 1B](#)). These results suggest that high A_{2B}AR expression might be associated with progressive malignancy in gliomas and is specific to GBM.

A_{2B} adenosine receptor upregulation under hypoxic GSCs promotes *in vitro* invasiveness

Searching for A_{2B}AR expression sites in GBM tissue according to the Ivy database, we found that A_{2B}AR was predominantly expressed in GBM tissues located in invasion-related hypoxic regions such as the pseudopalisades and perinecrotic areas ([Figure 2A](#)). To confirm this, we evaluated mRNA and protein levels in GSCs obtained from three different cell types cultured under hypoxia: U87MG and two primary cultures (GBM38 and GBM27). A_{2B}AR mRNA ([Figure 2B](#)) and protein ([Figure 2C](#)) levels increased under hypoxia, suggesting its importance in GSC maintenance under low O_2 pressure.

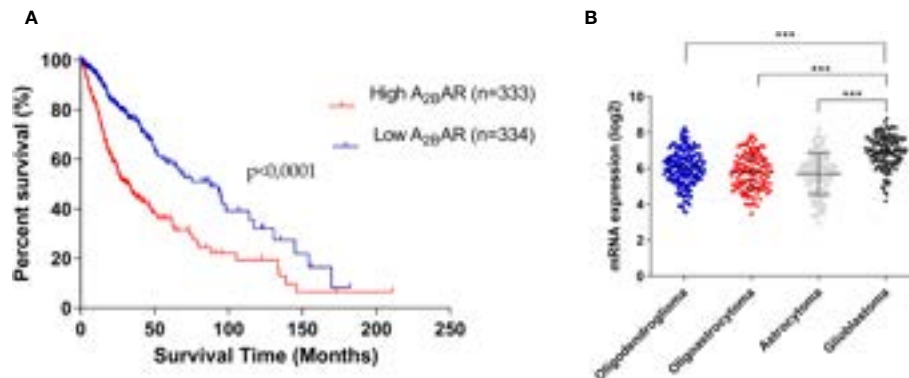


FIGURE 1

Overexpression of $A_{2B}AR$ is associated with worse GBM prognosis. **(A)** Kaplan–Meier analysis of the association between $A_{2B}AR$ expression and glioma patient survival (Median survival: low $A_{2B}AR$ expression 87.50 months vs high $A_{2B}AR$ expression 32.50 months. Cut-off used was the median of all samples (6.5722). p -value = 0.0001; n = 667). **(B)** $A_{2B}AR$ expression according to glioma grade (oligodendroglioma (n = 189), oligoastrocytoma (n = 126), astrocytoma (n = 191), and glioblastoma (n = 149). Statistical significance was determined by one-way ANOVA, *** p -value < 0.001.

Hypoxia has been identified as responsible for high GBM recurrence by enhancing infiltration into healthy brain tissue (25). For this reason, we evaluated the impact of hypoxia on GSC adhesion, migration, and cell invasion. To analyze cell adhesion, we performed a fibronectin-coated plate assay with U87MG, GBM38, and GBM27, demonstrating that hypoxia induced *in vitro* cell adherence of all cells (Figure 3A). To

assess migration and invasion capacities, transwell and transwell-matrigel assays were performed, respectively. As expected, migration and invasion of GSCs increased under hypoxia (Figure 3C). These results confirm that hypoxia, which is related to $A_{2B}AR$ overexpression, is also related to the GSC infiltrative phenotype. To further investigate the role of $A_{2B}AR$ signaling in GSCs, we used MRS1754 (50 nM), a

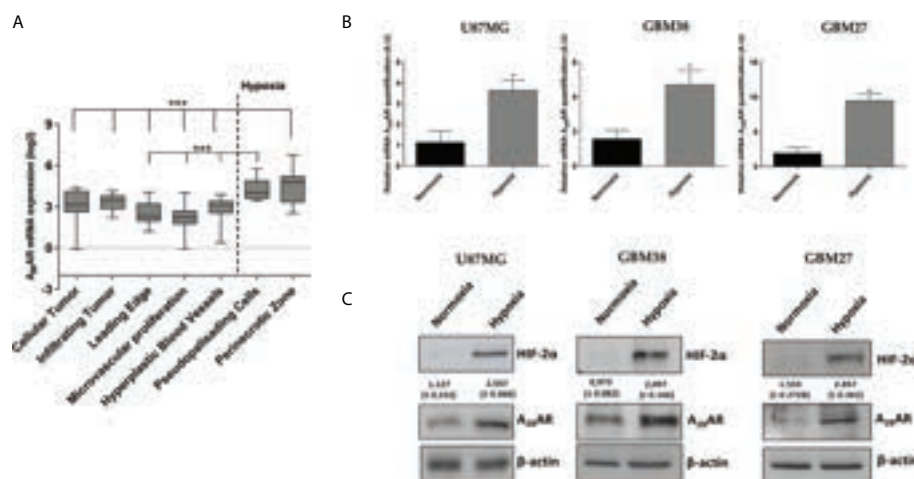


FIGURE 2

$A_{2B}AR$ expression increases under hypoxia conditions. **(A)** mRNA expression data were retrieved from the IVY Glioblastoma project and analysed for $A_{2B}AR$ expression (n = 55 samples). $A_{2B}AR$ mRNA was increased in the hypoxic pseudopalisades and peri-necrotic areas of glioblastoma (GBM) samples. Statistical significance was calculated by one-way ANOVA. (*** P < 0.001). **(B)** $A_{2B}AR$ mRNA levels were analyzed in three GSC lines (U87MG, GBM38, and GBM27) under normoxia and hypoxia conditions for 24 h. Values were normalized to β -actin mRNA expression (* p -value < 0.05; n = 3). Y-axis values are represented in arbitrary units (A.U.). **(C)** Representative Western blot of HIF-2 α , $A_{2B}AR$ and β -actin of the three GSC lines under normoxia and hypoxia for 24 h.

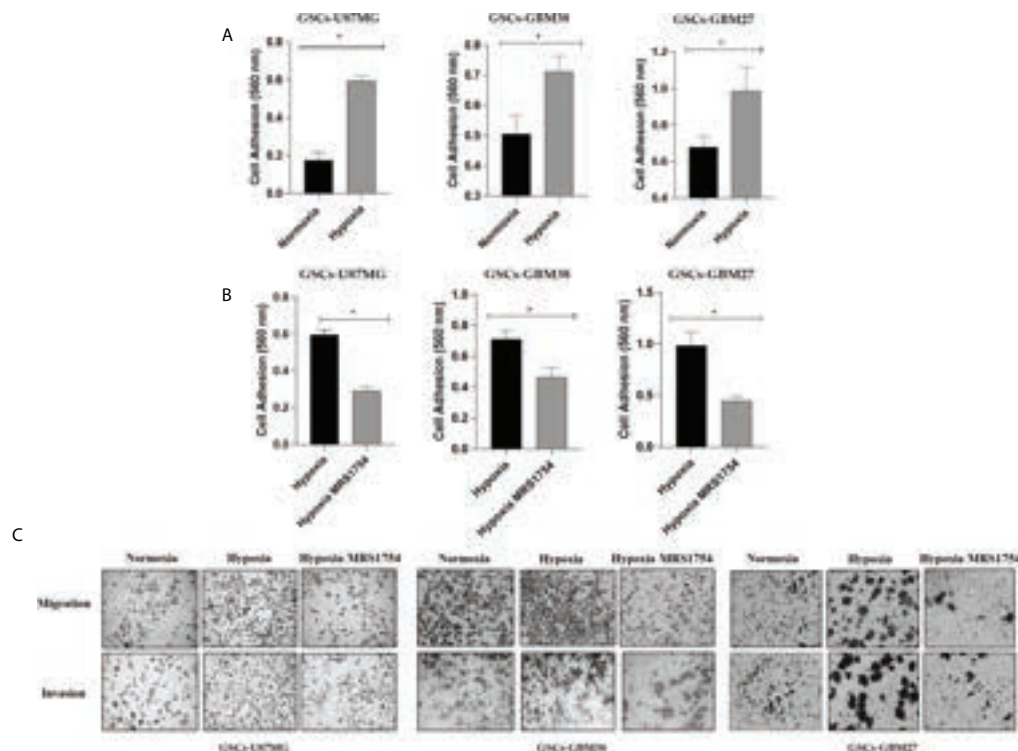


FIGURE 3

Hypoxia induces adhesion, migration, and invasion of GSCs. (A) Cell adhesion at 2 h in U87MG, GBM27, and GBM38 under normoxia (black box) and hypoxia (gray boxes) pre-incubated under hypoxia for 24 h. (B) Cell adhesion at 2 h in U87MG, GBM27, and GBM38 under hypoxia (black box) and hypoxia MRS1754 (gray boxes) pre-incubated under hypoxia for 24 h. Statistical significance was determined by Student t-test, *p-value <0.05. (C) Migration and Invasion (transwell and transwell matrigel-coated assay, respectively) in U87MG, GBM38, and GBM27 under hypoxia for 24 h.

selective $A_{2B}AR$ -antagonist (26). $A_{2B}AR$ signaling blockage under hypoxic conditions impaired cell adhesion, migration, and invasion of U87MG, GBM38, and GBM27 (Figures 3B, C). To rule out a cytotoxic effect of MRS1754 on GBM cells, we performed an MTS assay. We found that MRS1754 had no effect on GSC viability (Figure 4). All together, these results suggest that $A_{2B}AR$ signaling is a suitable target for decrease GSC hypoxia-related invasion.

$A_{2B}AR$ blockage decreases endothelial-mesenchymal transition markers and MMP9 activity *in vitro*

Invasiveness progression is associated with endothelial-mesenchymal transition (EMT) in different types of cancers, including GBM (27, 28). To study the role of $A_{2B}AR$ signaling on EMT, we evaluated mRNA expression of *SNAIL*, *TWIST1*, *CDH-1*, *CDH-2*, and *VIMENTIN* in all cell lines grown under hypoxia and treated with MRS1754. $A_{2B}AR$ blockage reduced mRNA levels of *TWIST1* and *SNAIL* in all GSCs under hypoxic conditions and decreased *VIMENTIN* levels and increased *CDH-1* in GSCs-

U87MG. Finally, *TWIST1* and *SNAIL* mRNA levels were downregulated under $A_{2B}AR$ blockage in hypoxic GSCs-GBM38 and GSCs-GBM27 (Figure 5). Matrix-metalloproteinase-9 (MMP-9) is relevant to GBM infiltration. As expected, $A_{2B}AR$ blockage decreased MMP9 protein expression (Figure 6A), which was related to downregulation of gelatinase activity (Figure 6B) under hypoxic conditions in all GSCs. All together, these results suggest that the invasion/migration of GBM cells through EMT gene expression and MMP9 activity is $A_{2B}AR$ -dependent under hypoxic conditions.

$A_{2B}AR$ blockage reduces *in vivo* GBM angiogenesis expression marker

Finally, we evaluated the blockage of $A_{2B}AR$ by MRS1754 in a heterotopic xenograft model subcutaneously injected with GBM38-derived GSCs. Like proliferation *in vitro* results, we did not observe any differences in tumor volume mass (Supplementary Figure 2) (Figure 7A). As we observed differences in the expression of *TWIST1* and *SNAIL* mRNA levels in GBM38s *in vitro*, we evaluated the expression of these same genes in our *in vivo* model. In contrast to our previous

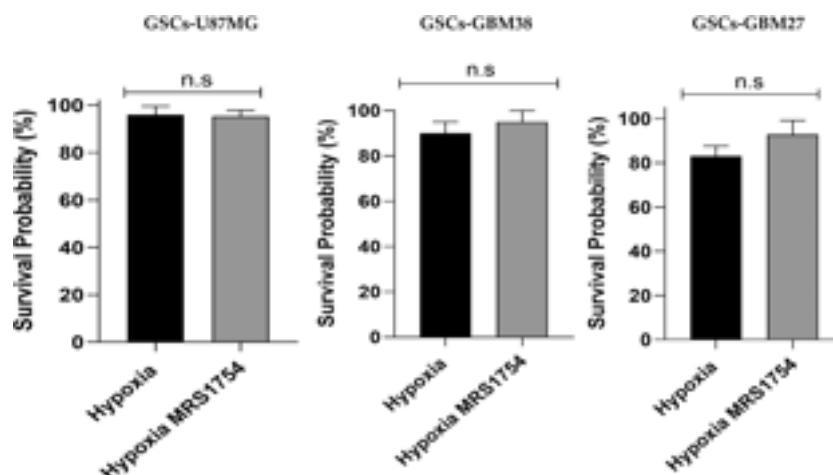


FIGURE 4

Effect of $A_{2B}AR$ pharmacological blockade on cell viability of glioblastoma stem-like cells under hypoxic conditions. The cell viability of U87MG, GBM38, and GBM27 was determined through the MTS assay. Ten thousand cells from each cell culture were seeded and cultured in the experimental conditions under which the transwell and transwell-matrigel assays were performed. The absorbance of the MTS was read at 490 nm on an ELISA plate reader, with values expressed as percentages. The graphs represent the mean $\pm$ SD. n.s., non-significant.

results, we did not observe differences in mRNA expression levels of *TWIST1* and *SNAIL* in the tumoral tissue treated with MRS1754 (Figure 7B). Despite this, due to the importance of $A_{2B}AR$ in the regulation of angiogenesis, which is strongly related to tumor maintenance and recurrence, we evaluated the expression of angiogenesis markers by immunofluorescence. Interestingly, CD105⁺ [Endoglin, associated with microvascular proliferation in GBM (29)] blood vessel formation and VEGFA marker decreased under $A_{2B}AR$ blockade *in vivo* (Figures 8A–E). Also, we observed a decrease in VEGFA mRNA levels, along with a lower expression of VEGFR in humans and mice (Figure 8F). All together, these results suggest that adenosine through $A_{2B}AR$ is involved in the regulation of angiogenesis in GBM tumors *in vivo*.

Discussion

In the last few years, a subpopulation called GSCs has been identified as being responsible for the aggressiveness of GBM. These GSCs present stem-cell properties like self-renewal and multi-lineage differentiation, but GSCs can initiate the formation of the tumour. Different studies have implicated these GSCs in the relapse of GBM patients due to their high infiltrative capability (30). GSCs reside in a specific tumor microenvironment (TME), which promotes the maintenance of this type of cell. We previously reported that extracellular adenosine levels increased in GSCs when under hypoxia conditions (14). Adenosine can activate A_1 , A_{2A} , and A_3 receptors, which are classified as high affinity

receptors that activate at mM adenosine concentrations, but it has been reported that $A_{2B}AR$ is only a low affinity receptor (12), which is activated by high extracellular adenosine levels observed under hypoxia (31, 32). Despite the importance of $A_{2B}AR$ signaling on cell adaptation to low oxygenation levels, the role of $A_{2B}AR$ on GSC invasiveness under hypoxia has not been described. Our analyses showed $A_{2B}AR$ levels correlated with shorter survival times in patients, and that $A_{2B}AR$ was overexpressed in GBM, especially in hypoxic regions like pseudopalisades and the perinecrotic zone, which have been associated with a high invasive potential of the GBM cells (33, 34). To validate this analysis, we incubated GSCs derived from three different GBM cells under hypoxic (0.5% O_2) conditions. We observed that mRNA and protein levels of $A_{2B}AR$ increased in GSCs cultured under hypoxic conditions. These results are consistent with those previously described by other authors, which indicate that the expression of $A_{2B}AR$ is associated with stressful and physio-pathological conditions such as low oxygen tension, which induces an increase in adenosine levels (35). As hypoxic niches have been considered a poor prognostic marker for GBM (36), it has been demonstrated that cells found in areas with low oxygenation increase their invasiveness by 60% compared to cells located in areas of higher oxygenation (37), and similar results were observed in our experimental assay. In this work, we show that pharmacological blockade of $A_{2B}AR$ significantly reduces the adhesion of GSCs, suggesting that the signaling of this receptor could have an impact on the regulation of integrins. In fact, there is some evidence suggesting that adenosine can regulate the expression and

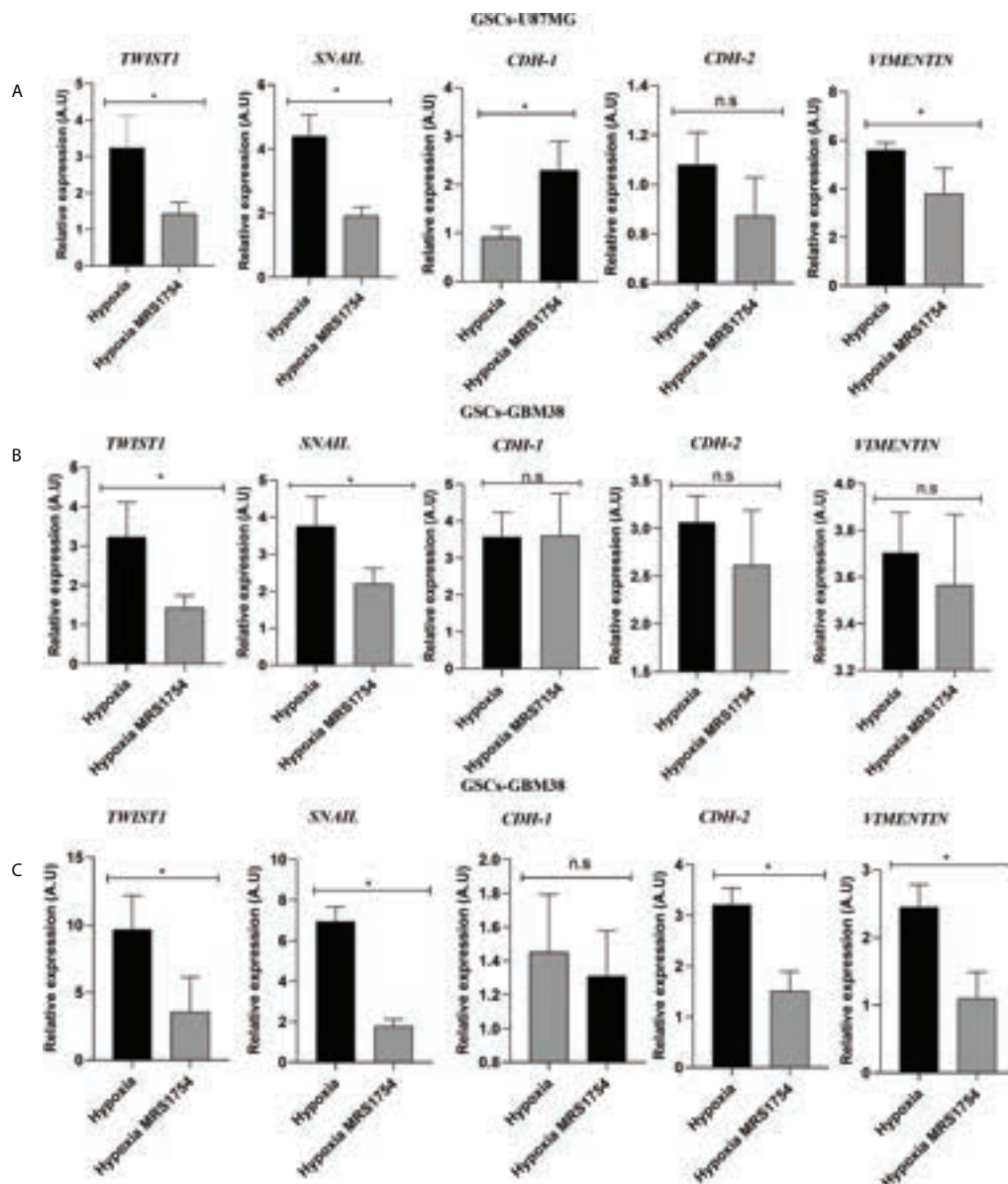


FIGURE 5

The A2B Adenosine Receptor controls the expression of Epithelial–Mesenchymal Transition markers in Glioblastoma Stem-like Cells under hypoxia. RT-qPCR of *TWIST1*, *SNAIL*, *CDH-1*, *CDH-2*, and *VIMENTIN* in GSCs derived from (A) U87MG, (B) GBM27, and (C) GBM38 under hypoxia and hypoxia MRS1754 from 24 h. Values were normalized to β -actin mRNA expression. Y-axis values are represented in arbitrary units (A.U.). Statistical significance was determined by the Student t-test; *p-value <0.05; n = 3.; n.s., non-significant.

localization of this type of surface protein in different cellular models (38). Yi et al. showed MRS1754 reduces migration and invasion of renal carcinoma (39). Meanwhile, increasing evidence suggests Ecto-5'-nucleotidase (CD73) derived adenosine also enhances tumor cell migration via $A_{2B}R$ (40). Also, the expression of $A_{2B}R$ in a metastatic colorectal cancer cell line (SW620) is significantly higher than in a non-metastatic model (SW480) (41), showing the regulatory role of $A_{2B}R$ in cancer cell invasion.

In GBM, despite being a non-epithelial neoplasia, there is clear evidence that cells comprising that type of malignant brain tumor are capable of an EMT-like process (27, 28). Mikheev et al. evaluated the gene expression of 85 biopsies derived from patients diagnosed with GBM, where the transcription factor Twist, which regulates the expression of genes involved in EMT, was overexpressed compared to healthy tissue sections, demonstrating the importance of this transcription factor as a modulator of the cell invasion

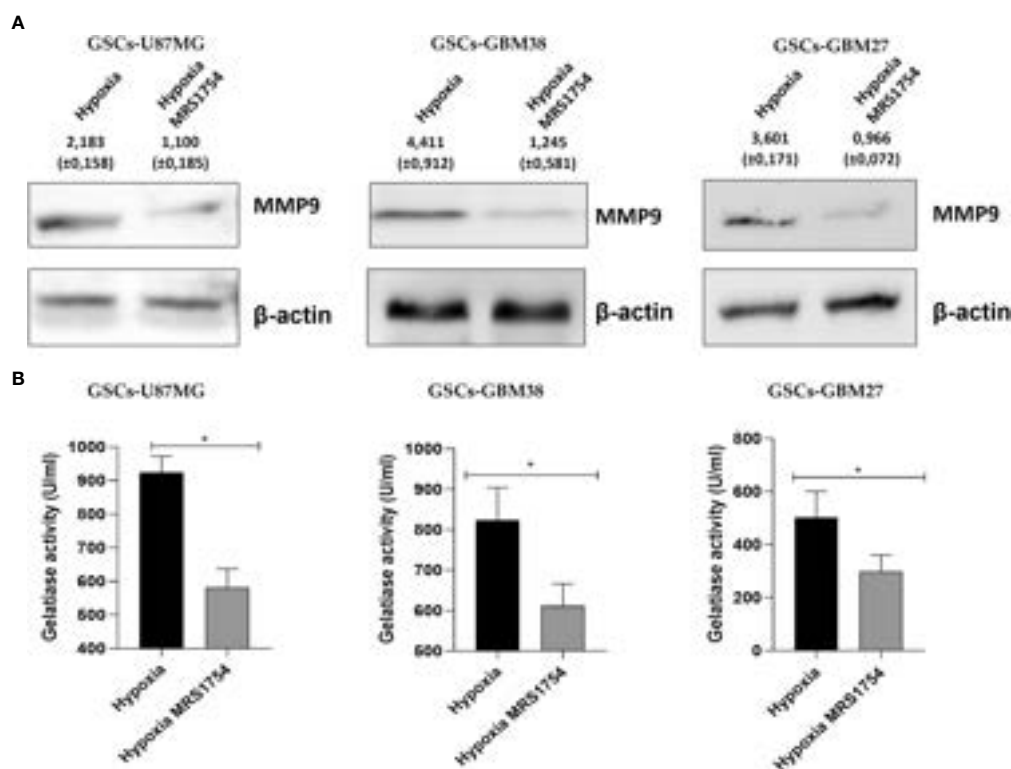


FIGURE 6

MRS1754 reduces MMP9 protein levels and gelatinase activity in GSCs treated with MRS1754 under hypoxia conditions. (A) Immunoblot of MMP-9 from total lysate of U87MG, GBM27, and GBM38 under hypoxia and hypoxia MRS1754. β-actin was used as a loading control. (B) Quantification of gelatinase activity from U87MG, GBM27, and GBM38 incubated under hypoxia and hypoxia MRS1754. The assays were performed according to the kit protocol. Statistical significance was determined by the Student t-test, * $p < 0.05$; $n = 3$.

process in GBM (42). It has been previously described that adenosine may participate in promoting GBM invasion (14, 43, 44). However, the role of A_{2B} AR signaling on EMT marker expression has not yet been described. These results indicate that A_{2B} AR can regulate the expression of Twist1 and Snail, but we can appreciate different impacts on other EMT markers like *CDH-1* and *CDH-2*. Also, A_{2B} AR blockage decreases the expression and activity of MMP-9, which is important for GBM infiltration of non-GSCs GBM cells into healthy brain tissue (45). This evidence and our results suggest a role for A_{2B} AR in enhancing the infiltrative phenotype of GBM and GSCs. Although this is the first evidence of the role of A_{2B} AR in the EMT process in GSCs, the role of this receptor in the regulation of EMT has been previously reported. It has been previously described that A_{2B} AR activation decreased the expression of epithelial markers (E-cadherin) and enhanced mesenchymal markers (Vimentin, N-cadherin) during pulmonary fibrosis (46). In the case of GSCs, our research group has already demonstrated that adenosine can regulate the expression of some EMT markers, impacting on the invasive capacity of

GSCs, specifically through A_3 AR (14). In the same way, it has been described that adenosine, through A_3 AR activation, regulates the EMT process in head and neck cancers because the blockade of this adenosine receptor decreased the expression of proteins such as Vimentin, Zeb, Slug, and Twist1, which are essential during the EMT process and the invasiveness of this type of neoplasia (47). A similar effect was reported by Tsiampali et al. in which they described that adenosine production by Ecto-5'-nucleotidase/CD73 was capable of activating EMT through increased expression of Snail and ZEB1 via the activation of A_3 AR (48). The latter study only focused on differentiated cells, but nevertheless, it provides evidence for the importance of adenosine signalling in EMT.

Despite the heterogeneity presented by GBM cells, pharmacological blockage of A_{2B} AR decreased invasiveness in all three cell lines under hypoxic conditions. Clear evidence exists that hypoxia can provoke a molecular shift in GSCs toward the mesenchymal (MES) subtype. Bao et al. observed that GBM cell lines that do not have mesenchymal characteristics, when cultured under hypoxia, induce the

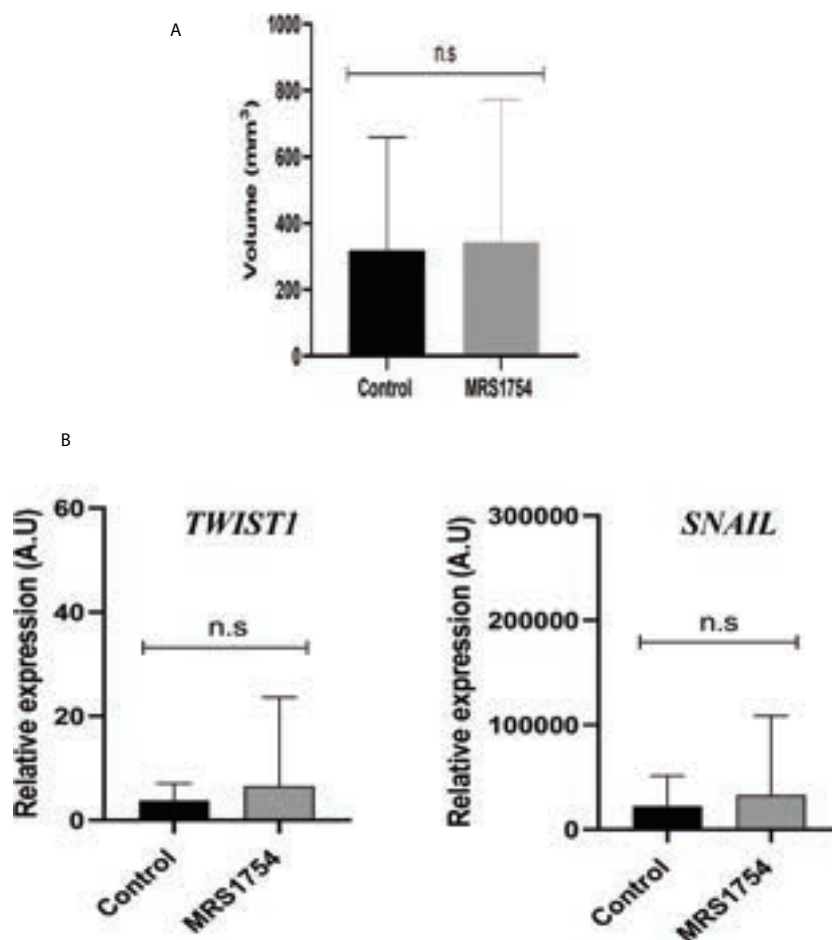


FIGURE 7

MRS1754 does not reduce the tumor volume and EMT marker expression *in vivo* on subcutaneous GBM38. Tumors were generated by subcutaneously implanting GBM38 GSCs in nude mice. (A) Graph of tumour size (mm³) of *in vivo* treatment. Treatments started when the tumour reached a size of >25 mm³, injecting 1x PBS-0.001% DMSO (Vehicle) or MRS1754 160 ng/kg/48 h in 0.01% DMSO (treatment). n.s., non-significant. (B) RT-qPCR of *TWIST1* and *SNAIL1* from tumor tissue. Values were normalized to β -actin mRNA expression.

expression of the MES gene within the higher invasive capacity (49). It is possible to suggest that hypoxia, through A_{2B}AR, induces an invasive phenotypic change, such as the mesenchymal type. In fact, the increased expression of A_{2B}AR was related to the MES subtype of GBM (Supplementary Figure 2).

Although we did not observe differences in the expression of EMT markers, we observed that tumors treated with MRS1754 exhibited a reduced formation of blood vessels, in addition to a lower expression of VEGF and VEGFR. Endothelial cells express high levels of A_{2B}AR, suggesting its potentially critical role in promoting angiogenesis (50). Du et al. showed that the activation of A_{2B}AR by NECA significantly increased the intracellular cAMP levels and concomitant CREB phosphorylation, eventually leading to the production of VEGF in HMEC-1 (50). Also, in the

tumoral microenvironment, A_{2B}AR activation is involved in the synthesis of VEGF and IL-8 in endothelial cells and stromal tumor cells (51). In melanoma tissues, A_{2B}AR receptor activation induces an increase in VEGF by activating the transcription factor STAT3 (52). In a GBM cell line derived from a mouse line expressing A_{2B}AR, this receptor is highly upregulated, leading to angiogenesis (52).

Our results suggest an eventual therapy focused on adenosine signaling, mainly through blocking A_{2B}AR, since its low affinity for adenosine postulates it as a potential target for pathological conditions, such as cancer (52). In addition to our observations, the blockade of A_{2B}AR signaling significantly decreases the invasive and angiogenic potential of GSCs, it making it an attractive therapeutic target to decrease the high recurrence of GBM. However, we must emphasize that our study has some limitations, mainly

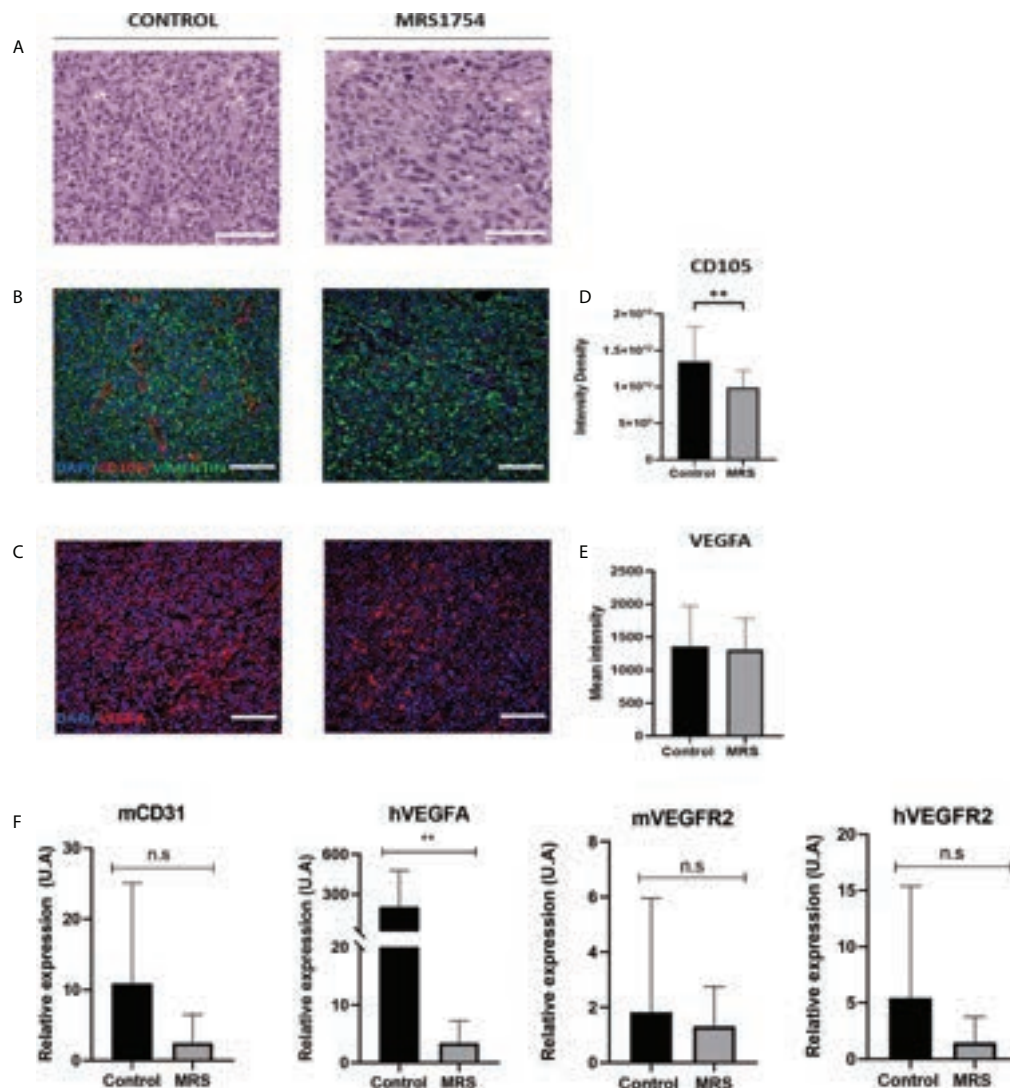


FIGURE 8

MRS1754 reduces tumor blood vessel formation and angiogenesis marker expression *in vivo* on GBM38. (A) H&E staining of representative sections of tumors from control and MRS treatment groups. Scale bar is 200 μ m. (B) Immunofluorescence images of representative areas from control and MRS treatment groups. Stained with DAPI (in blue), CD105 (in red), and Vimentin (in green). The scale bar is 100 μ m. (C) Immunofluorescence images of representative areas from control and MRS treatment groups. Stained with DAPI (in blue) and VEGFA (in red). The scale bar is 100 μ m. (D) Quantification of CD105 levels in immunofluorescence images. Shown here is the integrated intensity density of CD105 in the control and MRS treated groups. **p value < 0.01. (E) Quantification of VEGFA levels in immunofluorescence images. Shown here is the mean intensity of VEGFA in the control and MRS treated groups. (F) mRNA expression levels of mCD31, hVEGFA, hVEGFR2, and mVEGFR2 in both control and MRS treatment groups. **p value < 0.01. n.s., non-significant.

focusing on our subcutaneous models because they do not represent the tumor context of GBM. For this reason, it is of utmost importance to use GBM models such as the intracranial mouse model or the use of organoid models (53, 54). This will allow us to have a more complete view of the role of A_{2B}AR on the invasiveness of GSCs and to design a more effective treatment. As a projection, we propose an eventual therapy targeting the extracellular adenosine axis, which would not only affect the cells that surround the tumor

but also GSCs in the hypoxic niches that are responsible for the progression and recurrence of GBM.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the ANID.

Author contributions

JE conceived the project and designed all the experiments. JE, AU-O, and MT carried out most *in vitro* experiment. NR, JN, AMS, and AAS carried out the *in vivo* experiment. JE, IN, CQ, RS, and NR wrote and revised the manuscript. Data analyses were performed by JE, AU-O, MT, IN, and CQ. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

The study was supported by the Fondecyt regular 1200885, (CQ-M) Agencia Nacional de Investigación y Desarrollo (ANID)—Millennium Science Initiative Program—ICN09_016/ICN 2021_045: Millennium Institute on Immunology and Immunotherapy (ICN09_016/ICN 2021_045; former P09/016- F), (CQ-M), Vicerrectoría de Investigación, Desarrollo y Creación artística VIDCA-UACH and the CONICYT Postgraduate scholarship N° 21181983 (JIE). Also, this work was supported in part by grants from the

“Fondo de Investigaciones Sanitarias” (FIS) (PI21/01353) and the Ministerio de Economía y Competitividad–FEDERER (RTC-2019-6918-1)(AA-S).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2022.969993/full#supplementary-material>

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Infectious bursal disease virus (IBDV) as a novel oncolytic virotherapy in glioblastoma

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To cite: Tur-Planells V, Bykov Y, Dawodu G, *et al.* Infectious bursal disease virus (IBDV) as a novel oncolytic virotherapy in glioblastoma. *Journal for ImmunoTherapy of Cancer* 2025;13:e011741. doi:10.1136/jitc-2025-011741

► Additional supplemental material is published online only. To view, please visit the journal online (<https://doi.org/10.1136/jitc-2025-011741>).

Accepted 13 October 2025



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ABSTRACT

Background Glioblastoma (GBM) is the most aggressive form of cancer of the central nervous system. Despite advances in immunotherapies and standard-of-care treatments for GBMs, clinical outcomes remain limited—owing to the immunosuppressive tumor microenvironment and the intrinsic resistance of GBM to conventional approaches. As a result, there is growing interest in rational combination strategies, particularly those pairing oncolytic viruses with immune-based therapies or established treatment modalities. Oncolytic viruses, by displaying conditionally enabled tumor cell-restricted replication, while stimulating antitumor immune responses and leaving healthy tissue unharmed, have the potential to reshape the therapeutic landscape in GBM and aid in achieving more durable benefits for patients. This study investigates the use of infectious bursal disease virus (IBDV) as a potential virotherapy for GBM.

Methods and results In vitro, IBDV infects and replicates within murine GBM cells and patient-derived GBM stem cells, inducing direct oncolysis and activating proinflammatory gene expression programs. IBDV also enhances the cytolytic activity of temozolomide (TMZ) in treated GBM cells, complementing TMZ chemotherapeutic activity. In vivo, treatment with IBDV in CT-2A GBM-bearing syngeneic mice significantly reduced tumor growth and improved survival compared with control mice. Intratumoral administration of IBDV induces a deep remodeling of the tumor immune microenvironment, reducing immunosuppressive M2-like macrophages and increasing the ratio of CD8+T cells to regulatory T cells. This reversion of immunosuppression linked to monocyte-derived macrophages has been confirmed on experimental ex vivo infections of explants derived from human GBM donors.

Conclusion These findings support further consideration of IBDV as a novel virotherapeutic agent for GBM.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Glioblastoma (GBM) is the most aggressive primary brain tumor, with limited treatment options and a median survival of only 15 months despite standard care with surgery, radiotherapy, and temozolomide (TMZ). Resistance to TMZ and the immunosuppressive tumor microenvironment (TME) present significant challenges to current treatments. Oncolytic viruses (OVs) have shown promise as a therapeutic strategy by selectively infecting tumor cells and activating potent immune responses.

WHAT THIS STUDY ADDS

⇒ This study introduces infectious bursal disease virus (IBDV) as a novel oncolytic virotherapy for GBM. IBDV effectively infects and replicates in patient-derived glioblastoma stem cells and murine GBM cells, inducing tumor cell death and immune activation. The virus synergizes with TMZ to enhance cytotoxic effects and remodels the TME by reducing immunosuppressive cells, such as M2-like macrophages and regulatory T cells, while increasing cytotoxic CD8+T cells. These findings establish IBDV as a safe and potent virotherapy candidate.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ IBDV's ability to complement TMZ, reduce immunosuppression, and activate antitumor immunity positions it as a promising addition to GBM treatment regimens. Its non-human origin and that avoids pre-existing immunity issues seen with other OVs in the general population. This study lays the groundwork for further preclinical and clinical research on IBDV, potentially leading to innovative combination therapies that improve patient with GBM outcomes and inform policies on oncolytic virotherapy integration into standard cancer care.

INTRODUCTION

Glioblastoma (GBM) is a diffuse astrocytic-oligodendroglial tumor. It is the most aggressive malignant primary brain tumor, which accounts for 77%–81% of all primary malignant central nervous system (CNS) tumors.¹ It is classified as a Grade 4 glioma in the latest version of the WHO (CNS5), based on molecular and histopathological features.² GBM is characterized by cytologically malignant, highly mitotic, and necrosis-prone neoplasms, typically associated with a high proliferation rate and rapid disease progression both before and after surgery, leading to a fatal outcome. Current treatment consists of a combination of surgery, radiotherapy, and chemotherapy with the DNA alkylating agent temozolomide (TMZ). However, despite advances in clinical diagnosis and the development of novel therapies, no effective treatment exists, and the overall survival remains at 15 months.³ Several factors contribute to the failure of current treatments, including TMZ resistance pathways,⁴ high invasive and infiltrative potential, significant intratumoral and intertumoral heterogeneity, limited access to chemotherapeutic drugs like TMZ to the CNS due to the presence of the blood-brain barrier, low permeability of blood vessels within the tumor mass,⁵ a highly immunosuppressive tumor microenvironment (TME),⁶ and the presence of a tumor cell subpopulation called cancer stem cells (CSCs).⁷

The study of CSCs has gained relevance due to their stem cell-like properties and their implications in tumor development and cancer prognosis. CSCs are responsible for GBM tumor dissemination through brain parenchyma,⁸ contributing to glioma genesis, as well as drug resistance, metastasis, and tumor recurrence.⁹ Additionally, various mutations have been identified in these tumors. Particularly interesting are the heterozygous or homozygous deletions at Ch9p21.¹⁰ These deletions involve the genetic loss of the cyclin-dependent kinase inhibitor 2A gene (CDKN2A).¹¹ The type I interferon (IFN) gene cluster is in the same chromosomal region as CDKN2A, and co-deletion of both, CDKN2A and the type I IFN cluster, is common.^{12,13} IFN activity functions as one of the primary immune defense mechanisms against viral infections. The absence of IFN type I cluster may render these tumors more permissive to viral replication and potentially to oncolytic therapies.¹⁴ Therefore, it is crucial to develop new therapeutic approaches that address both the limited access to the CNS and the genetic characteristics of the tumors.

Oncolytic viruses (OVs) represent a novel class of multi-mechanistic therapeutic agents for cancer treatment. OVs display conditionally enabled, tumor cell-restricted replication while stimulating antitumor immune responses and leaving healthy tissue unharmed.¹⁵ The susceptibility of cancer cells to support viral replication depends on unique molecular features mainly related to dysfunction in antiviral signaling pathways, while OVs remain inactive or unable to replicate in normal, healthy cells—where the antiviral defense machinery remains intact.^{16,17} The death

of tumor cells leads to the release of tumor antigens, cytokines and chemokines that stimulate the recruitment and activation of innate cells, leading to a deep remodeling of the immunosuppressive TME; importantly, the recruitment and activation of antigen-presenting cells (APCs) capable of presenting both tumor and viral epitopes is critical to achieve T cell-specific tumor elimination.¹⁸ Numerous preclinical and early-phase clinical studies (phase I and II) have explored both DNA and RNA OVs.¹⁹ A recent example is the conditional approval of an attenuated type I herpes simplex virus (Delytact) in Japan to treat GBM.²⁰ As of today, the major challenges facing OVs in GBM treatment are linked to the highly immunosuppressive microenvironment of GBM often limits viral infection, spread, and the induction of robust antitumor immune responses, as well as pre-existing antiviral immunity that can lead to rapid immune clearance of the virus and reduce treatment efficacy.^{21,22}

Infectious bursal disease virus (IBDV) is a bi-segmented double-stranded RNA virus that belongs to the genus *Avibirnavirus* within the family Birnaviridae. IBDV primarily infects young domestic chickens, targeting immature IgM-bearing B lymphocytes localized in the bursa of Fabricius through several proposed surface proteins, including surface immunoglobulin M, integrin $\alpha 4 \beta 1$, and heat shock protein 90,^{23,24} which facilitate viral attachment, internalization, and uncoating. IBDV can also infect macrophages and monocytes, although to a lesser extent.²⁵ As a result, chickens develop immunosuppression, making them more susceptible to opportunistic and secondary infections that can exacerbate disease severity.

To date, there have been no reports of IBDV causing infections in mammals, including humans.²⁶ Previous studies have explored whether IBDV can infect and replicate in mammalian cell lines—under laboratory conditions—demonstrating that IBDV typically does not establish productive infection or cytopathic effect in mammalian cells, and many IBDV strains require adaptation through serial passages to replicate efficiently in mammalian cells.^{27–29} In the case of IBDV, this restriction is likely due to the incompatibility of viral entry mechanisms, the absence of the required host factors for replication, and strong mammalian antiviral defense. Yet, to date, only one report has shown IBDV ability to infect human cervix adenocarcinoma HeLa cells.³⁰

In this work, we investigate the potential of IBDV as OV. As of today, several avian viruses are under investigation for cancer therapy, including Newcastle disease virus (APMV-1, Avian avulavirus 1, aka Avian paramyxovirus 1),^{31,32} avian reoviruses,³³ avian avulavirus (APMV-4)³⁴ and certain avian influenza strains.³⁵ Research into avian viruses as cancer therapeutics is robust and expanding, as their inherent oncolytic features, combined with host restrictions and lack of pre-existing immunity in humans, are major advantages to be used as therapeutics in human cancer. Furthermore, IBDV has also been investigated for its potential broad-spectrum antiviral effects

in non-oncologic contexts, including the post-infection treatment of acute and chronic viral diseases, reinforcing its safety and therapeutic versatility beyond cancer.³⁶

Here, we have studied responses elicited by IBDV in different models of GBM. In addition to inducing tumor cell oncolysis, IBDV stimulates antiviral and proinflammatory immune responses in different glioma cells. In vitro, the susceptibility of GBM cells to IBDV infection and killing is further boosted in combination with the chemotherapeutic TMZ—current standard of care in GBM management. In preclinical studies in mice, intratumoral administration of IBDV delays tumor growth, concomitant to a robust immune cell remodeling at the TME. In human explant studies, short exposure to IBDV demonstrates a reduction of the immunosuppressive nature of GBM TME.

MATERIAL AND METHODS

Cell lines and viruses

Murine glioma cell lines CT-2A (ATCC cat# SCC194) and GL261 (ATCC cat# ACC 802) were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F-12 supplemented with 10% FBS (Fetal Bovine Serum), 1% antibiotics, and 2mM of L-glutamine. Human patient-derived primary GBM cells (GBM18 and GBM27) were obtained according to García-Romero, González-Tejedo *et al*, and subcultured in supplemented DMEM/F-12 (Gibco, 11039) containing non-essential amino acids (10mM; Gibco, 11140), Hepes (1 M; Gibco, 15630), D-glucose (45%; Sigma, St. Louis, Missouri, USA; G8769), BSA-F5 (7.5%; Gibco, 15260), sodium pyruvate (100mM; Gibco, 11360), L-glutamine (200mM; Gibco, 25030), antibiotic-antimycotic (100×; Gibco, 15240), N2 supplement (100×; Gibco, 17502), hydrocortisone (1 µg/µL; Sigma, H0135), tri-iodothyronine (100 µg/mL; Sigma, T5516), EGF (25 ng/µL; Sigma, E9644), bFGF (25 ng/µL; Sigma, F0291), and heparin (1 µg/µL; Sigma, H3393). QM7 cells (ATCC cat# CRL-1962) were cultured in DMEM supplemented with 10% FBS. Murine BMDMs (Bone Marrow-Derived Macrophages) were isolated from the femur, tibia, and fibula of bone marrow of 8-week-old C57BL/6 mice. Bone marrow was liberated through the injection of phosphate-buffered saline (PBS) and clogs were disaggregated mechanically by a syringe. Afterwards, cells were centrifuged for 5 min at 1,500 rpm and the supernatant was discarded. Finally, cells were resuspended in RPMI (Roswell Park Memorial Institute medium) medium supplemented with GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor) (Gibco, Waltham, Massachusetts, USA) (30 ng/mL) before plating. After 4 days, cell medium was replaced with a fresh stock for 3 more days to complete cell differentiation before viral infections. The human microglia HMC3 cell line (ATCC cat# CRL-3304) was maintained in DMEM supplemented with 10% FBS. Cells were maintained at 37°C with 5% of CO₂ and 90% humidity.

IBDV, Soroa strain, and serotype I, being low pathogenic in birds, were provided by Professor José F Rodríguez (CNB-CSIC, Spain); viral stocks were propagated in QM7 cells and cleared-purified following the protocol previously described.³⁷ The recombinant Newcastle disease virus LaSota strain NDV-LS-L289A, was propagated in 9-day-old chicken-embryonated eggs and clear-purified by ultracentrifugation in 30% sucrose gradient.

Fluorescence microscopy and image acquisition

Cancer cells were infected at the indicated multiplicity of infection (MOI) for 24 hours. Cell fixation was performed using 2% paraformaldehyde in PBS for 10 min. Cell membrane permeabilization was carried out using 0.2% Triton-PBS for 10 min and blocked in PBS 1% BSA (Bovine Serum Albumin) for 1 hour. Specific antibodies used for indirect immune detection are described in online supplemental table 1. Images were taken using an EVOS FL cell imaging system (Thermo Fisher Scientific).

Virus replication kinetics and titrating assays

Supernatants of IBDV-infected GBM cells at corresponding time points were titrated

in QM7 cells plated the day before in 96-well plates in DMEM supplemented medium. IBDV supernatants were diluted by 1/10 dilutions in DMEM supplemented medium and left on QM7 cells for 72 hours. Negative control wells had GBM supernatants without IBDV infection. The cytopathic effect was analyzed by optical microscopy 72 hours post-infection. Tissue culture infectious dose 50 (TCID₅₀/mL) viral titers were calculated using the Reed-Muench method.³⁸

Viability assays

An (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) MTS assay was used to assess cell viability. A total of 1×10⁴ cells per well were plated in a 96-well plate containing complete medium and allowed to adhere for 24 hours. After attachment, cells were treated with TMZ or infected with IBDV or NDV-LS viruses for the corresponding experimental conditions. PBS was used as a control, with five replicate wells per condition. After the designated treatment time, 20 µL of MTS solution (5 mg/mL) was added to each well. The plates were then incubated at 37°C, protected from light, for 1.5 hours. Following incubation, the solutions in the wells were mixed, and absorbance was measured at 450 nm (for tetrazolium salts) and 630 nm (for background) using a microplate reader (Varioskan Lux). Relative cell viability was calculated as the percentage of absorbance compared with the control group.

Western blots

Protein was extracted from the cultured cells with radio-immunoprecipitation lysis buffer on ice and centrifuged at 12,000×g for 15 min at 4°C to eliminate cell debris. The total protein concentration was determined using a Bicinchoninic Acid (BCA) protein assay kit, and whole lysates mixed with 5×sodium dodecyl sulfate (SDS)

loading buffer were denatured with a 5 min incubation at 95°C. Then, equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Blocked membranes were then incubated with their corresponding antibodies described in online supplemental table 1. The horseradish peroxidase immune complexes were detected using an enhanced chemiluminescence kit (Alkali Scientific) in a ChemiDoc imaging system (Bio-Rad).

Transcription analysis by qRT-PCR

Cells were mock treated or infected with the specified virus at an MOI of 3 Plack formation units (PFU)/cell in 500 µL of OptiMEM-I. After allowing virus adsorption for 1 hour, the cells were incubated with an additional 1,500 µL of supplemented media. Total RNA was isolated using a Qiagen RNeasy Minikit (catalog no.74106, Qiagen) at the indicated time post-infection. Complementary DNA (cDNA) synthesis was performed using the Maxima First Strand cDNA Synthesis Kit for quantitative Real-time reverse transcription-polymerase chain reaction (RT-PCR) (catalog no. K1671, Thermo Fisher Scientific). Mean n-fold expression levels of cDNA from three individual biological samples were normalized to housekeeping gene levels and calibrated to mock-treated samples according to the $2^{-\Delta\Delta Ct}$ method.³⁹ Results are expressed as relative cycle threshold (Ct) values to the mock-treated group. Human and murine primer sequences are compiled in online supplemental table 2.

Histological staining

Spleen, bone marrow, lymph nodes, and liver were harvested in 9-week-old C57BL/6 mice after 24 hours of one IBDV (10^7 PFU) tail vein injection for systemic administration (n=3). PBS was used for negative controls. Tissue fragments were fixed in a 4% buffered formalin solution, pH 7.2, for 24 hours. The buffer was changed to ethanol solution 70% and organs were embedded in paraffin. Paraffin blocks of each organ were cut in a microtome (3 µm slices) and mounted on slides prior to routine histological analysis (H&E). Histological slides were qualitatively analyzed under light microscopy.

Systemic and intracranial virus delivery studies

9-week-old C57BL/6 mice (Charles River Laboratories, Wilmington, Massachusetts, USA) were stereotactically injected with viral doses of 10^4 , 10^5 , and 10^6 PFU into the striatum of the right hemisphere (0 mm anterior and 2.5 mm lateral to the bregma; 3.5 mm intraparenchymal). PBS was used as a control vehicle. Mice weight was monitored weekly for 6 weeks. Procedures used on mice were approved by and performed according to the guidelines of the institutional animal-care committee of Universidad Francisco Vitoria under the accession PROEX 142.8/24.

The protocol for biodistribution studies was approved by Universidad de Leon under the access OEBA-ULE-001-2023.

Tumor model

Tumor progression and survival studies were performed following Institutional Animal Care and Use Committee (IACUC) guidelines and have been approved by the IACUC of Icahn School of Medicine at Mount Sinai (IACUC-2014-0234). 6–8 weeks old of age female C57BL/6J mice used in our in vivo studies were purchased from Jackson Laboratory.

1.5×10^6 CT-2A cells were intradermally engrafted on the right hind leg and tumors were allowed to grow to 50 mm^3 before treatment initiation. Tumor-bearing mice were treated by intratumoral injection of IBDV, NDV, or PBS with viral doses of 1×10^7 PFU/50 µL PBS. Intratumoral injections were administered every other day for a total of four treatment doses or as specified for downstream analysis or tissue samples. Tumor volume was monitored every 48 hours, and every 24 hours as tumor volumes approached the experimental endpoint of $1,000 \text{ mm}^3$. Mice were humanely euthanized on the day when the volume exceeded the predefined endpoint or at any sign of distress, including tumor ulceration. Tumor measurement was determined using a digital caliper, and the total volume was calculated using the formula: tumor volume (V)= $L \times W$, where L (tumor length) is the larger diameter and W (tumor width) is the smallest diameter.

Human GBM explants study

Human GBM-isocitrate dehydrogenase (IDH) wild-type tumor biopsy was obtained from the Biobank of Hospital Universitario Puerta de Hierro Majadahonda/Instituto de Investigación Sanitaria Puerta de Hierro-Segovia de Arana. Ethical approval for the use of these samples was granted by the institutional review board of HM Hospitals (CEIm No: 23.06.2206-GHM). All patients provided written informed consent prior to enrollment, and all procedures were conducted in accordance with the principles of the Declaration of Helsinki. Fresh tumor samples were placed in ice-cold Hibernate-A medium supplemented with penicillin-streptomycin immediately after resection and processed within 2 hours of surgery. GBM tissue was cut into $20\text{--}30 \text{ mm}^3$ fragments under a dissection microscope, washed three times with ice-cold PBS, and then placed in 24-well plates containing specialized growth medium (DMEM-F12, Neurobasal, non-essential amino acids, N2 supplement, B27 supplement, human insulin, penicillin-streptomycin, and 2-mercaptoethanol). The tissue slices were maintained at 37°C in a humidified atmosphere with 5% CO_2 . Explants were subjected to TMZ treatment (1 mM) and/or IBDV infection (10^7 PFU) and cultured for 72 hours prior to downstream processing and flow cytometry analysis.

Flow cytometry

Murine tumors and tumor-draining lymph nodes (tdLN) were excised from terminated animals at the indicated time points and tissues were processed for downstream analysis by flow cytometry. Briefly, samples were incubated in FC-blocking antibody

(BD #553141) diluted in Fluorescence-Activated Cell Sorting FACS buffer (1% BSA, 0.5 mM EDTA in PBS) for 20 min on ice, then resuspended in Fixable Viability Dye eFluor 780 (Thermo Fisher #65-0865-14; 1:3000) in PBS for 10 min at room temperature. Samples were incubated with corresponding extracellular antibodies prepared in FACS buffer for 30 min. Samples were then fixed according to manufacturer's instructions using the eBioscience Fop3/Transcription Factor Staining Buffer Set (Thermo Fisher, Ref. 00-5523-00). Intracellular staining was performed in permeabilization wash buffer (eBioscience, Ref. 00-8333-56/88-8824-00). Antibodies used for intracellular and extracellular staining of isolated immune cells are itemized in (online supplemental table 1). Samples were run on an LSRFortessa X-20 flow cytometer (BD); immunophenotyping analysis was performed using FlowJo V.10 software.

Single-cell isolation from GBM explants for spectral flow cytometry analysis

GBM tumor biopsy specimens were preserved in MACS Tissue Storage Solution (Miltenyi Biotec, Bergisch Gladbach, Germany), maintained at 48°C, and processed within 3 hours after collection. To obtain single-cell suspensions, samples were enzymatically digested in 10 mL of RPMI with Ca²⁺/Mg²⁺ (Gibco) containing 2% FBS (ATCC, Manassas, Virginia, USA), 0.5 mg/mL DNase I (grade II, from bovine pancreas; Roche, Penzberg, Germany), and 0.5 mg/mL collagenase IV (Gibco) for 40 min at 37°C with continuous shaking of 150 rpm. Tissues were disrupted, passed through a 70 mm cell strainer, and washed two times with RPMI medium (Gibco). Isolated cells were centrifuged and directly used for phenotyping. Immune phenotyping of GBM biopsy specimens was performed by staining the cells for spectral flow cytometry analyses using the flow antibodies listed in online supplemental materials, and samples were acquired with a Cytex Aurora 5-laser cytometer (Cytex Biosciences, Fremont, California, USA). Immunophenotyping analysis was performed using FlowJo V.10 software.

Statistical analysis

Data analysis was performed using GraphPad Prism V.10 package. One-way analysis of variance (ANOVA) or two-way ANOVA was used to compare multiple groups with one or two independent variables, respectively. Results are expressed as mean value±SEM or ±SD as indicated. Comparisons of survival curves were performed using the log-rank (Mantel-Cox) test. Survival analysis was carried out using the Kaplan-Meier method. P values>0.05 were considered statistically non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

RESULTS

Patient-derived GSCs and murine GBM models support IBDV replication

While the cell cycle and immune responses to IBDV have been vastly covered in chicken models, our current understanding of the capacity of IBDV to infect and drive antiviral responses in mammalian cancer cells is limited to a single study performed using HeLa cells.⁴⁰

To assess the ability of IBDV to infect brain cancer cells, murine gliomas (CT-2A, GL261) and patient-derived glioblastoma stem cells (GSCs), monolayers were exposed to IBDV for 24 hours. Indirect immunodetection targeting the viral protein VP3 revealed that IBDV is capable of efficiently infecting both human and murine cancer cells (figure 1A). Furthermore, all tested cancer cell types supported productive viral replication, as demonstrated by the cumulative recovery of infectious viral particles over the course of 120 hours (figure 1B,C). Additionally, IBDV displayed differences in replication kinetics and viral titers in a cell-specific manner: in murine GBMs, earlier accumulation of VP3 protein in GL261 cells (online supplemental figure S1) correlated with an increase in viral titers over time when compared with CT-2A cells (figure 1B). In the case of human GSCs (figure 1C), there was a strong correlation between the capability of the infected cells to induce type-I IFN responses during viral infection and limit IBDV production (GBM18), as compared with GBM 27, which lacked the type I IFN cluster¹⁴ and rendered a higher viral yield. These data present evidence of the distinct cell-dependent behavior of IBDV in different mammalian brain cancer cells.

Additionally, the cytopathic effect of IBDV infection was evaluated in different GBM cells, including the GSCs GBM18 and GBM27. Microscopy analysis at 120 hours post-inoculation revealed a direct correlation between higher MOIs and an increased cytopathic effect, noticed by the presence of cellular debris and small vesicles (figure 1D,E). Remarkably, IBDV effectively disrupted GBM27 sphere architecture at an MOI as low as 1.

MTS assay was used to evaluate the impact of the infection on cell viability at 24, 48, and 72 hours post-infection (hpi) with cells now exposed to a fixed MOI of 3 (figure 1F-I). The kinetics of the cytopathic effect of IBDV were compared with those of the NDV-LS as a reference.⁴¹ The results showed that IBDV significantly reduced the viability of human and murine GBM cells at 72 hpi compared with NDV-LS in murine CT2A and GL261. In CT-2A and GL261 cells, a rebound effect was observed, where the cell viability increased at this time point, allowing for resumed growth and counteracting the effects of NDV-LS infection (figure 1F,G). In GBM 18 and GBM 27 cells, both viruses reach their peak cytotoxic effect at 72 hpi, with similar kinetics (figure 1H,I). In contrast, experimental infection of HFF-1 human fibroblast cell line cells showed no detectable viral replication at 72 hpi, with titers remaining low (10¹–10² TCID₅₀/mL), as shown in online supplemental figure S2B. Viability assays conducted at 48 and 72 hpi—time points when

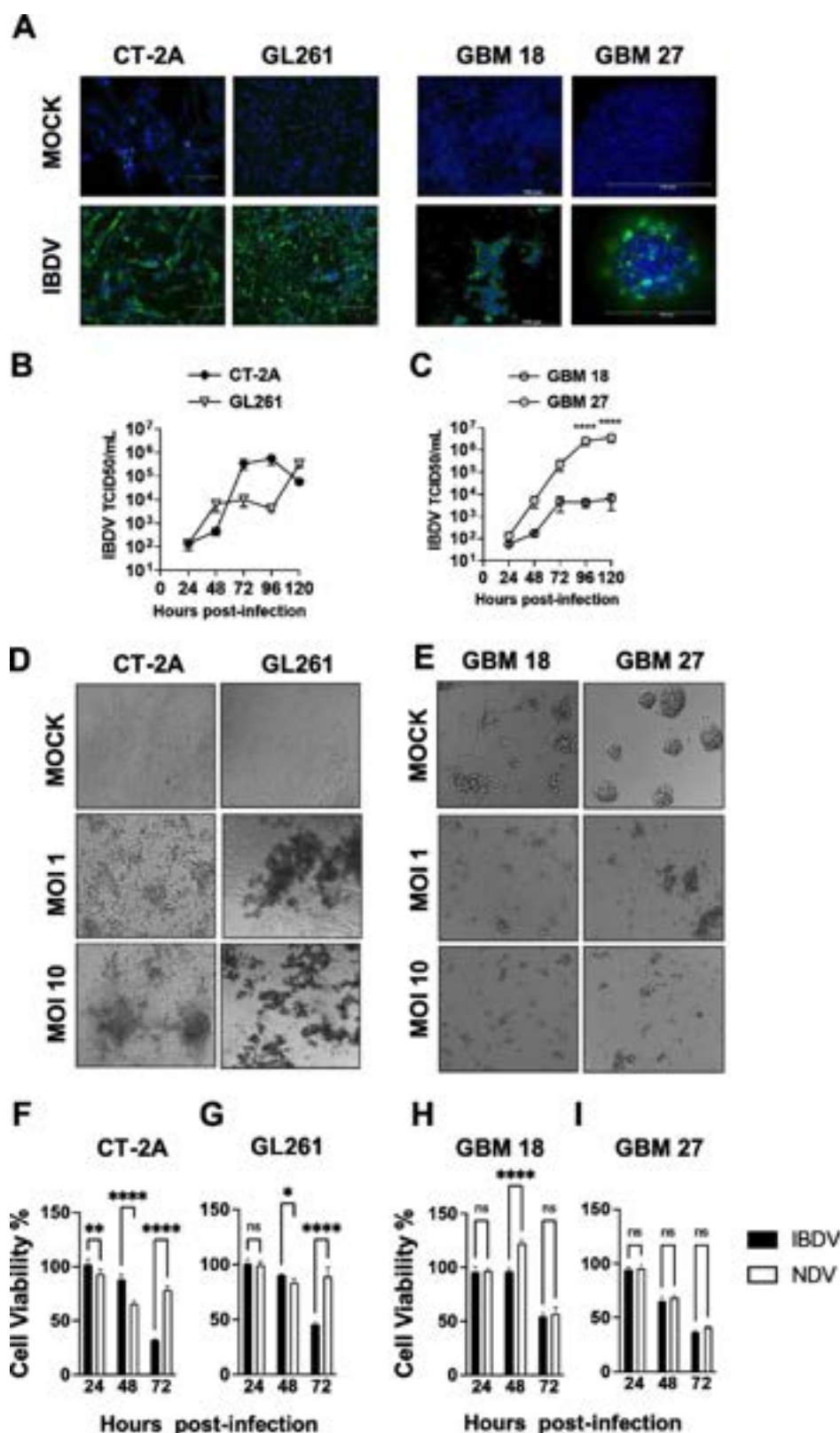


Figure 1 Infectivity and replication capacity of IBDV in murine GBM and patient-derived GSC cells. (A) Indirect Immunofluorescence microscopy: immunodetection of IBDV's VP3 protein on GBMs 24 hours post viral exposure (MOI=3). Images were taken at 40× magnification. (B, C) IBDV's replication kinetics—growth curves: viral titers were calculated by TCID₅₀/mL method; initial input=MOI 0.01 (n=3). (D, E) Cytopathic effect: brightfield microscopy of GBMs monolayers 120 hours post-infection with IBDV at specified MOI. 20× magnification. (F, G, H, I) Viability analysis: cell viability was assessed via standard MTS assay at indicated times post infection (24, 48, and 72 hours). Two-way ANOVA analysis: *p<0.05; ***p<0.001; ****p<0.0001. ANOVA, analysis of variance; GBM, glioblastoma; GSC, glioblastoma stem cell; IBDV, infectious bursal disease virus; MOI, multiplicity of infection; ns, non-significant; TCID₅₀, tissue culture infectious dose 50.

IBDV induces cytotoxic effects in GBM cells—showed no decrease in fibroblast viability (online supplemental figure S2C). These findings confirm IBDV's capability to effectively infect, replicate and induce oncolysis in vitro across different brain cancer cells, including patient-derived GSCs and murine GBM cell lines with minimal impact in survival on healthy cells.

IBDV infection triggers type-I interferon and pro-inflammatory cytokine release in GBMs

Viruses with therapeutic potential against diverse tumors have been demonstrated to stimulate antiviral responses in infected cells. This primarily involves the induction of type-I IFN and cytokine release and ultimately promotes the activation of cell death programs.^{42 43}

To study the intrinsic immunological response of GBM cells to IBDV infection, murine and human GBMs, including the human GSCs GBM18 and 27, were infected with IBDV at an MOI=3 for 16 hours. Subsequently, we analyzed the expression of IFNs (*IFNB1*, *IL28*) and the IFN-stimulated gene *MX1* as well as pro-inflammatory (*IL1B*, *IL6*) by RT-qPCR. The clinical candidate NDV-LS virus was included as reference for comparative purposes. Both murine and human GBM infected cells trigger a moderate type I IFN response, characterized by the expression of *IFNB1* and *MX1* genes, together with induction of type-III IFN (*IL28B*) cytokines and pro-inflammatory genes (figure 2A–D), similarly to the effect elicited by NDV-LS infection.

During natural infection, IBDV has been shown to exert modulatory effects on cells of the monocyte-macrophage lineage (Mo-Macs), with IBDV-infected Mo-Macs releasing chemotactic, proinflammatory and immunoregulatory cytokines.⁴⁴ This delays the resolution of the viral infection and associated pathology in its natural host. In GBM, tumor-associated macrophages (TAMs) and microglia are the most abundant immune cell populations in the TME and the major drivers of immunosuppression and progression of the disease.⁴⁵ Due to their relevance in GBM biology, IBDV-triggered pro-inflammatory gene expression signatures in mammalian murine bone-marrow-derived macrophages (M1-Mo) and HCM3 human microglia myeloid-derived cells were analyzed by RT-qPCR 72 hours after exposure to the virus (figure 2E,F). IBDV stimulates *MX1* gene expression in both cell types and, consistently with the responses observed in GBMs, to a lower extent than NDV-LS, highlighting IBDV's moderate inflammatory behavior. Gene expression analysis of infected HFF-1 human fibroblast cell line revealed that it activates an antiviral response, with expression of IFN- β and *Mx1* detected at 24 hpi (online supplemental figure S2D). These latest results further support the lack of antagonism of type-I IFN signaling of IBDV in healthy human cells.

Overall, these findings indicate that IBDV infection induces a moderate immunostimulatory effect across different GBM cells, including patient-derived and murine lines, as well as in mouse and human myeloid

cells and that mammalian healthy cells can efficiently counteract the replication of IBDV through the activation of antiviral signaling pathways.

Combination of IBDV and TMZ exhibits synergistic impact on GBMs survival

TMZ is the standard first-line treatment for patients newly diagnosed with gliomas.⁴⁶ However, approximately 50% of GBMs exhibit resistance to TMZ, which can be attributed to both intrinsic and acquired resistance mechanisms.⁴ TMZ-induced cell death presents differences in the murine and human GBM models under the current study (figure 3A–C). Specifically, the human GBM27 cell line was the most refractory to TMZ treatment, while GBM18 exhibited a dose-dependent response. In contrast, murine GBM cells (CT-2A) showed the greatest reduction in cell viability at lower TMZ doses.

Considering these phenotypes—the differential sensitivity to TMZ by GBMs, we next investigated whether TMZ treatment could alter the susceptibility of GBM cells to IBDV infection (figure 3D–F). At 0.5 mM, the viability of GBM cell lines and GSCs decreases to around 60% with TMZ administration alone, having a similar impact on cell viability as IBDV infection only. Interestingly, concomitant administration of TMZ and IBDV significantly reduced viability when compared with each treatment individually. Follow-up highest single-agent statistical analysis confirmed a synergistic effect of both treatments reducing GBM cell viability, with a remarkable impact on GBM27 cells. Data to assess the combinatory effect of both treatments is compiled in (figure 3G–I and online supplemental figure S3A). Furthermore, sustained exposure to TMZ and IBDV demonstrated a dual effect in GBMs: the reduction in cell viability exerted by the combination was accompanied by enhanced IBDV replication (online supplemental figure S3B and C), as indicated by a significant increase in viral release at the peak of replication (72 hpi). This boost in viral production was not a reflection of a diminished antiviral responses, as indicated by the levels of expression of IFN- β and *MX1* genes (online supplemental figure S3D and E).

These results highlight the complementary and synergistic interaction between TMZ and IBDV in GBM models that exhibit different sensitivity to the chemotherapeutic TMZ. The clear effects demonstrated in vitro merit further investigation to clarify its mechanisms and clinical implications.

Intratumoral administration of IBDV results in control over tumor growth and extended survival in CT-2A tumor-bearing mice

Our in vitro studies demonstrated that IBDV can induce both immunostimulatory and cytotoxic responses in various GBMs, exhibiting a profile similar to that of the clinical OV candidate NDV-LS. To assess the feasibility of using IBDV as a therapeutic

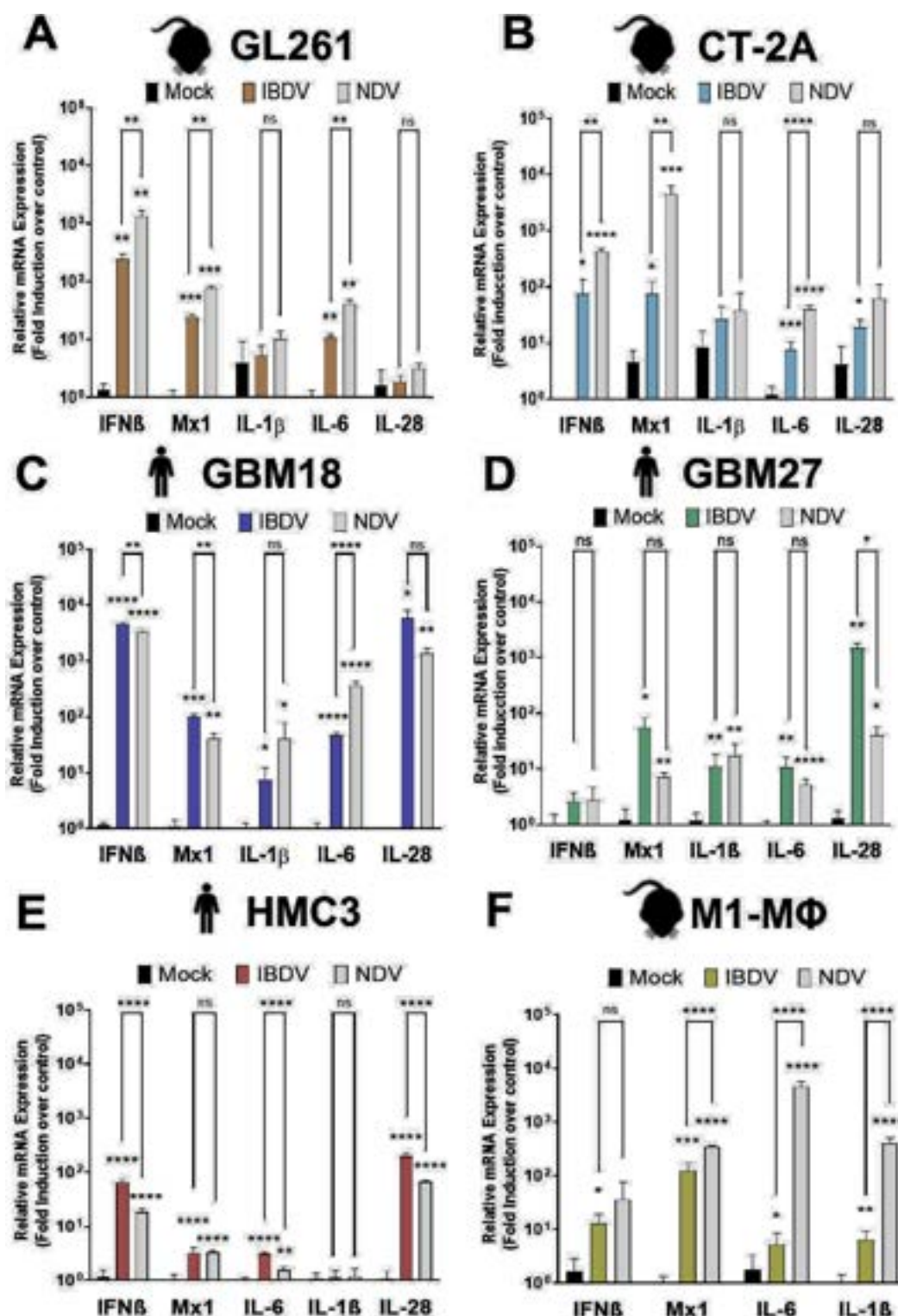


Figure 2 Antiviral and pro-inflammatory gene expression triggered by IBDV infection. Gene expression analysis (RT-qPCR). Cancer cell monolayers (A–D), bone-marrow-derived macrophages and human microglia HCM3 (E–F) cells were infected with IBDV and NDV-LS at an MOI=3 for 16 hours. Expression levels for each individual gene were calculated as log10 of fold induction over mock-infected cells. Two-way ANOVA analysis: * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$. ANOVA, analysis of variance; IBDV, infectious bursal disease virus; IFN, interferon; IL, interleukin; MOI, multiplicity of infection; mRNA, messenger RNA; ns, non-significant.

for GBM, we first conducted safety studies in immuno-competent C57Bl/J6 mice through both systemic and intracranial administration of IBDV (figure 4A,B).

Histological analysis of tissue samples, 24 hours after systemic inoculation of a high dose of IBDV (10^7 PFU), revealed no virus-associated pathology in any

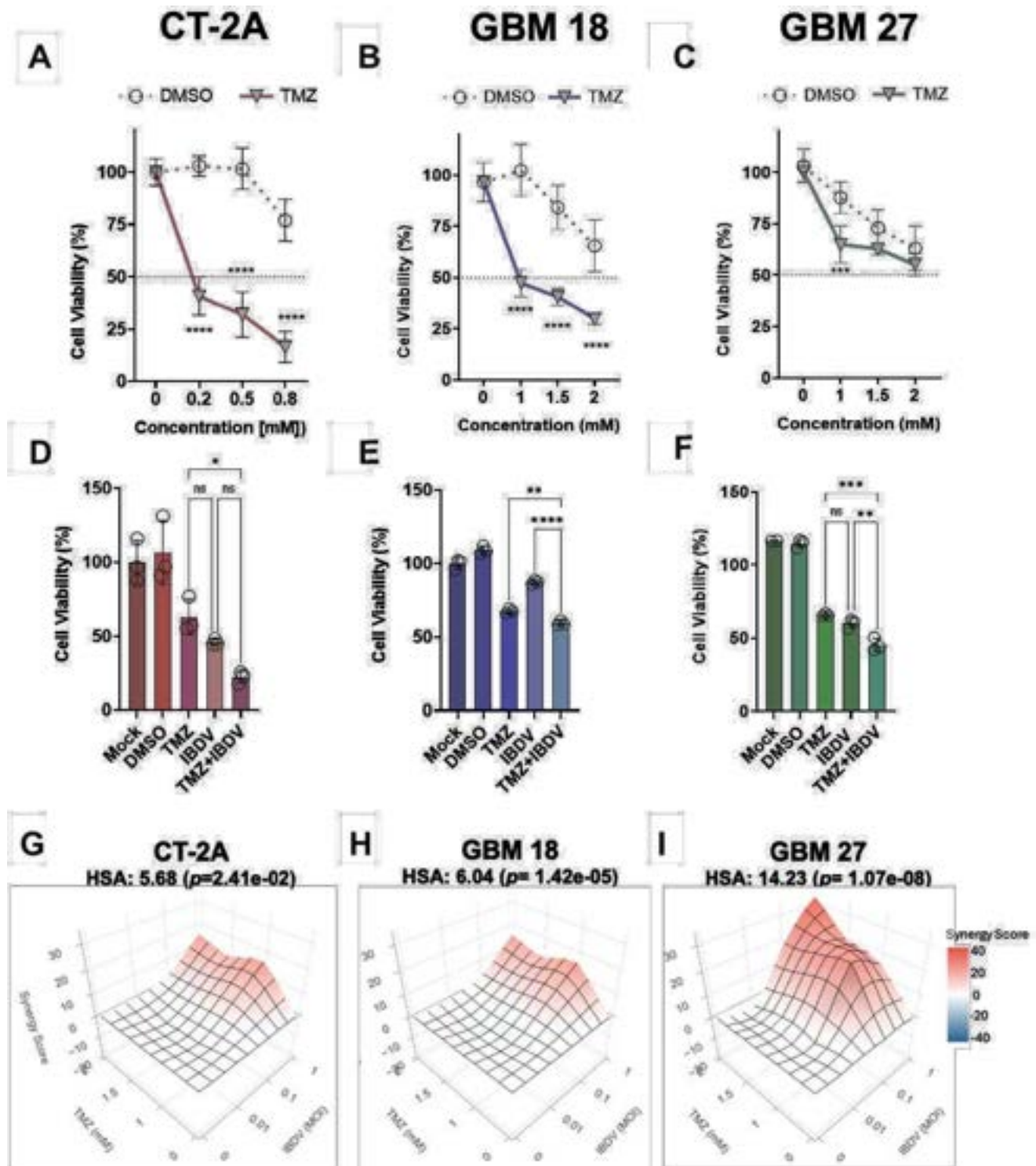


Figure 3 Combinatory effect of TMZ with IBDV. (A, B, C) TMZ cytotoxicity analysis: at 72 hours, cell viability was measured via standard MTS assay compared with a Mock control after treatment with different concentrations of TMZ (DMSO control concentrations correspond to the final concentrations of TMZ used in each condition). (D, E, F) Combination effect of TMZ and IBDV: GBM cell viability was measured after treatment with 0.5mM of TMZ, IBDV infection (MOI 1), or both conditions together. (G, H, I) HSA statistical test: the HSA statistical analysis was performed based on the effect of drug combinations relative to the effect of each treatment alone to assess additive, synergistic or antagonistic effects. DMSO was used as the vehicle for TMZ, and final DMSO concentrations in each well correspond to those of the TMZ treatments (eg, 2 mM TMZ corresponds to 2% DMSO). Equivalent DMSO concentrations were included in vehicle controls. Two-way ANOVA analysis: * $p<0.05$; *** $p<0.001$; **** $p<0.0001$. ANOVA, analysis of variance; GBM, glioblastoma; HSA, highest single agent; IBDV, infectious bursal disease virus; MOI, multiplicity of infection; ns, non-significant; TMZ, temozolomide.

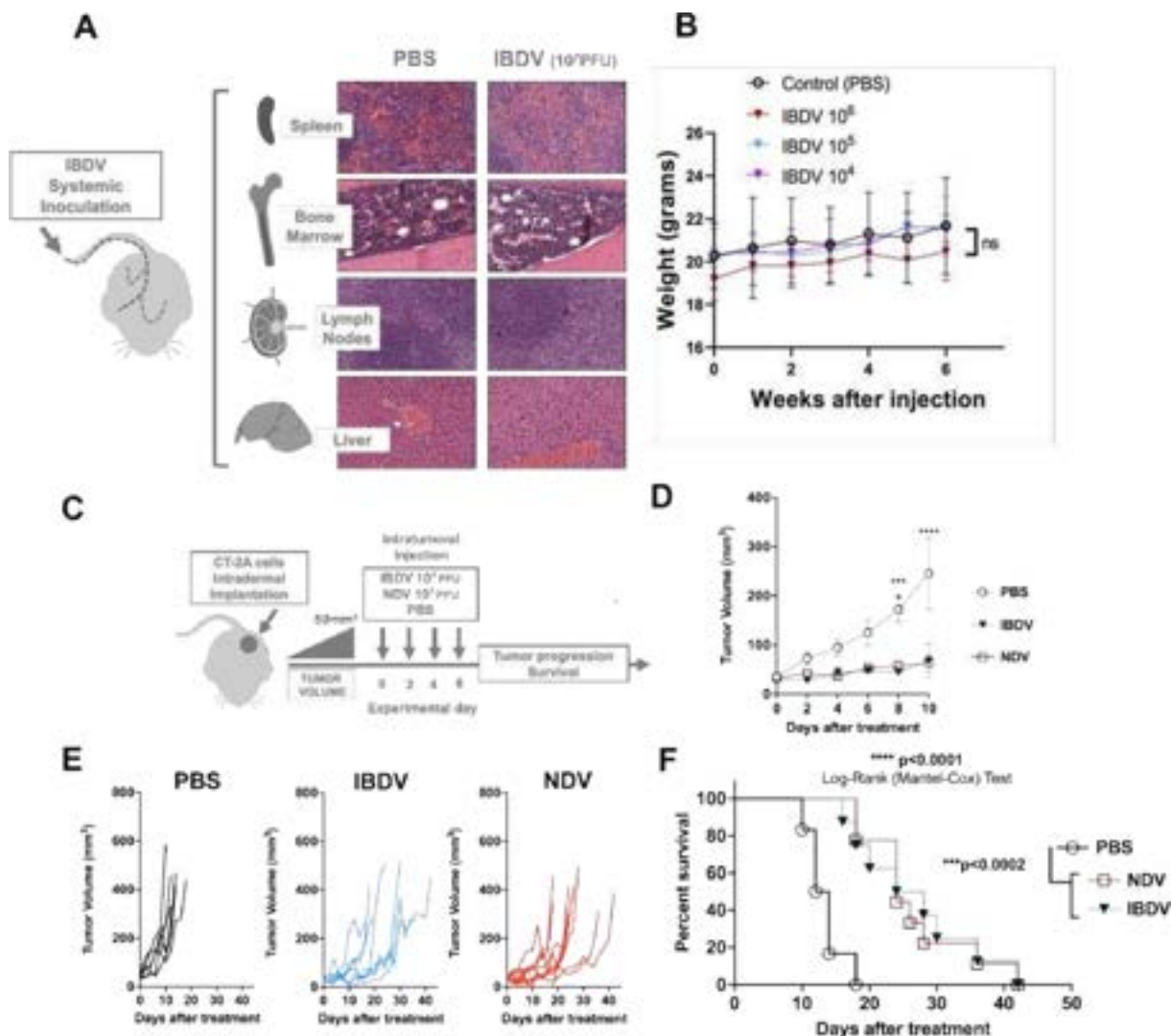


Figure 4 In vivo antitumor effect of IBDV. (A–B) Pathogenicity studies. (A) 6–8 weeks old C57BL/6 mice (N=3) were intravenously administered with either PBS or 10^7 PFU of IBDV; H&E histochemistry of indicated organs processed 24 hours post systemic viral dissemination. (B) IBDV intracranial toxicity: body-weight progression of C57BL/6 mice (N=3) after exposure to a single intracranial injection of IBDV at the indicated dose. (C–E) Antitumor capacity of IBDV. (C) Schematic representation of the study. CT-2A tumor-bearing mice were intratumorally administered 10^7 PFU of IBDV/NDV or PBS, once every other day, for a total of four doses. Tumor volume was monitored every 48 hours. Experimental end-point: 1,000 mm³ tumor volume. (D) Average of tumor volume per experimental group at the time of first death. Two-way ANOVA. * $p<0.05$, ** $p<0.01$. (E) Individual tumor growth curves. (F) Survival analysis: log-rank (Mantel-Cox) test. *** $p<0.001$, **** $p<0.0001$. ANOVA, analysis of variance; IBDV, infectious bursal disease virus; PBS, phosphate-buffered saline.

organ or tissue processed. Additionally, no signs of distress were detected in any of the experimental animals. Similarly, exploratory intracranial administration of IBDV at high doses resulted in absence of morbidity or mortality over the 6-week study period (figure 4B).

Next, we investigated if IBDV could drive antitumor responses in a syngeneic CT-2A experimental tumor model developed in the flank of immunocompetent mice. As a reference and for comparative purposes, we included an experimental group treated with NDV-LS virus (figure 4C). Results showed that both IBDV and NDV-LS treatments can control tumor growth (figure 4D) compared with untreated control

mice. Individual tumor growth volume curves indicated that while none of the viruses could completely inhibit tumor progression, both treatments significantly delayed tumor growth compared with PBS-treated controls (figure 4E). Moreover, both IBDV and NDV-LS treatments significantly extended survival in treated mice compared with the control group (figure 4F). No signs of distress or morbidity associated with IBDV treatment were observed in any of the experimental animals.

Overall, these results confirmed that IBDV is safe in immune-competent mice and exhibits significant oncolytic activity, comparable to NDV-LS, in delaying CT-2A GBM progression and improving mouse survival.

IBDV virotherapy remodels TME reducing immunosuppression priming tumors for cytotoxic responses

The architecture and immune landscape of the TME are critical determinants of the efficacy and durability of targeted antitumor responses.⁴⁷

Given that IBDV demonstrated a beneficial antitumor effect in GBM *in vivo*, we assessed changes in immune cell populations within the TME. Mice were divided into two groups: those receiving either a single injection (Day 1) or three injections (Day 5) of IBDV (10^7 PFU), compared

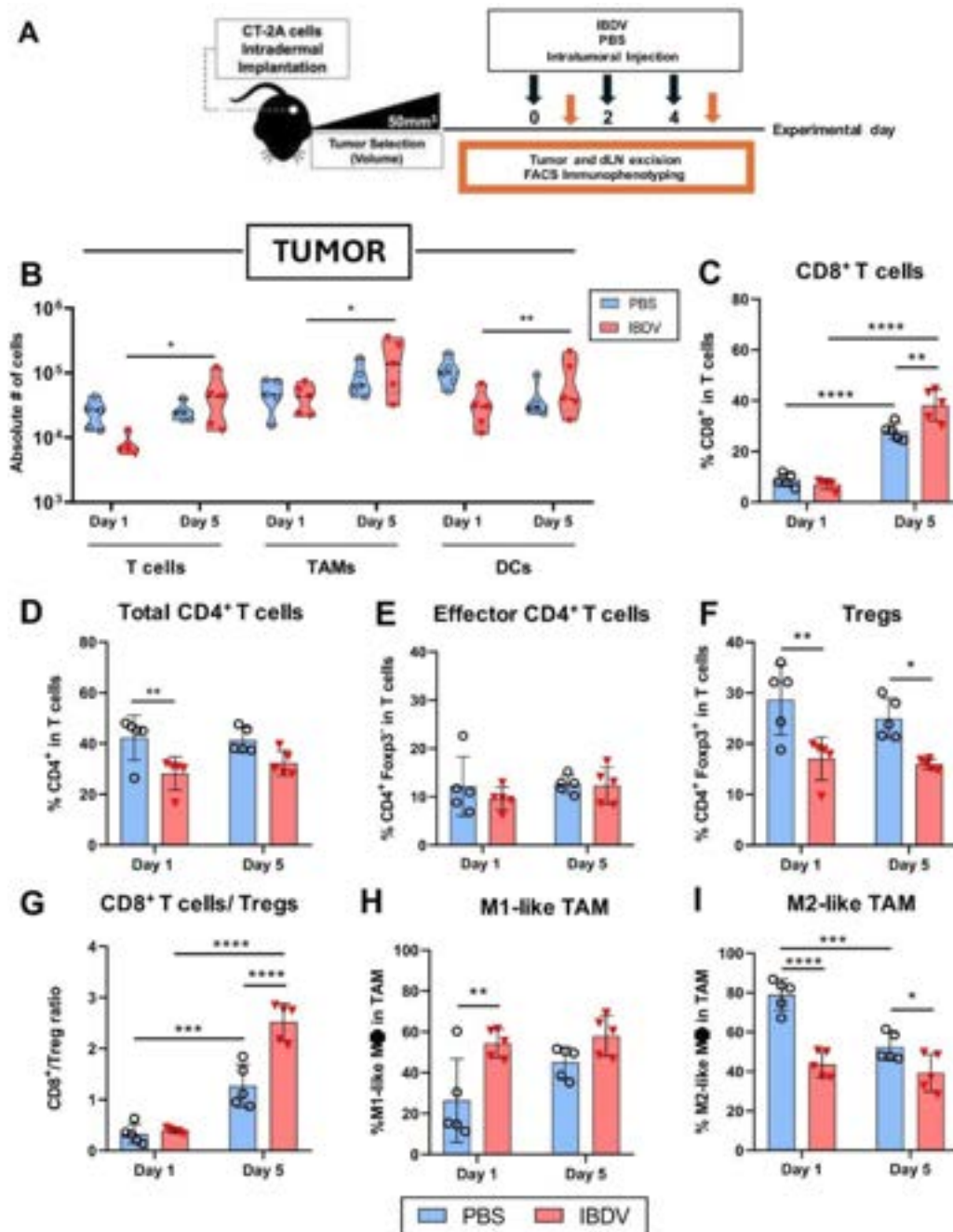


Figure 5 Immunostimulation of innate and adaptive immune response in TME on IBDV treatment in CT-2A murine GBM model. (A) Schematic representation of the study: CT-2A tumor-bearing mice were intratumorally treated every other day with a total of either one or three doses of 10^7 PFU of IBDV. Mice were sacrificed 1 day after the first or third injection in which tumor masses (TME) were harvested and probed with primary-conjugated antibodies against main biomarkers of immune cells. (B) Analysis of main immune populations in TME: temporal distribution of immune cell populations within TME of IBDV/PBS-treated mice. (C–J) Analysis of myeloid and lymphoid immune cell populations in TME: changes in innate and adaptive immune response in IBDV-treated compared with PBS-treated mice were analyzed in both groups (day 1 and 5). Two-way ANOVA analysis: * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$. ANOVA, analysis of variance; dLN, draining lymph nodes; GBM, glioblastoma; IBDV, infectious bursal disease virus; PBS, phosphate-buffered saline; TAM, tumor-associated macrophage; TME, tumor microenvironment; Treg, regulatory T cell.

with PBS-treated controls (figure 5A), to identify earlier (Day 1) and delayed (Day 5) immunological changes in the tumor composition induced by IBDV treatment. Flow cytometry was employed to analyze lymphoid and myeloid immune cell populations, using the gating strategy outlined in online supplemental figure S4.

Analysis of treated tumors revealed differential enrichment of T cells, macrophages, and Dendritic cells (DCs) in IBDV-treated tumors when compared with control mice (figure 5B). Specifically, mice receiving three doses of IBDV exhibited an enrichment in CD8+T (figure 5C), while those treated

with a single injection showed a decrease in total CD4+T cells (figure 5D) compared with controls. This reduction in total CD4+T cells was associated with a decrease in immunosuppressive regulatory T cells (Treg) (figure 5F), rather than effector T cells (figure 5E). These findings suggest that IBDV breaks TME immunotolerance, facilitating cytotoxic T-cell functions, as defined by a higher CD8+T cell/Treg ratio after repeated doses of IBDV (figure 5G).

Analysis of the myeloid compartment showed a reduction in the presence of immunosuppressive M2-like macrophages (CD11c-, CD11b+, Ly6G-, Ly6C-,

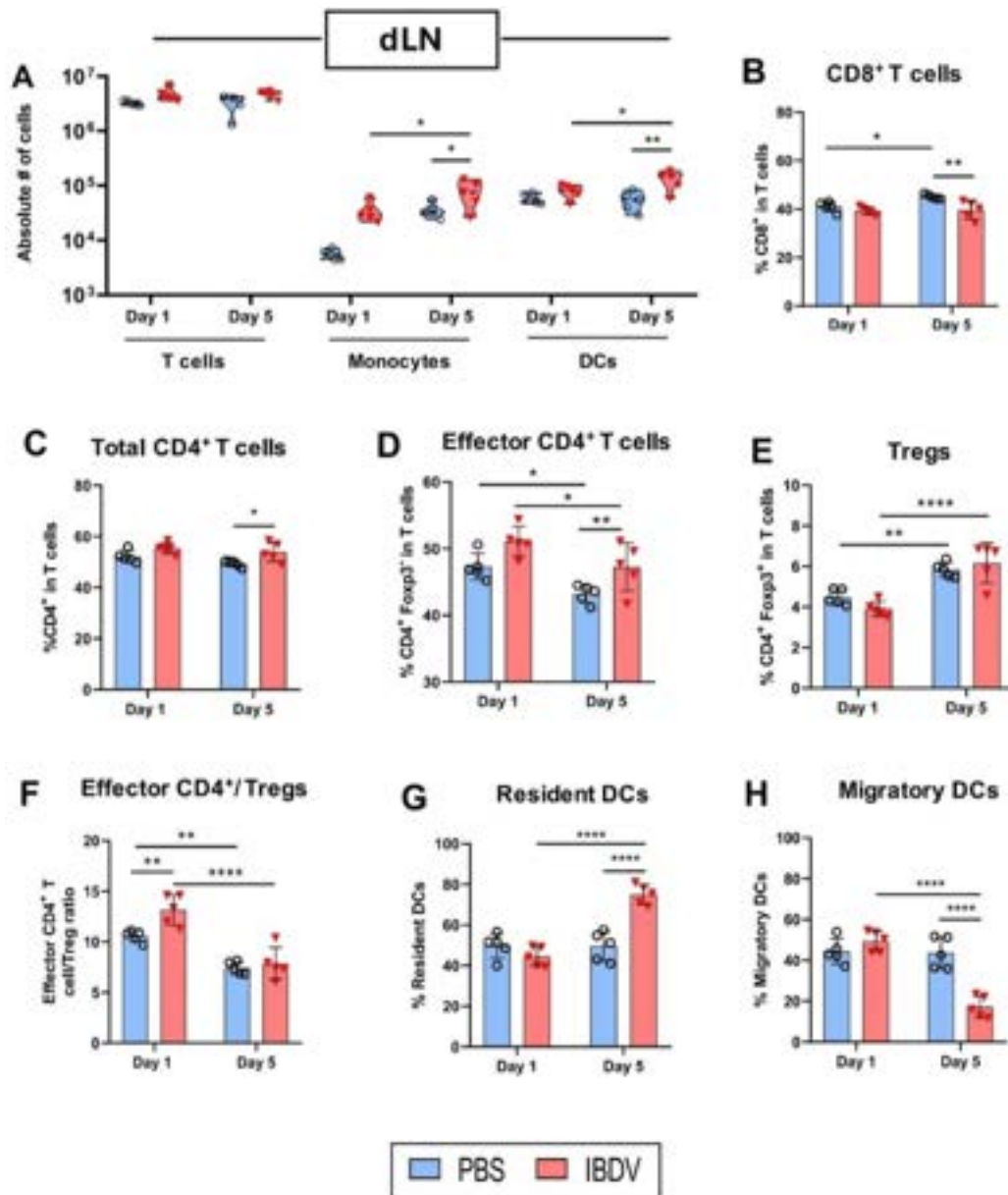


Figure 6 Immunophenotyping of tdLN in CT-2A murine GBM model. (A) Analysis of immune populations in tdLN: temporal distribution of immune cell populations within tdLN of IBDV/PBS-treated mice over the course of the treatment. (B–H) Analysis of myeloid and lymphoid immune cell populations in tdLN: changes in lymphoid and antigen-presenting cells were analyzed in IBDV-treated mice compared with PBS-treated mice in both groups (day 1 and 5). Two-way ANOVA analysis: * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$. ANOVA, analysis of variance; tdLN, tumor-lymph nodes; GBM, glioblastoma; IBDV, infectious bursal disease virus; PBS, phosphate-buffered saline; Treg, regulatory T cell.

MHC-II+) (figure 6I; online supplemental figure S5A-C). This effect was accompanied by an increased presence of inflammatory M1-like macrophages (CD11c⁻, CD11b⁺, Ly6G⁻, Ly6C⁺, MHC-II⁺) in mice treated with a single dose of IBDV (figure 5H).

Overall, IBDV virotherapy demonstrated significant impact on TME remodeling, reducing the presence of immunosuppressive populations and promoting T-cell functions.

IBDV therapy induces CD8⁺ T-cell migration and expansion of effector CD4⁺ T cells in tumor-draining lymph nodes

As tdLNs are pivotal hubs for initiating adaptive immune responses against tumors, their immune makeup was analyzed in the context of IBDV treatment by flow cytometry. Results presented no variation in the number of total T cells, but a higher infiltration of monocytes and DCs in the tdLN of mice treated with three injections of IBDV as compared with the PBS-treated control mice (figure 6A). Furthermore, the abundance of conventional type II DCs (cDC2) increased with repeated IBDV treatment (online supplemental figure 6D) denoting a sustained stimulation of DC migration.

T-cell analysis revealed a modest reduction in the percentage of cytotoxic CD8⁺T cells (figure 6B) accompanied by a significant increase in total CD4⁺T cells in IBDV-treated mice following three injections (figure 6C). The increase in total CD4⁺T cells was mainly due to effector helper T cells (figure 6D) rather than Tregs (figure 6E). Notably, the proportions of Tregs were already influenced by a higher ratio of effector CD4⁺T cells to Treg in mice treated with a single injection of IBDV (figure 6F). This suggests that, at this stage, helper T cells outnumbered compared with the immunosuppressive Tregs. Lastly, mice treated with three injections of IBDV showed an increase in resident DCs (intermediate major histocompatibility complex (MHC)-II) (figure 6G) while migratory DCs (high MHC-II) were significantly reduced (figure 6H) in tdLNs.

These findings highlight IBDV's capacity to reshape key immunological hubs characterized by increased resident DCs, increased effector CD4⁺T cell responses, and reduced immunosuppressive Tregs.

IBDV virotherapy converts immunosuppressive macrophages into immunoreactive effectors in murine and human tumors

GBMs are among the so-called immune desert tumors, characterized by minimal, if any, cytotoxic CD8⁺T cells, dominance of myeloid immunosuppressive populations and poor antigen presentation, with DCs often preserved in a dysfunctional state due to the lack of inflammatory signaling.⁴⁸ In GBMs, glioma-associated macrophages and microglia (GAMs) are central regulators of GBM TME. Presenting a phenotype skewed towards an M2-like immunosuppressive state, GAMs could represent up to 40–50% of the TME and conditionate tumor progression, invasion, angiogenesis, immunosuppression and resistance to therapy.⁴⁹ M2-like GAMs presence is associated

with poor clinical outcome and shorter survival and many ongoing works to improve these outcomes are focused on shifting this restrictive phenotype into an immunoreactive pro-antitumoral one.⁵⁰

Our analysis of the myeloid compartment in mice carrying CT-2A tumors showed a rapid inflammatory response characterized by a reduction in the presence of immunosuppressive M2-like TAMs (CD11c⁻, CD11b⁺, Ly6G⁻, Ly6C⁻, MHC-II⁺). This effect was accompanied by an increased presence of inflammatory M1-like TAMs (CD11c⁻, CD11b⁺, Ly6G⁻, Ly6C⁺, MHC-II⁺) after a single dose of IBDV (figure 5H,I; online supplemental figure 5A-C).

Due to the importance of these findings, we wanted to further investigate whether this early effect induced by IBDV can also be translated into human immune cells. To do so, we carried out a study of ex vivo exposure to IBDV of tissue explants generated from a human GBM-IDH wild-type tumor biopsy (supplementary figure 7A). A TMZ-only (1 mM) and a combination TMZ-IBDV conditions (1 mM / 10⁷ viral particles) were also included in the experimental set-up, aiming to identify possible synergetic effects and for comparative purposes. Due to the characteristics of the model and the aim of the study, experimental conditions were set-up to 72 hours of sustained exposure, to warranty tissue stability, and we focused the analysis on the myeloid populations—myeloid-derived monocytes (MDMs) and DCs—as shown in the gating (supplementary figure 7B).

Explants exposed to IBDV showed a significant reduction in immunosuppressive M2-like macrophages (HLA-DR⁺, CD64⁺, CD14⁺, CD11b⁺, arginase I high) accompanied by a marked increase in inflammatory M1-like macrophages (HLA-DR⁺, CD64⁺, CD14⁺, CD11b⁺, arginase I low) (supplementary figure 7D). Compared with other treatment modalities, IBDV induced the most significant shift in MDMs polarization. Further analysis of the M1-like MDMs population revealed an immunoreactive phenotype, as indicated by the expression of CD80, confirming the functional reprogramming (supplementary figure 7E).

IBDV-treated samples exhibited a higher prevalence of DCs at the time of the analysis (supplementary figure 7F). Notably, while the proportion of activated DCs—characterized by elevated expression of CD80 and HLA-DR high—was greater than in both TMZ and control groups. Interestingly, the combination of TMZ and IBDV further enhanced APC reprogramming, resulting in a larger proportion of activated DCs (supplementary figure 7G). Given the observed synergistic effect on cell viability, this phenotype might be associated with a more immunostimulatory mode of cancer cell death induced by the combined treatment.

Overall, these results demonstrate that IBDV effectively reprograms the myeloid compartment in GBM, both in murine and human model systems, promoting a shift toward an immunoreactive phenotype and enhancing dendritic cell activation. This last effect is further

amplified when combined with TMZ, suggesting a synergistic immunostimulatory response.

DISCUSSION

IBDV is introduced here as a potential oncolytic virotherapy candidate with attributes that address key challenges in GBM, including immune evasion and resistance to chemotherapy. As an alternative to current treatments, it shows promise in activating both innate and adaptive immune responses and serving as a complementary agent in combination therapies.

Efficient innate immune activation is essential for a successful adaptive antitumor response.⁵¹ The data presented here show the induction of some key gene markers of innate immune activation programs following IBDV infection in human and murine GBM cells, as well as myeloid cells. A notable feature of IBDV infection is its moderate pro-inflammatory response compared with NDV, as observed in some GBM cell lines (figure 2A–C) and M1-like BMDMs (figure 2F). This characteristic could potentially minimize the risk of excessive inflammation in specific CNS tumor contexts, where excessive inflammation can damage neural tissues.⁵²

IBDV infection can induce type III IFN-mediated immune responses, stimulating *IL28B* and *Mx1* expression in GBM27 cells, which lack the type I IFN gene cluster.¹⁴ This presents an opportunity to explore the priming of type III IFN-mediated immune responses in the context of type I IFN-deficient tumor cells as well as to compare these responses with cells possessing both type I and type III IFN pathways. Identifying factors that could promote an improved adaptive immune response while minimizing excessive inflammation will aid in reducing immunosuppressive effects associated with immunotherapeutic strategies.⁵³

While in vitro quantitative PCR analyses revealed differences in expression of key inflammatory genes between IBDV-infected and NDV-infected cells, these differences did not translate into significant therapeutic differences in vivo in the CT-2A GBM model (figure 4D). Both viruses showed comparable efficacy in controlling tumor growth and extending survival (figure 4,F). However, despite the observed improvement, all treated mice eventually reached the experimental endpoint and were terminated, highlighting the necessity for further refinement of IBDV's therapeutic strategy.

To gain a deeper understanding of the immune responses that made IBDV-treated mice outperform controls in restricting tumor growth, flow cytometry was used to analyze key immune populations in the TME and tdLN. In the TME, results revealed a reduction in immunosuppressive cells, including M2-like macrophages (figure 5I) and Tregs (figure 5E), and an increase in cytotoxic CD8+T cells (figure 5C) and M1-like macrophages (figure 5H), associated with a more favorable immune landscape to prime antitumor responses.⁵⁴

The analysis of tdLN revealed additional insights into the immune dynamics induced by IBDV treatment. A marked increase in effector CD4+T cells (figure 6D) suggests an enhanced priming and activation within the lymph nodes.⁵⁵ Concurrently, there was a reduction in Tregs (figure 6E), indicative of a shift away from immunosuppressive conditions.^{56–57} Notably, a lower presence of CD8+T cells in the tdLN (figure 6B) implies a potential migration of these cytotoxic cells toward the tumor, reinforcing the idea of a coordinated systemic immune response being directed against the GBM tumor.^{58–59}

While these results demonstrate the robust immunostimulatory effect of intratumoral administration of IBDV, the treatment failed to elicit long-lasting responses that resolve in tumor elimination. For instance, the increase in migratory cDC2 rather than cDC1 suggests a priming of T cells that could lead to an antitumoral Th2 response or, alternatively, the priming of Tregs, counteracting an effective cytotoxic immune response by inducing immune tolerance (online supplemental figure S6).⁶⁰ Furthermore, while IBDV treatment induces a shift towards M1-like polarization in tumor macrophages (figure 5H), this does not translate into an activation of those macrophages, potentially limiting their contribution to the antitumoral immune response (online supplemental figure S5A). Similarly, while our results support an active T-cell engagement in response to IBDV, a deeper understanding of T-cell functionality could help to narrow down mechanisms of resistance due to T-cell exhaustion. Chronic inflammation within the TME can impair immune cell functionality.^{61–62} Understanding how these immunological shifts contribute to tumor control and survival will be important to fully unlock IBDV potential as a virotherapeutic agent.

Early administration of IBDV and NDV treatments led to significant control of tumor growth and extended survival; however, complete tumor elimination was not observed, indicating the presence of resistance mechanisms that warrant further investigation. The inability to achieve sustained therapeutic responses, such as long-term survivors or complete responders, limited the scope of deeper analyses like tumor-specific and virus-specific T-cell profiling, which are essential for understanding the balance between antiviral and anti-tumor immune responses as well as the durability of IBDV-induced immunity. Nonetheless, this initial demonstration of the oncolytic and immunomodulatory potential of IBDV in GBM provides a solid foundation for future studies. The integration of translational strategies—optimized dosing, combinatorial regimens, and advanced responsive models—is anticipated to unlock conditions that enable comprehensive investigation of T-cell specificity and the mechanisms underpinning long-lasting antitumor immunity, advancing both mechanistic understanding and clinical application in the field of cancer virotherapy.

Our studies have demonstrated that IBDV can replicate and complement TMZ-induced cytotoxicity in vitro (figure 3; online supplemental figure S3). Moreover, exposure to TMZ did not impair GBMs' susceptibility to be infected by IBDV but potentiated the replication capacity and viral release of

the virus without diminishing antiviral responses. Furthermore, in our ex vivo GBM's explants study (supplementary figure 7), we detected a trend towards increased activation of APCs on the combination IBDV+TMZ treatment, as demonstrated by the percentage of CD80⁺-HLA-DR (high) DCs, initially supporting the idea that the combination might be more immunogenic than the monotherapies. Yet, although our data provide insights into the multimodal advantage of pairing IBDV and TMZ, additional in vivo research is needed to explore the full scope and molecular basis of these findings to confirm the feasibility of this combination and support its clinical application.

In this regard, and based on similar observations, multiple studies aiming to overcome resistance to TMZ and to improve therapeutic outcomes have been focused on the combination of TMZ with different OV's.^{63–65} the oncolytic adenovirus DNX-2401 has been tested alongside TMZ in patients with recurrent GBM (NCT01956734), showing enhanced survival benefits compared with monotherapy.⁶⁶ Similar synergistic effects have been observed in preclinical studies combining TMZ with oncolytic herpes simplex virus (G47Δ).^{67,68} Chinese clinical trials have also used H101, an adenovirus-based OV, in combination with TMZ and chemoradiotherapy across various tumor types (ChiCTR2400085353). Overall, our data concurred with these advanced studies supporting that TMZ can enhance viral replication or immune activation, improving therapeutic outcomes.

In conclusion, IBDV presents an oncolytic profile that combines direct oncolysis, TME remodeling, and immune system activation. Its non-human origin further adds to its appeal, potentially enabling it to evade pre-existing immune responses that might limit the efficacy of other virotherapies.⁶⁹ These attributes make it a promising candidate for addressing unmet needs in GBM treatment. Further preclinical and clinical evaluations are essential to fully unlock its potential to expand its application in oncolytic virotherapy.

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Acknowledgements We would like to express our deepest gratitude to José F Rodríguez and Dolores Rodríguez for their support and guidance in Infectious Bursal Disease Virus (IBDV). We also sincerely thank Granja Rodríguez Serrano, located in Alba de Tormes, Salamanca, Spain, for generously providing chicken embryonated eggs for this research. The authors wish to express their deepest gratitude to the "Fundación Nemesio Díez" for its generous support and commitment to advancing meaningful and high-quality scientific research.

Contributors VT-P, SC-C: experimental design, methodology, investigation, analysis, writing, review and editing. YB, GD, LP-R, SI-L, SR-R, IP-A, JA-H, AM-S, IM-L: investigation and analysis. NG-R, DL-O: experimental design, methodology, review and editing. AG-S, JC-O, M-LdR, J-IR-B: methodology, review and editing. EN-V, AA-S: experimental design, review and editing. Guarantor: SC-C.

Funding This study has been funded by Instituto de Salud Carlos III (ISCIII) through the project "PI21/01353" and co-funded by the European Union.

Competing interests The AG-S laboratory has received research support from GSK, Pfizer, Senhwa Biosciences, Kenall Manufacturing, Blade Therapeutics, Avimex, Johnson & Johnson, Dynavax, 7Hills Pharma, Pharmamar, ImmunityBio, Accurius, Nanocomposix, Hexamer, N-fold LLC, Model Medicines, Atea Pharma, Applied Biological Laboratories and Merck. AG-S has consulting agreements for the following companies involving cash and/or stock: Castlevax, Amovir, Vivaldi Biosciences, Contrafect, 7Hills Pharma, Avimex, Pagoda, Accurius, Esperovax, Applied Biological Laboratories, Pharmamar, CureLab Oncology, CureLab Veterinary, Synairgen, Paratus, Pfizer, Virofend Therapeutics and Prosetta. AG-S has been an invited speaker in meeting events organized by Seqirus, Janssen, Abbott, AstraZeneca and Novavax. AG-S is an inventor on patents and patent applications on the use of antivirals and vaccines for the treatment and prevention of virus infections and cancer, owned by the Icahn School of Medicine at Mount Sinai, New York, USA, outside of the reported work. SC-C is the inventor on patents on the use of different viral platforms to treat cancer, owned by the Icahn School of Medicine at Mount Sinai, New York, USA. SC-C is co-founder of ViroFend Therapeutics SL.

Patient consent for publication Not applicable.

Ethics approval Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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RESEARCH

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Selection of glioma organoids for drug discovery: a patient-derived model to study glioma biology and drug response

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Abstract

Background Gliomas are the most common primary brain tumors in adults and encompass a spectrum from IDH-mutant gliomas to highly aggressive IDH-wild type glioblastomas (GBM). Despite advances in our understanding of glioma biology, GBM remains largely incurable, with a median overall survival (mOS) of approximately 15 months and minimal improvements over recent decades. The current standard of care, maximal surgical resection followed by radiotherapy and temozolomide (TMZ) chemotherapy, has proven insufficient, and experimental approaches such as immune checkpoint inhibitors and CAR-T cell therapies have also failed to provide meaningful survival benefits. These limitations highlight the urgent need for innovative, patient-specific platforms to evaluate novel therapeutic strategies.

Methods In this study, we established patient-derived glioma organoids (GOs) from 61 patients, including both IDH-mutant gliomas (GOMut) and IDH-wild type glioblastoma (GOWt). These organoids were characterized by a variety of techniques against their parental tumors. Afterwards, we performed *in silico* drug discovery with paired RNA-seq data and validated the results with diverse glioma models.

Results GOs recapitulated the cellular heterogeneity, histological architecture, and molecular signatures of their parental tumors, including the preservation of clinically relevant features such as immune profiles, vascular structures, and tumor subtype. Notably, GOMuts retained the IDH mutation over extended culture periods. Importantly, GOs maintained resistance to standard chemotherapeutic agents, mirroring the limited response of gliomas to conventional chemotherapy. Our unbiased *in silico* screening via the DiSCoVER platform applied to RNA-seq data from paired tumors and GOs identified alectinib, dabrafenib, and ruxolitinib as promising candidates. All three compounds

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proved effective in *in vitro* cytotoxicity assays, with alectinib and ruxolitinib displaying significant antitumoral effects *in vitro*.

Conclusions Our findings establish GOs as a clinically relevant and biologically faithful platform for therapeutic screening in glioma. By integrating transcriptomics-based drug discovery with a patient-specific organoid model, we present an easy to implement platform for more effective and personalized treatments for glioma patients. Our findings also support the inclusion of two compounds, alectinib and ruxolitinib, in future clinical investigations.

Keywords Glioma, Glioblastoma, Organoids, Personalized therapies

Background

Malignant gliomas are classified based on their histological characteristics and the presence or absence of isocitrate dehydrogenase (IDH) mutation into IDH-mutant gliomas and IDH-wild type glioblastoma (GBM). GBM is the most prevalent and aggressive form, with an incidence of 3.22 cases per 100,000 people [1]. GBM is further marked by significant inter- and intra-tumoral heterogeneity [2] contributing to its poor prognosis, with a median overall survival (mOS) of only 15 months and a 5-year survival rate of around 5% [3]. Despite these challenges, the standard of care (SOC) therapy, comprising maximal surgical resection followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ) chemotherapy, has remained largely unchanged for decades, with limited improvements in patient outcomes [4, 5]. On the other hand, studying IDH-mutant gliomas presents additional challenges due to their lower proliferation rates and heterogeneity. Moreover, traditional models fail to maintain the clinically relevant IDH mutation, which hinders the development of conventional preclinical models such as established cell line cultures [6, 7].

The urgent need for new therapeutic approaches is underscored by the persistent failure of current treatments to significantly improve survival outcomes for glioma patients. Despite decades of research, GBM remains a largely incurable disease, with standard therapies offering only marginal benefits. Moreover, alternative experimental strategies such as immune checkpoint inhibitors and CAR-T cell therapies have also failed to yield meaningful clinical success in this context [8, 9]. These challenges highlight the need for the development of innovative, patient-specific strategies to identify effective therapeutic options. Given the variability in tumor characteristics, tumor-derived preclinical models present an opportunity to screen novel pharmaceutical options in a personalized, patient-specific manner, especially in incurable malignancies like gliomas [10]. In this context, patient-derived organoids have emerged as a highly promising tool. Unlike traditional 2D cultures, glioma organoids retain key features of the original tumor, including cellular diversity, tissue architecture, and the tumor microenvironment. These characteristics allow them to more accurately replicate patient-specific drug

responses, making them ideal platforms for therapeutic screening and disease modeling [11, 12]. Additionally, organoids are faster to establish and more cost-effective to maintain than animal-based patient-derived xenografts (PDX) [13].

In this study, we generated malignant glioma-derived organoids (GOs) from 61 patients, encompassing both IDH-mutant (GOMut) and GBM (GOWt), to identify novel therapeutic options. GOs were used to evaluate responses to standard chemotherapeutic agents commonly used in glioma treatment, like TMZ, which proved to be ineffective, mirroring the clinical relapse of the original patients. Additionally, we employed the DiSCoVER platform, an RNA-seq-based tool that predicts drug sensitivity by contrasting tumor gene expression data with three reference databases (CTRP, CCLE, and GDSC), which contain drug response profiles for over 600 compounds [14–17], to perform an unguided and unbiased *in silico* drug screening, which identified 3 promising compounds; alectinib, ruxolitinib and dabrafenib. These candidates were subsequently validated in a prospective GOWt cohort, glioma stem-like cells (GSCs), glioma cell lines, and both a classical (U87) and a novel GO xenograft model, confirming their antitumoral activity across multiple glioma models.

Altogether, our work highlights the potential of GOs as predictive, patient-specific models that preserve the intricate biological features of malignant gliomas. By combining high-fidelity *in vitro* systems with advanced bioinformatics, this approach not only enhances our understanding of glioma biology but also sets the groundwork for their integration into clinical decision-making pipelines, potentially informing therapeutic choices, guiding clinical trial design, and ultimately contributing to improved patient outcomes.

Methods

Human samples

Tumor specimens and peripheral blood samples were obtained from patients undergoing surgery at Hospital Universitario Puerta de Hierro (Madrid, Spain) via its biobank, as well as from Hospital Universitario Quirónsalud Madrid (Madrid, Spain) and Hospital Universitario

12 de Octubre (Madrid, Spain) immediately following resection.

GOs generation, culture, and biobanking

GOs were generated, maintained, biobanked and recovered based on a previously described protocol [18, 19], with the only modification being the absence of agitation. GOs were cultured with M-GO, containing DMEM/F12, Neurobasal, non-essential amino acids, GlutaMAX supplement, N2 supplement, B27 Supplement, Penicillin-Streptomycin, 2-Mercaptoethanol and human insulin (all provided by GIBCO, USA). GOs were tested routinely for mycoplasma.

Tissue processing for downstream applications

Tumor samples and GOs were collected, washed in 1X PBS, and fixed in 4% paraformaldehyde (PFA) overnight under constant agitation. Samples designated for hematoxylin and eosin (H&E) staining were stored in 70% ethanol at 4 °C until further processing. Samples intended for immunofluorescence (IF) and immunohistochemistry (IHC) were embedded in optimal cutting temperature compound, snap-frozen in cryomolds, and stored at -80 °C.

Cryostat sectioning was performed to obtain 10 µm sections from the frozen samples, which were then mounted on poly-lysine-coated glass slides by the Histology Unit at San Pablo CEU IMMA (Madrid, Spain).

Hematoxylin and eosin (H&E) staining and imaging

Tissue dehydration and paraffin embedding were performed manually by the Histology Unit at San Pablo CEU IMMA (Madrid, Spain). Standard H&E staining was conducted on 5 µm sections. Slides were imaged using a Leica Aperio VERSA pathology scanner equipped with a 20x objective.

Immunofluorescence (IF) and immunohistochemistry (IHC) staining and image acquisition

Tissue sections were washed, permeabilized with 0.1% Triton-X, and incubated in a 10% blocking solution of specific serum for 1 hour. Sections were incubated overnight at 4° C with primary antibodies (Supplementary Table 1) and washed. Then, for IF staining, tissues were incubated with Alexa Fluor-conjugated secondary antibodies for 1 hour at room temperature (RT) (Supplementary Table 2). Sections were subsequently counterstained with DAPI. All fluorescence images were captured using the Leica Thunder Imager system with a 20x apochromatic objective. Raw images were processed with the Thunder deconvolution function in Leica LAS X software and assembled using Fiji software version 2.3.0.

For IHC staining, tissues were incubated with a biotinylated secondary antibody (Supplementary Table 2) for

1 hour at RT. After washing, sections were treated with the VECTASTAIN® Elite® ABC Kit (Vector Laboratories, USA), developed with DAB, and counterstained with hematoxylin. Coverslips were mounted with Eukitt® quick hardening mounting medium following dehydration. Brightfield images were acquired with a Leica Aperio VERSA pathology scanner.

Periodic acid-schiff (PAS) staining

FFPE GOs were subjected to standard IHC protocols. Before counterstaining, slides were washed and standard PAS staining was performed using the Periodic Acid-Schiff Kit (epredia, USA) following the manufacturer's protocol. Brightfield images were acquired with a Leica Aperio VERSA pathology scanner.

Bromodeoxyuridine (BrdU) staining

GOs were incubated overnight in M-GO medium containing 20 µM BrdU. Following incubation, GOs were collected, washed, and processed according to the IF staining protocol described above.

GO growth measurement

GOs were seeded in 6-well ultra-low attachment plates at varying densities: low (10 GOs per well), medium (20 GOs per well), and high (40 GOs per well) immediately after establishment. Media was refreshed every 48 hours, and widefield images were captured weekly using a Motic binocular stereomicroscope (Motic, China). Diameters were measured in Fiji software (version 2.3.0) by recording two perpendicular diameters per GO; the average of these measurements was then compared to the baseline diameter average recorded at week 0.

Quantitative real time PCR (qRT-PCR) of tissue and GOs

Fragments of tissue and paired GOs were dissociated, and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. One microgram of purified RNA was used for complementary DNA (cDNA) synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real time PCR (qRT-PCR) was conducted in an optical 96-well plate using a Bio-Rad CFX Connect Real-Time PCR system. SYBR Green dye (Takara Bio USA, Inc.) was used for detection. Gene expression levels were analyzed with specific primers (Supplementary Table 3), and *β-actin* was used as a reference gene for normalization.

Droplet digital PCR (ddPCR)

DNA was extracted from GOs and liquid biopsy samples using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. Droplets were generated with the Qx200 Droplet Generator (Bio-Rad,

Spain) using DNA at a concentration of 10 ng/ μ L (quantified by Nanodrop) per sample, combined with ddPCR Droplet Reader Oil (Bio-Rad, Spain). Plates were sealed with the P $\times$ 1 PCR Plate Sealer (Bio-Rad, Spain), and amplification was performed using the C100 Touch Thermal Cycler (Bio-Rad, Spain) with ddPCR Supermix (Bio-Rad, Spain) and the ddPCR Mutation Assay IDH1 R132H probe (Bio-Rad, Spain). Droplet reading was conducted with the Q $\times$ 200 Droplet Reader (Bio-Rad, Spain), and data analysis was performed using QuantaSoft™ software version 1.7.

D-2-Hydroxyglutarate (D-2HG) measurement

The concentration of D-2HG in GOs and their corresponding culture media was quantified using the PicoProbe D-2-Hydroxyglutarate (D2HG) Assay Kit (BioVision, USA). Frozen samples were thawed, homogenized, and centrifuged at 10,000 $\times$ g for 5 minutes at 4 °C to remove cellular debris. The resulting supernatants were then processed according to the manufacturer's instructions.

Sanger sequencing of IDH1 G395A

Genomic DNA was extracted from GOs using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The extracted DNA was amplified by conventional PCR using the following primers: forward, CGGTCTTCAGAGAAGCCA TT; reverse, GCAAATCACATTATTGCCAAC. PCR products were purified, and standard Sanger sequencing was performed by the Genomics Service at the Instituto de Investigaciones Biomédicas Sols-Morreale (IIBM, Madrid, Spain). Resulting sequences were aligned and compared using the BLAST algorithm. Chromatograms were inspected with FinchTV (version 1.4.0).

Flow cytometry (FC)

Tissue fragments and GOs were dissociated into single-cell suspensions using a combination of mechanical dissociation and the Neural Tissue Dissociation Kit (Miltenyi Biotec, Germany). Cell suspensions were rinsed with 1X PBS, centrifuged, passed through a 70 μ m strainer, washed again, and incubated with specific antibodies (Supplementary Table 4). After antibody staining, cells were washed with 1X PBS, and dead cell exclusion was performed using either propidium iodide (Sigma-Aldrich, USA) or Viability 488 (Miltenyi Biotec, Germany).

Flow cytometric analysis was conducted on a Miltenyi MACSQuant 10 flow cytometer. Results were analyzed using FlowJo software, version 10, with non-labeled controls, compensation controls, and fluorescence minus one controls included.

Transmission electron microscopy (TEM)

Tissue fragments (1 mm³) and GOs were fixed overnight at 4 °C in a solution containing 2% PFA and 2.5% glutaraldehyde. Samples were washed with phosphate buffered saline (1X PBS), post-fixed (1 h, 4 °C) with 1% osmium tetroxide (TAAB Laboratories) in potassium ferricyanide 0.8% (Sigma) and incubated with 2% aqueous uranyl acetate (Electron Microscopy Sciences) for 1 h at 4 °C. Subsequently, samples were dehydrated with increasing concentrations of acetone (anhydrous, VWR) and embedded in epoxy resin TAAB 812 (TAAB Laboratories). Polymerization was carried out for 2 days, 60 °C. Resin blocks were trimmed and ultrathin 70 nm-thick sections were obtained with the UC6 ultramicrotome (Leica Microsystems), transferred to 200 mesh nickel grids (Gilder) and stained with saturated aqueous uranyl acetate (25 min) and lead citrate 0.2% (1 min) at room temperature. Sections were visualised on a JEOL JEM 1400 Flash electron microscope (operating at 100 kV). Micrographs were taken with a Gatan One View digital camera at various magnifications.

Assessment of chemotherapeutic compounds in GOwts

To evaluate GO responses to standard chemotherapeutics, 20 GOs per condition were incubated for 48 hours with either TMZ (250 μ M), irinotecan (250 μ M), vincristine (10 μ M), gefitinib (25 μ M), etoposide (5 μ M), doxorubicin (0.2 μ M), dabrafenib (100 μ M), ruxolitinib (120 μ M) or alectinib (50 μ M) each dissolved in M-GO medium. Dosage was selected based on the average IC50 values of the selected compounds in glioma cell lines from the Genomics of Drug Sensitivity in Cancer database [16].

After 48 hours, GO viability was assessed using the MTS assay. Selected GOs were then processed for H&E staining, IF staining for GFAP and Vimentin, and IHC for γ H2AX.

RNA extraction, RNA sequencing and analysis

For RNA sequencing (RNA-seq), GOwts or tissues were collected, rinsed with 1X PBS, and stored in RNAlater at -80 °C. RNA was extracted, and RNA concentration was measured with a Nanodrop spectrophotometer. RNA integrity was assessed using a Bioanalyzer (Agilent Technologies, Spain), with only samples exceeding a RIN value of 7 used for sequencing.

Sequencing was carried out on the DNBSEQ™ platform (BGI TECH SOLUTIONS, Poland) with 150 bp paired-end reads and a sequencing depth of 20 million clean reads per sample, sufficient for downstream differential gene expression analysis.

RNA-seq data analysis was performed with the Dr. Tom bioinformatics platform from BGI. To assess the statistical significance of clustering patterns in heatmaps,

we used a multiscale bootstrap resampling approach implemented in the *pvcust* R package [20]. Clustering was performed using Pearson correlation distance ($1 - r$) and average linkage, consistent with the metrics used to generate the heatmaps. The resulting Approximately Unbiased (AU) p-values provide a robust and statistically validated measure of cluster reliability, as it estimates the probability that a given cluster is supported by the data beyond random chance. Clusters with $AU \geq 95\%$ were considered statistically significant ($p < 0.05$).

Samples subtype classification

GBM molecular subtypes were assigned using the GlioVis application [21], which integrates three complementary classification algorithms: support vector machines (SVM), k-nearest neighbors (k-NN), and gene set enrichment analysis (GSEA). Each sample received an individual prediction from each method, and a consensus subtype was assigned when at least two of the three classifiers agreed.

Single-cell RNA-seq analysis (scRNA-seq)

scRNA-seq datasets were downloaded from GEO (GSE117891) [22] and GBmap [23]. All analyses were conducted in R (v4.4.1) using the Seurat package (v5.3.0) [24].

After merging raw matrices, cells with $< 1,000$ UMIs, < 250 genes, or $> 15\%$ mitochondrial reads were discarded. Mitochondrial, ribosomal (RPL/RPS) and hemoglobin genes were removed to reduce technical noise. Doublets (DoubletFinder) [25] and ambient-RNA-contaminated cells (decontX) [26] were excluded; genes expressed in $< 1\%$ of cells were filtered out.

Data were log-normalised, the 2,000 most variable genes were selected, and counts were scaled. We computed 30 principal components, corrected patient-specific batch effects with Harmony [27], built a shared-nearest-neighbour graph and applied Louvain clustering; 2-D visualisation used UMAP.

Malignant versus non-malignant cells were identified with SCEVAN [28], which infers large-scale copy-number alterations from transcriptomes. Cells with GBM-typical aneuploidies were labelled “tumor”, diploid cells “non-tumor”. Broad lineages (tumor-associated macrophages, microglia, glia, vasculature, T cells) were assigned by SingleR [29], label transfer from multiple reference atlases, then harmonized at cluster level by majority rule.

RNA-seq deconvolution

Genes not present across all samples were filtered out, and raw read counts were normalized to counts per million. Deconvolution was performed using CIBERSORTx [30] in R (version 4.4.1) applying a custom signature matrix derived from Couturier et al. [31]. The analysis

was conducted with batch correction enabled in S-mode, 100 permutations, and quartile normalization disabled.

MTS viability assay and IC50 determination

To assess viability, six GOs of similar size per condition were selected, washed with 1X PBS, and dissociated using a combination of manual and enzymatic digestion (TrypLE Express, Gibco). Cell suspensions were centrifuged, resuspended, and seeded in 96-well plates in 200 μ L of M-GO medium. To determine IC50 of GOs, ten GOs per concentration were collected after 48 h treatment, washed with 1X PBS, dissociated manually and enzymatically (TrypLE Express, Gibco), single cell suspensions were centrifuged, washed and seeded in 96 well plates with 200 μ L of M-GO. Cell lines were passaged and seeded at a density of 3000 cells per well in a 96-well plate with 100 μ L of their respective growth media. Cells were left overnight and subsequently treated for 48 hours. Afterwards, 30 μ L of MTS reagent (Promega Biotech Ibérica, Spain) was added to each well, and plates were incubated at 37 °C in the dark for 2 hours. Absorbance was measured using a TECAN Sunrise microplate reader at 492 and 620 nm. Readings were normalized against the control condition. IC50 values were calculated by fitting a nonlinear variable slope curve in Prism software version 10.

Annexin V/propidium iodide (PI) apoptosis assay

Ten GOs per condition were collected after 48 h treatment, washed with 1X PBS, dissociated in collagenase IV (20 min, 37 °C), filtered through a 70 μ m strainer, and resuspended in 100 μ L Annexin V Binding Buffer (BioLegend, USA). Samples were stained with 5 μ L Annexin V-FITC (BioLegend, USA) and 5 μ L PI (Sigma-Aldrich, USA) for 15 min at RT, diluted with 400 μ L Binding Buffer, and analyzed immediately on a MACSQuant 10 flow cytometer (Miltenyi, Germany). Unstained and compensation controls were included. Data analysis was performed using Kaluza V2.1.

In silico drug sensitivity screening via DiSCoVER

To assess potential drug sensitivities in our samples, RNA-seq gene expression data was analyzed using the DiSCoVER platform (version 1.1) [17]. Bulk expression data from healthy brain samples from the GTEx portal [32] served as reference datasets. The analysis generated a ranked list of potential drug sensitivities for each organoid and its paired parental tissue. For *de novo* drug discovery, the top 20 predicted candidates from each sample were selected. These results were compared across all samples, resulting in two refined lists containing the top 6 compounds for parental tumor tissues and derived GOwts, respectively. From these lists, the top 3

overlapping compounds were identified and selected for subsequent functional validation studies.

Cell lines culture

The primary glioma stem cell (GSC) line GBM38 was generated and maintained following the protocol previously described [33]. Immortalized GBM cell lines U87, LN229, and U373 were cultured in DMEM/F-12 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 100 U/mL of penicillin/streptomycin (Gibco, USA). All cell lines were maintained under standard cell culture conditions at 37 °C in a humidified atmosphere with 5% CO₂. All cell lines were routinely tested for mycoplasma.

GOWts transwell migration assays

To evaluate migration capabilities, seven similarly sized GOs were dissociated into single-cell suspensions. The resulting solutions, containing M-GO and their respective treatments, were added to the top chamber of an 8 µm pore transwell insert (Corning, USA) placed in a 24-well plate containing 1 mL of M-GO supplemented with heparin, fibroblast growth factor (FGF), and epidermal growth factor (EGF). The setup was maintained under standard growth conditions for 6 hours.

After incubation, transwells were washed with 1X PBS, fixed in 4% PFA, and stained with 0.1% crystal violet. Transwell membranes were cut from the insert and mounted on a standard microscope slide. Images of migrated cells were captured immediately using a Nikon Eclipse microscope (Nikon, Japan).

GBM cell lines wound healing assay

To assess the migration capabilities of GBM cell lines, 500,000 cells per well were seeded in a 6-well plate (Corning, USA) with 3 mL of DMEM/F-12 medium (Gibco, US) supplemented with 10% FBS (Gibco, US). After overnight incubation to achieve full confluency, a wound was created manually using a 200 µL pipette tip. The wells were gently washed twice with 1× PBS to remove cell debris, and fresh medium containing 10% FBS, 100 U/mL of penicillin/streptomycin and the respective drug concentration was added.

Wound areas were imaged immediately after scratching and again after 24 hours using a Leica Thunder Imager system equipped with a 5× apochromatic objective (Leica, Germany). Wound closure was quantified using an automated image analysis macro [34], to minimize bias introduced by manual region selection.

Mice experiments

For in vivo drug studies, whole GOs or 1×10^6 U87 cells were resuspended in cold liquid Matrigel (Corning, USA) and subcutaneously injected into the flank of athymic

nude mice. Mice were anesthetized using 5% isoflurane for induction and maintained at 2% during the procedure.

Tumor growth was monitored daily with caliper measurements. Treatments commenced when tumors reached a volume of approximately 200 mm³. Mice were treated daily via intraperitoneal injections of vehicle, alectinib (25 mg/kg), ruxolitinib (50 mg/kg) or dabrafenib (20 mg/kg). Mice were euthanized either when tumors reached 1500 mm³ or when tumor burden became apparent, in accordance with ethical guidelines.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 10). Sample normality was assessed with either the Shapiro-Wilk or Kolmogorov-Smirnov test, depending on sample size. For normally distributed data, comparisons among groups were conducted using ANOVA with Welch's correction, while non-parametric data were analyzed with the Kruskal-Wallis test. Statistical significance was represented as follows: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Differences among groups were considered statistically significant at $p < 0.05$.

Results

Efficient generation of patient-derived glioma organoids (GOs)

Our cohort of GOs was derived from 61 patients, including IDH-wild type GBM (termed GOWt) and IDH-mutant (termed GOMut) cases, with the majority corresponding to GBM (Fig. 1A and B). Success in GO generation was confirmed by GOs reaching spherical morphology after 2 weeks in culture (Fig. 1C–F), increase in size and histological similarity confirmed via an independent attending pathologist. We also observed cycling cells shown as BrdU positive cells (Fig. 1G), confirming the macroscopic proliferation observations. Overall, we achieved a robust over 90% success rate in GO generation (Fig. 1B).

Seeding density and size severely impact GO growth rates

To determine the optimal conditions for GO growth, we first assessed the effects of culture density on GO expansion. GO diameters were measured in 9.6 cm² wells seeded at low (10 GOs per well), medium (20 GOs per well), and high (40 GOs per well) densities. Growth rates were comparable between medium and high densities, while low-density cultures exhibited significantly reduced growth, suggesting that an optimal seeding density of ~2 GOs per cm² maximizes growth efficiency. Across all conditions, growth rates declined after four weeks, defining this as a practical experimental timeframe (Fig. 2A).

We next investigated how GO size influences cellular distribution and proliferation. When GOs grew to diameters of ~3 mm, vimentin expression became restricted

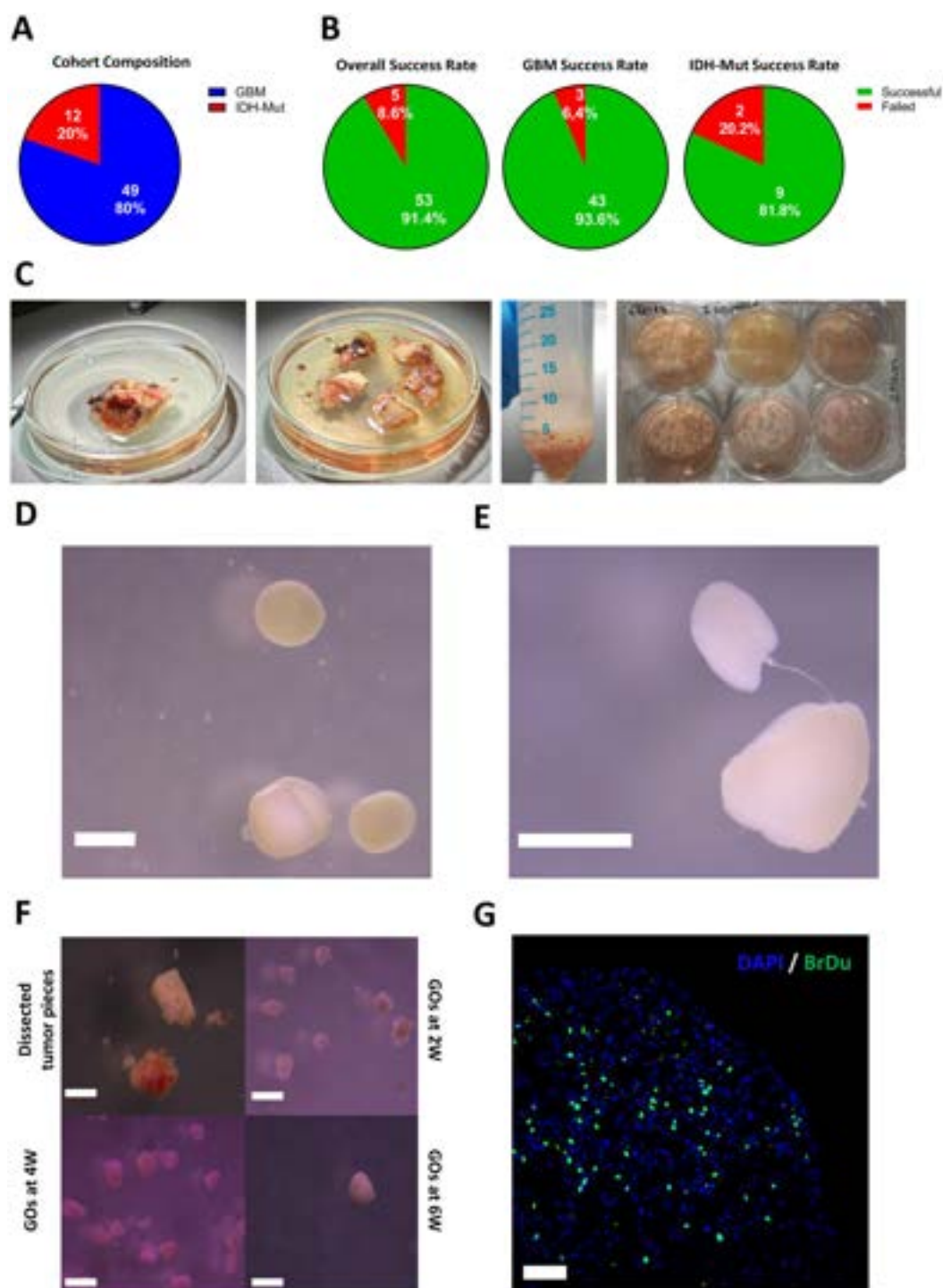


Fig. 1 Generation of patient-derived GOs: **(A)** Sample distribution used for organoid generation, with GBM patients shown in blue, IDH-mutant patients in red. **(B)** GO generation success rates, shown as the number of successful GOs (green) and unsuccessful attempts (red) across all samples (left), GBM samples (center), and IDH-mutant samples (right). **(C)** Images illustrating the organoid generation process, from left to right: original tumor sample, coarse sectioning, final wash of pre-organoids, and seeded pre-organoids. **(D)** Representative brightfield image of fully formed GOs after two weeks in culture. **(E)** Brightfield image showing two organoids in culture connected by a thin tube-like structure. **(F)** Brightfield images depicting organoid progression in culture, from initial tumor dissection to six weeks. Scale bar is 1 mm. **(G)** Immunofluorescence (IF) image of BrdU (green) incorporation in a representative GO, indicating cell proliferation; scale bar = 200 μ m

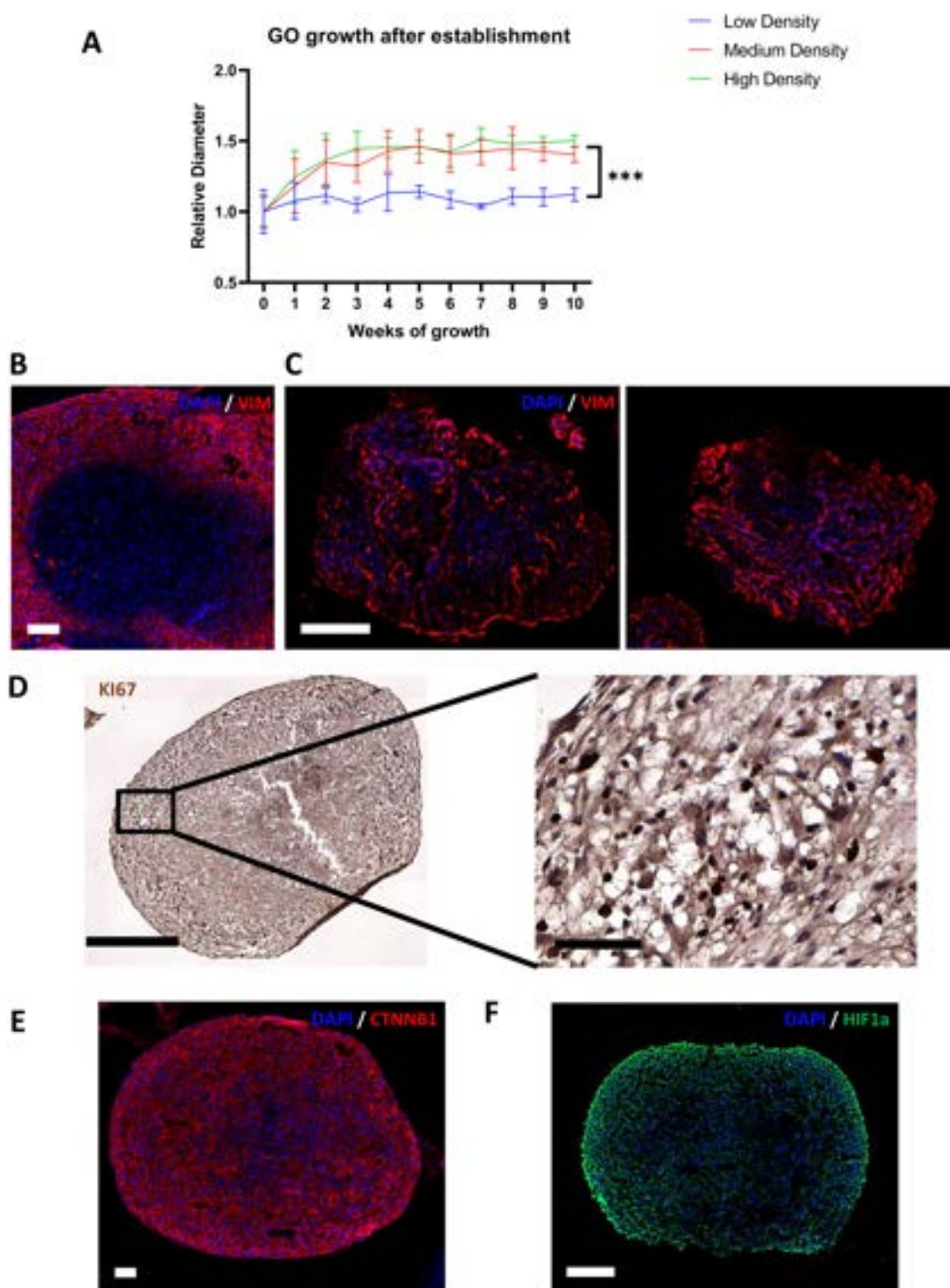


Fig. 2 Effects of seeding density and GO size on growth: **(A)** Relative diameters of a representative GO measured over 10 weeks, with low-density (10 organoids/well), medium-density (20 organoids/well), and high-density (40 organoids/well) culture conditions. Each time point represents the mean diameter, with vertical lines indicating standard deviation (SD). *** $p < 0.0001$. **(B)** IF staining for VIMENTIN (red) in representative GO; scale bar = 200 μ m. **(C)** IF staining for VIMENTIN (red) in two different GOs; scale bar = 200 μ m. **(D)** IHC staining for Ki67 (dark brown) in GO, with a close-up of the GO periphery (right). Scale bar = 1 mm (left) and 0.1 mm (right). **(E)** IF staining for CTNNB1 (red) in GO; scale bar = 100 μ m. **(F)** IF staining for HIF-1 α (green) in a representative GO; scale bar = 200 μ m

to the periphery, despite nuclei still being present in the core (Fig. 2B). In contrast, smaller GOs displayed a more homogeneous vimentin distribution (Fig. 2C). A similar core-periphery pattern was observed for Ki-67, indicating that proliferative activity is concentrated in the periphery of larger GOs (Fig. 2D). Interestingly, this pattern was not observed for β -catenin (Fig. 2E) or HIF1 α (Fig. 2F), suggesting that oxygen and nutrient diffusion limitations in larger GOs primarily impact proliferation rather than other cellular functions.

Finally, we examined the impact of biobanking on GO growth and histology. While post-biobanked GOs retained their histological characteristics, closely resembling both the original tumor tissue and pre-biobanking GOs (Fig. S1A), they did not increase in size, indicating severely impaired proliferation (Fig. S1B). Next, we wondered whether we could improve the proliferation rates of recovered GOs in an *in vivo* model. To do so, we selected GOs that had been cryopreserved for longer than 6 months and performed subcutaneous xenografts in athymic mice. We observed that these GOs maintained their original tumorigenic properties (Fig. S1C and D), and we were even able to derive a GSC line from them (Fig. S1E). Together, these findings emphasize that seeding density and GO size significantly influence growth, proliferation, and structural organization. While biobanking preserves histological integrity despite halting proliferation, GOs exceeding 3 mm in diameter develop core-periphery segregation, which may impact their functionality. Maintaining optimal culture conditions is crucial for maximizing GO utility in preclinical studies.

GOs conserve key tumoral histological features and elements of tissular and cellular microarchitecture

To characterize the structure of our GOs, we first performed H&E staining on successfully established GOs. GOWt (Fig. 3A and S1F) and GOMut (Fig. 3B). GOs were evaluated by an independent attending pathologist, who confirmed their resemblance to the original tumor tissue. GOs also displayed diverse tumor cell phenotypes and hallmark glioma features, such as necrotic areas (GO30) and abundant microvasculature (GO8). These histological characteristics remained stable over several weeks and passages in culture (Fig. 3C and S1G).

To further confirm that GOs closely replicate the microstructure of the original tumoral tissue, we examined GOs and their corresponding parental tumors using TEM after two weeks in culture. Initial observations revealed the presence of myelin sheaths in the GOs, consistent with the tissue of origin, although early signs of degeneration were observed (Fig. 3D). We also observed areas of necrosis and edema in both parental tumors and GOs (Fig. 3E), reflecting classic histological features of GBM.

Within the GOs, neurons were readily identified based on nuclear shape and electron density (Fig. 3F). These neurons retained healthy mitochondria (Fig. S2A) and an intact reticulum network, and they also engaged in interactions with nearby cells. This is noteworthy given the challenges of sustaining neurons *in vitro* [35]. Glial cells were abundant, with close to 25% containing large glycogen deposits (Fig. 3G, S2B, S2C and S2D) and a significant number (50–60%) in both tumors and GOs presented lipid droplets (Fig. 3H, S3A). Oligodendrocytes could be identified close to myelin sheaths (Fig. S3B). Close examination of the cytoplasm of these glial cells showed intact nuclear membranes and well-preserved organelles (Fig. S3C). Cellular projections loaded with ribosomes were observed, suggesting that these are not phagocytic processes (Fig. S3C).

Furthermore, we observed multiple cell-cell interactions, primarily between neurons and glial cells (Fig. 3I and S3D), indicating preserved cellular roles and interactions in the GO model. Tumor cells displayed a range of morphologies and sizes, with healthy mitochondria and enlarged reticulum (Fig. 3J, S3E and S3F). Around 7% of cell projections contained mitochondria, with a density of 5–6 mitochondria per μm^2 , consistent with previously described tumor microtube-like structures in gliomas (Fig. S4A and S4B) [36, 37] and matching our macroscopic observations (Fig. 1E). Finally, extracellular matrix (ECM) components, including collagen, were also identified in the GOs (Fig. S4C).

Overall, these findings demonstrate that GOs faithfully replicate the complex histological and ultrastructural features of gliomas, including diverse cellular populations, cell-cell interactions, ECM components, and classical glioma features.

GOs contain heterogeneous cell populations and conserve vascular microstructure

After our general characterization, we decided to focus on their molecular properties. To that end, we began by analyzing protein distribution by IF. We first identified mature astrocytes, differentiated oligodendrocytes and neurons (Fig. 4A and S5). Additionally, we observed the conservation of vascular microarchitecture, including CD31+ structures resembling microvessels and a prominent presence of VEGFA and its receptor VEGFR2 (Fig. 4B). Furthermore, we detected glioma-associated stemness markers CD90, Vimentin, and Nestin (Fig. 4C).

The close resemblance of parental tumors and GOs was confirmed by the expression of stemness markers (Fig. 4D), cell-type markers (Fig. 4E), and vasculature markers (Fig. 4F). Linear regression analysis of these values highlighted a very strong relationship, with R^2 values of 0.79 for GBM and 0.83 for IDH-mutant gliomas (Fig. 4G). Supporting these results, the specific gene expression

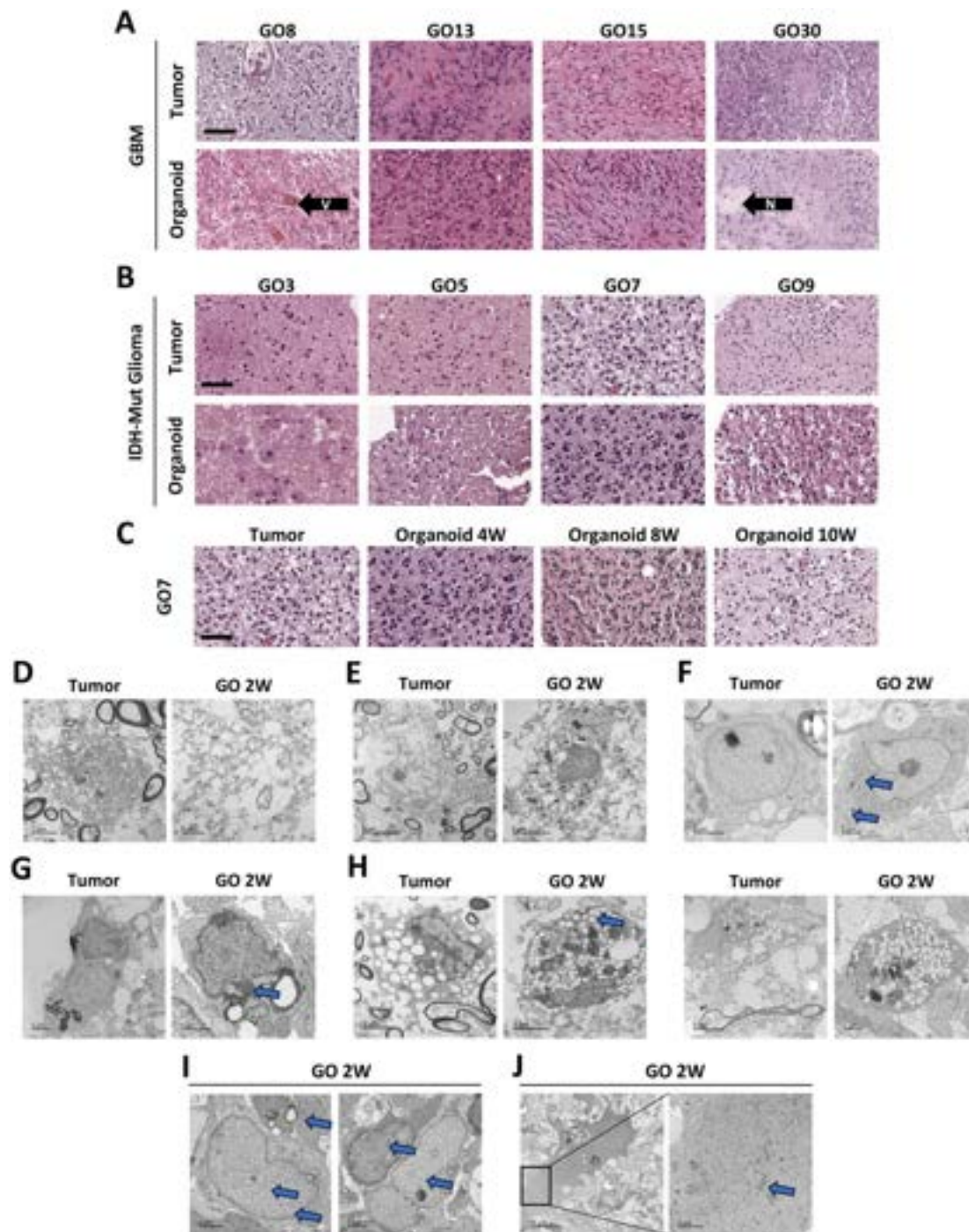


Fig. 3 General characterization of patient-derived GOs: H&E staining of representative sections from original tumor tissue (top) and GOs (bottom) for GBM (**A**) and IDH-mutant glioma (**B**) patients; scale bar = 100 μ m. Microvascular areas are marked as "V", while necrotic parts are highlighted with "N" (**C**). Histological progression of a GO in culture, with H&E-stained sections of the original tumor tissue (left) and corresponding GO after several weeks in culture; scale bar = 100 μ m. TEM images of: (**D**) Myelin sheaths observed in the original tumor tissue (left) and the corresponding GO (right). (**E**) Necrotic areas in tumor tissue (left) and GO (right) after two weeks in culture. (**F**) Neurons in patient tumor tissue (left) and GO (right), with healthy mitochondria and reticulum network highlighted. (**G**) Oligodendrocytes in tumor tissue (left) and in the derived GO (right), showing glycogen deposits identified in the GO. (**H**) Lipid droplet-rich oligodendrocytes in original tumor tissue (left) and corresponding GO (right), shown in two different patients, with lipid droplets highlighted in blue. (**I**) Neuron-oligodendrocyte interactions observed in GOs after two weeks in culture; neurons (bottom) and oligodendrocytes (top) are indicated by blue arrows. (**J**) Tumor cell identified in a GO after two weeks in culture, with a magnified view of the cytoplasm (right) highlighting mitochondria and the Golgi apparatus

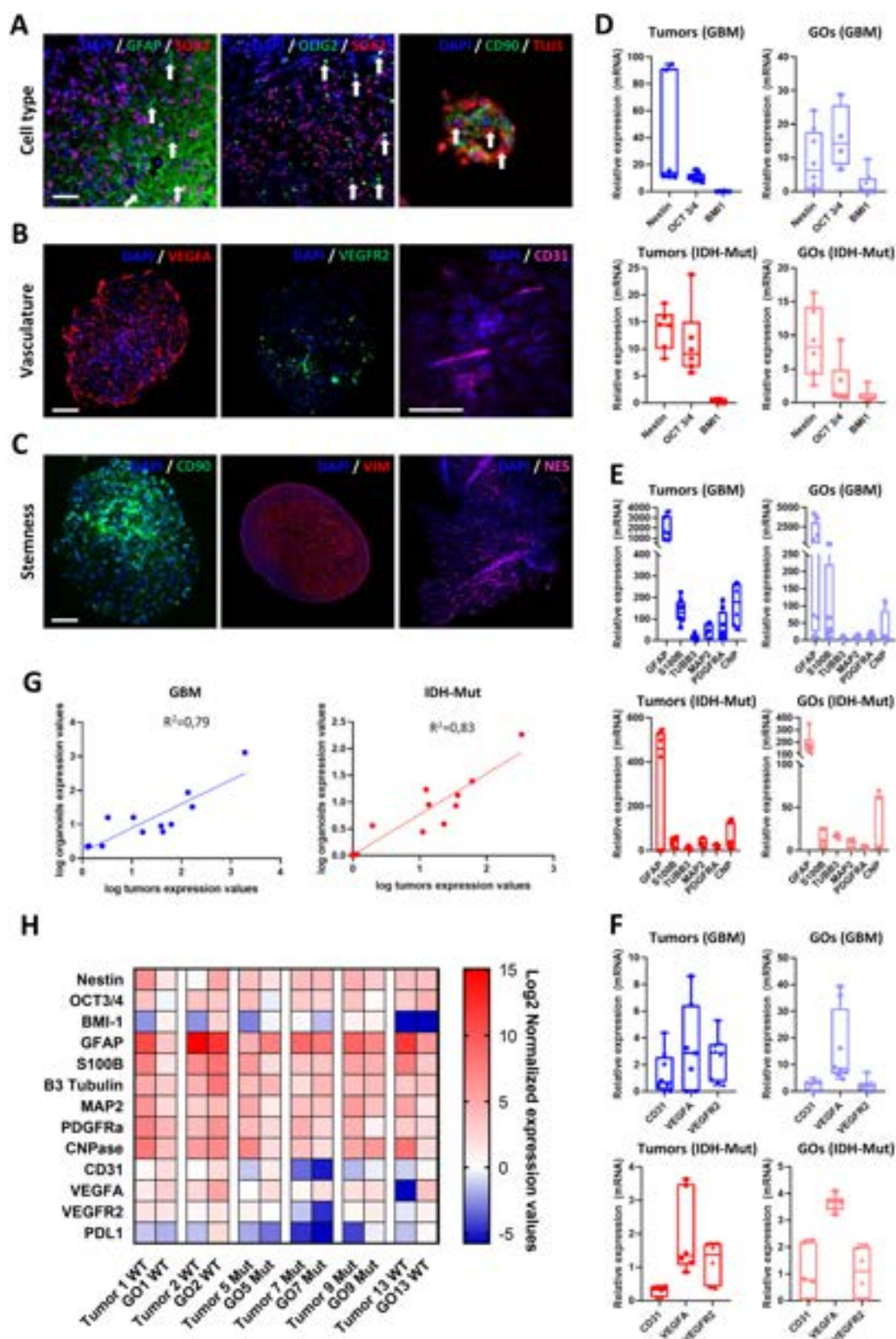


Fig. 4 (See legend on next page.)

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Fig. 4 Molecular characterization of GOs: **(A)** Immunofluorescence staining of cell type markers in patient-derived GOs: left GFAP (green) and SOX2 (red); center OLIG2 (green) and SOX2 (red); right TUJ1 (red) and CD90 (green), with nuclei counterstained using DAPI (blue); scale bar=50 µm. White arrows point to astrocytes, oligodendrocytes and neurons, respectively. **(B)** Immunofluorescence staining of microvasculature markers in GOs: VEGFA (red, left), VEGFR2 (green, center), and CD31 (magenta, right), with nuclei counterstained with DAPI (blue); scale bar=200 µm. **(C)** Immunofluorescence staining of stem cell markers in GOs: CD90 (green, left), VIMENTIN (red, center), and NESTIN (magenta, right), with nuclei counterstained with DAPI (blue); scale bar=200 µm. **(D–F)** Box plots showing qRT-PCR expression levels of **(D)** stemness markers (*NESTIN*, *OCT3/4*, *BM1*), **(E)** cell-type markers (*GFAP*, *SL100B*, *TUBB3*, *MAP2*, *PDGFRA*, *CNP*), and **(F)** microvasculature markers (*CD31*, *VEGFA*, *VEGFR2*) in glioma samples. Data is shown for tissue from GBM patients (dark blue) and IDH-mutant patients (dark red), as well as derived GOwts (light blue) and GOMuts (light red). Each point represents an individual organoid, with median, interquartile range (IQR), and standard deviation (SD) indicated. *N*=6 paired samples in duplicate. **(G)** Linear regression analysis of gene expression between tumor tissue and derived GOs from GBM (left, blue) and IDH-Mut (right, red) samples. Expression values are represented as log-transformed values, with the *R*² value of each regression displayed on the graph. **(H)** Heatmap of log₂-normalized qRT-PCR expression values for the assessed genes, with each pair of columns representing parental tissue (left) and the corresponding GO after two weeks in culture (right). *N*=6 paired samples.

patterns in each tumor sample were faithfully recapitulated in their paired GOs (Fig. 4H).

GOwts conserve molecular expression and key genetic alterations of parental tissues

To continue our GO molecular characterization, we performed RNA-seq on a representative cohort (*N*=11) of paired tumors and derived GOwts from GBM tissues, as these represent the majority of cases. First, we assessed differential gene expression (Fig. 5A).

We identified a subset of differentially expressed genes (DEGs) in GOs, primarily associated with metabolic pathways, disease adaptation, environmental response, and molecular transport (Fig. 5B). Moreover, GSEA analysis of these DEGs confirmed upregulation of signatures related to protein secretion, glycolysis and hypoxia in GOs (Fig. 5A). Additionally, we observed that 17088 genes were expressed in both tumors and GOs, constituting 96.2% of the total genes expressed. Conversely, only 287 (1.6%) genes were exclusively expressed in parental tumors and 396 (2.2%) were unique to GOs (Fig. 5C). Gene ontology (GO) analysis of these genes revealed both tumor and GOs exclusive genes related to mostly integral components of cell membranes as well as signaling and response pathways (Fig. 5D and E).

Complementary analyses using Pearson correlation (Fig. 5B) and hierarchical clustering (Fig. 5C) further underscored the strong molecular similarity between GOs and their parental tumors. These data suggest that GOs maintain expression of almost the full gene complement of parental tissues, and uniquely expressed genes may be related to adaptations to their new ex vivo culture environment.

We continued by examining the expression of stromal cell-associated markers and cell activity genes, which closely mirrored our previous results. Moreover, we observed that tumors and paired GOs tend to group together when examining both stromal and activity-related signatures, where we observed consistently high AU values within major tumor-GO clusters (Fig. 5F and G). These findings further reinforce the molecular similarities between GOs and their parental tumors

independent of the specific functional gene set analyzed, supporting their relevance as preclinical glioma models.

To evaluate the conservation of the molecular subtype [38] of our model, we analyzed RNA-seq data from paired tumors and GOs using the gene signature refined by Wang et al. [39]. Majority of GOs retained the molecular subtype of their parental tumor, while 4 evolved to the mesenchymal subtype and 1 shifted from proneural to classical (Fig. 5D), in addition, statistical analysis of subtype clustering showed a clear association between tumors and paired GOs (Fig. 5E). This transition may reflect sampling variability due to spatial heterogeneity within tumors or represent dynamic changes occurring in culture that parallel in vivo tumor evolution [40, 41].

We also examined the status of the IDH1 R132H mutation in our GOMut samples, which is currently the only clinically actionable mutation in gliomas and is associated with improved mOS [42, 43]. Unlike established glioma cell lines, which often lose this mutation in culture [44], our GOMuts maintained IDH1 R132H over time. We were able to detect the presence of the mutation in representative GOMuts by IHC, ddPCR, and Sanger sequencing, but not in GOwt controls. Consistent with this, GOMuts exhibited significantly elevated D-2HG levels. Moreover, we verified the long-term stability of this mutation in GO7mut after eight weeks in culture (Fig. 5F–I).

Altogether, these findings establish GOs as reliable models for preserving the molecular identity of gliomas, including key genetic mutations and subtype fidelity.

GOwts recapitulate myeloid-dominant immune profiles and low checkpoint expression found in parental IDH-wt GBM

Given the poor prognosis of gliomas, immunotherapy has been explored as a potential therapeutic option [45]. Several molecular approaches were performed to better understand and characterize the immune landscape of our samples in depth.

Transcriptomic analysis revealed elevated expression of myeloid lineage-associated genes, including microglial and macrophage markers (Fig. 6A), consistent with

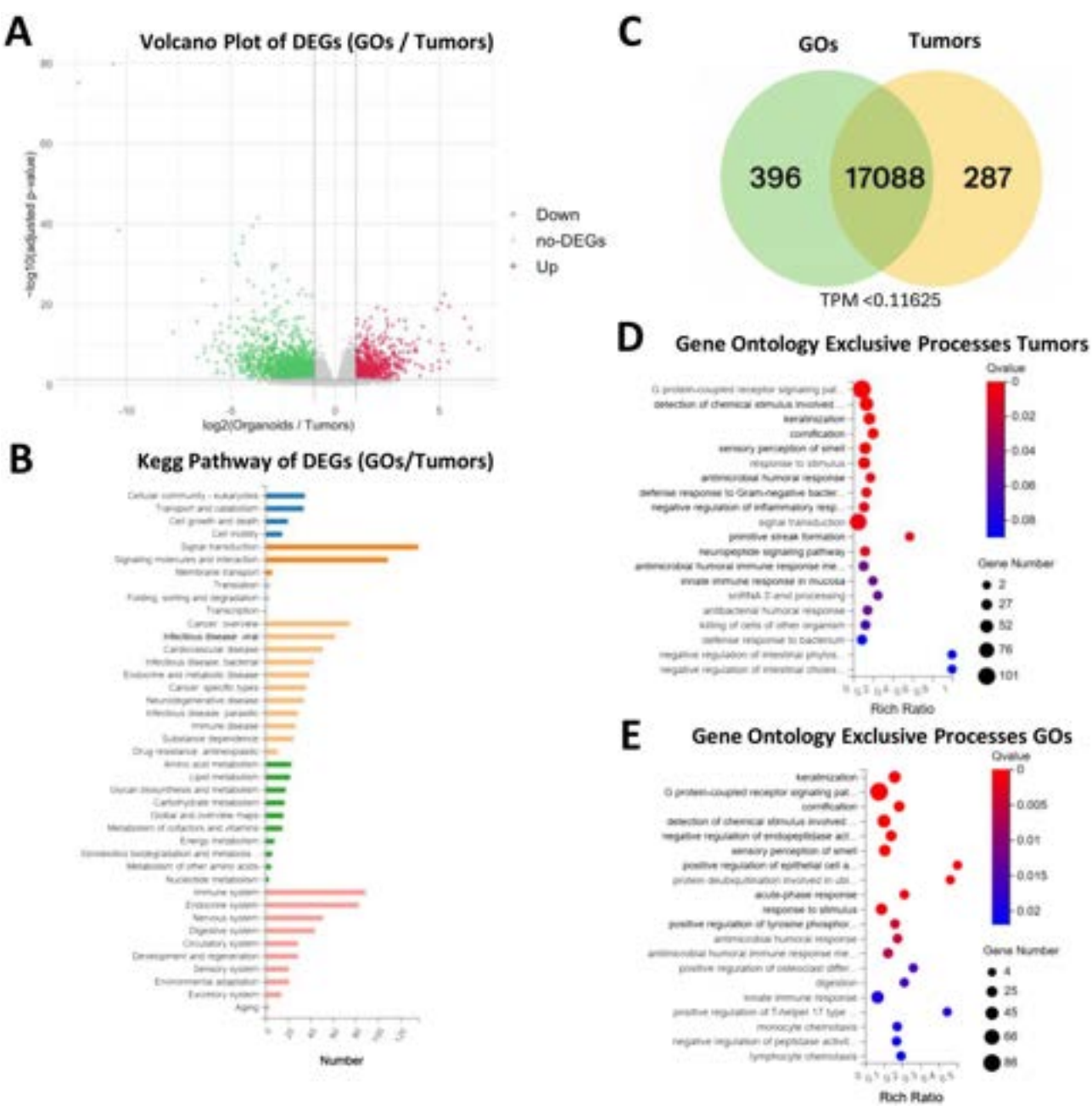


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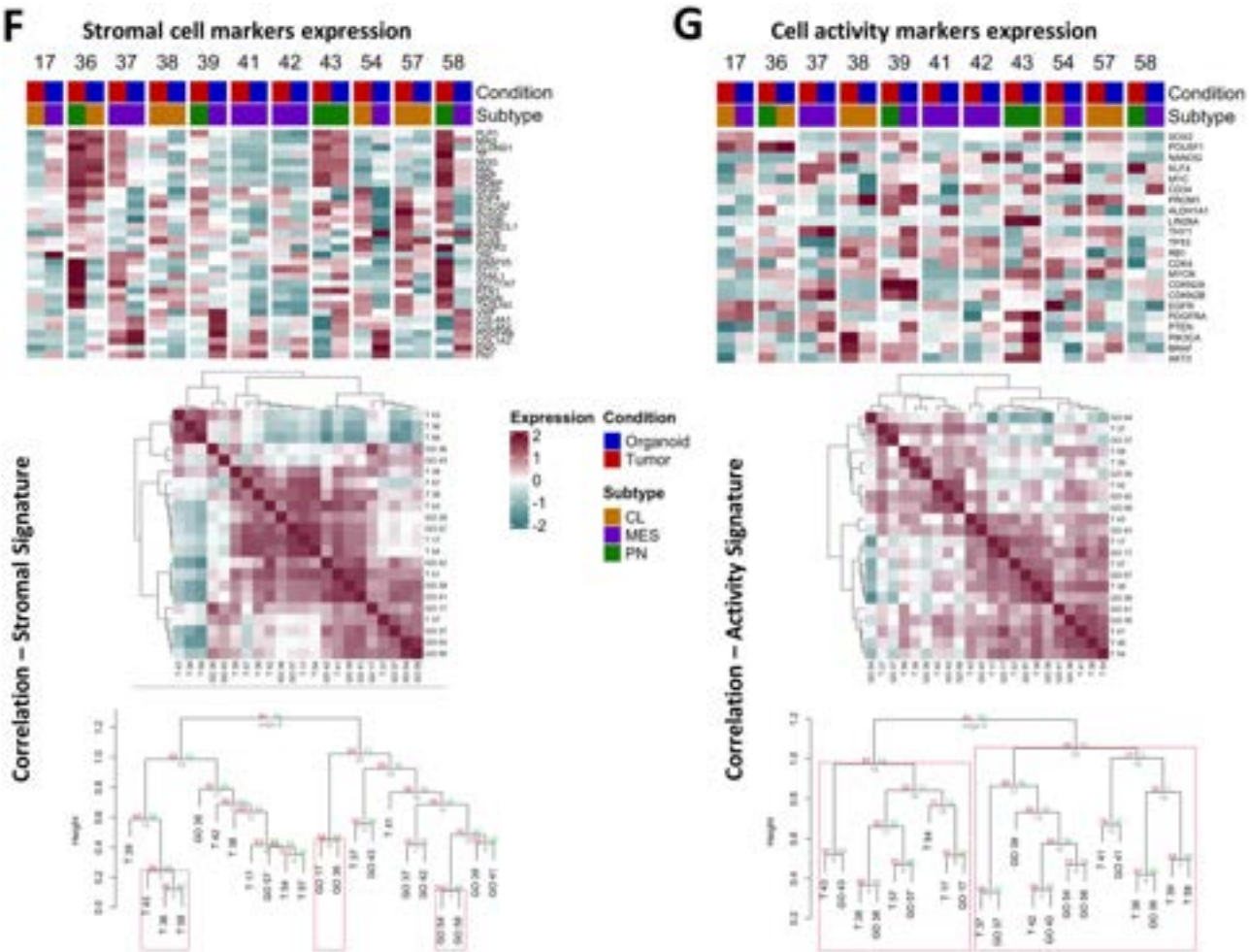


Fig. 5 RNA-seq analysis of GOs from IDH-wt GBM: **(A)** Volcano plot highlighting upregulated (red) and downregulated (green) genes in GOs compared to their parental tumors. Gene expression is represented as log₂ Fold change, with statistical significance shown as -log₁₀ q-value **(B)** Kegg pathway classifications of upregulated genes in GOs, with categories color-coded. **(C)** Venn diagram depicting genes uniquely expressed in GOs (green) and parental tumors (yellow). **(D-E)** Gene Ontology process classification of genes exclusively expressed in tumors **(D)** or GOs **(E)**. **(F-G)** Heatmaps displaying z-score normalized expression (top), sample-wise correlations (middle) and hierarchical clustering dendrograms with Approximately Unbiased (AU) p-values computed by pvclust (bottom) of stromal cell markers **(F)** and cell activity markers **(G)** in matched parental tissues (red) and GOs (blue). Samples are color coded according to their molecular subtype: classical (yellow), mesenchymal (purple) and proneural (green). TPM values were scaled by gene-wise z-score normalization (range -2 to 2) to standardize the expression levels across samples. *N* = 11 paired samples

previous observations in gliomas [46, 47]. These findings were validated by IF, where we readily detected IBA1+ microglia and CD68+ macrophages in both tumor tissues and paired GOs (Fig. 6B). Closer inspection of M1/M2 polarization markers [48] by IF identified the presence of M1 and M2 positive microglia in our GOs (Fig. 6C). These two subpopulations were also readily identified in our transcriptomic data in both tumors and derived GOs, with a slight prevalence of M1 markers (Fig. 6A). In contrast, RNA-seq analyses indicated a lack of lymphoid cells. T-cell markers, including CD3, CD4, and CD8, were expressed at negligible levels across samples (Fig. 6A) and were not detectable by IF (Figs. 6D). Mouse spleen samples were used as positive controls (Fig. S7A). Flow cytometry data confirmed this “cold” immune

profile, revealing that only approximately 10% of CD45+ cells were CD3+, with minimal CD4+ or CD8+ subpopulations present (Fig. 6E, S7B and C). This profile was conserved across GOs and their respective tumors. To further corroborate these observations, we analyzed two publicly available scRNA-seq cohorts (CGGA and Bhaduri), both of which confirmed a minimal presence of lymphoid lineage cells in glioma tissues (Fig. 6G). Additionally, immune cell deconvolution of our bulk RNA-seq data reinforced the prevalence of myeloid populations in both tumors and GOs, while lymphoid cell populations remained sparse, with no significant differences between the two sample types (Fig. 6H). Interestingly, principal component analysis (PCA) of our RNA-seq data using an immune cell-specific gene

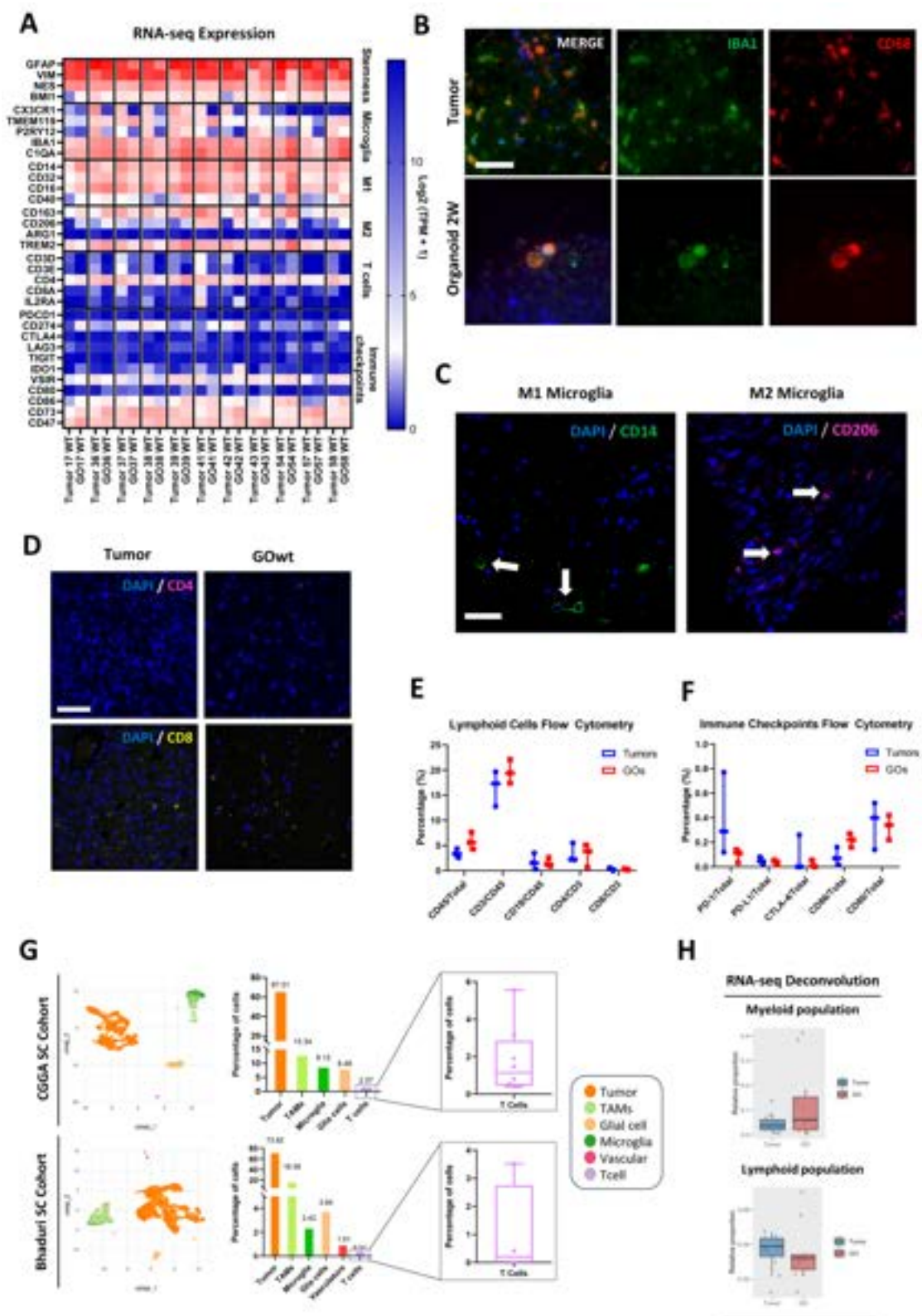


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Fig. 6 GOs derived from IDH-wt GBM replicate “cold” immune phenotype: **(A)** RNA-seq expression levels from 11 parental tumors (left, termed tumors) and their corresponding GOs after 2 weeks in culture (right, termed GO). Color represents log2 of TPM +1 values with a color gradient from blue (lowest expression) to red (highest expression). Selected genes are separated into their function, from top to bottom, stemness, microglia, M1 and M2, T cell and immune checkpoints markers. **(B)** Representative IF for CD68 (red) and IBA1 (green) in parental tumors (top) and derived GOs after 2 weeks in culture (bottom). Counterstained with DAPI (blue). Scale bar is 100 μ m. **(C)** Representative IF for M1 marker CD14 (left, green) and M2 marker CD206 (right, magenta) in GOs after 2 weeks in culture. Nuclei are counterstained with DAPI. Scale bar is 50 μ m. **(D)** Representative IF for CD4 (top), magenta) and CD8 (bottom, yellow), in both parental tumors (left) and derived GOs after 2 weeks in culture (right). Scale bar is 100 μ m. **(E)** Violin plot illustrating immune cell populations identified by flow cytometry in original tissue samples (left, blue) and corresponding derived GOs (right, red). **(F)** Violin plot showing percentage of cells expressing key immune checkpoint markers assessed by flow cytometry in tumor tissue (left, blue) and derived GOs (right, red). **(G)** scRNA-seq analysis of immune cell populations in glioma datasets from the CGGA (top) and Bhaduri (bottom) cohorts. UMAP plot of cell-type distribution (left), bar graph of immune cell proportions (middle), and box plot of T-cell percentages per patient (right). **(H)** Boxplot of RNA-seq deconvolution via CibersortX on myeloid cell population (top) and lymphoid population (bottom) of parental tumors (blue) and GOs (red)

signature revealed that GOs and their parental tumors form two distinct clusters (Fig. S7G). This suggests that while the overall immune landscape is preserved, certain immune components may diverge due to adaptation to ex vivo culture conditions, or due to the deterioration of immune cells after two weeks in culture.

We next investigated the expression of immune checkpoint (IC) proteins, which are often targeted in immunotherapy. RNA-seq and flow cytometry showed minimal expression of classical ICs, including PD-1, PD-L1, CTLA-4, CD80, and CD86, with fewer than 0.8% of total cells expressing these proteins (Figs. 6A, 6F and S7D). These findings were validated by IF, which confirmed the absence of PD-L1 in glioma samples but its presence in a primary B-cell lymphoma used as a positive control (Fig. S7E). qRT-PCR analysis also confirmed low PD-L1 transcript levels in both tumors and GOs (Fig. S7F and 4H). Notably, we did observe clear expression of macrophage-associated ICs CD47 and CD73 (Fig. 6A), which have been described in the GBM context [49].

Collectively, these findings demonstrate that GOs preserve the global immune composition of parental tumors, characterized by dominant myeloid infiltration and lymphocyte deficiency. While transcriptomic analyses reveal culture associated shifts in immune cell states, the conserved myeloid enrichment and low expression of classical immune checkpoints support the fidelity of GOs as a model of glioma immune evasion and help explain the limited efficacy of conventional immune checkpoint therapies in this tumor type.

GOs derived from IDH-wt GBM are resistant to standard chemotherapeutics

We next investigated the response of GOs to antineoplastic agents incorporated into glioma treatment regimens, including TMZ, vincristine, irinotecan, gefitinib, etoposide, and doxorubicin. For this analysis, we focused on GOwts, as GBM is the most prevalent form of glioma with the worst prognosis. To begin, we conducted an *in silico* analysis using our RNA-seq data. This data was analyzed with the bioinformatic tool DiSCoVER [17], which compares expression profiles of tumor samples to predict

potential drug sensitivities. The analysis suggested that GOs would be resistant to most of the selected compounds, except for gefitinib, which was predicted to elicit a potential response in all samples with a score of 0.3–0.4, and doxorubicin, with a lower score of 0.15–0.2 (Fig. 7A). These data were further validated in newly established GOs via MTS assay (Fig. 7B) and annexin V/PI apoptosis assay (Fig. 7C). We also observed a notable reduction in the number of cells per mL of gefitinib treated GOs (Fig. S9A)

Histological features of treated GOs remained unchanged when compared to untreated GOs, and we further examined the expression of classical GBM markers (Fig. 7D and E).

No significant vimentin (Fig. 7G) or GFAP (Fig. 7H) level changes were observed among treatments. However, we observed that TMZ and gefitinib treated GOs present a lower nuclei count (Fig. 7F) while TMZ, etoposide, doxorubicin and gefitinib presented significantly higher levels of DNA damage (Fig. 7I).

To assess clinical relevance, we analyzed the clinical progression of patients included in the *in silico* screen. Notably, almost 70% of patients experienced tumor recurrence within 12 months despite receiving SOC treatment (Fig. 7J), aligning with the predicted resistance to TMZ and reinforcing the predictive value of our GO-based model.

DiSCoVER-guided drug screening uncovers alectinib, ruxolitinib and dabrafenib as novel therapeutic candidates for GOs derived from IDH-wt GBM patients

Building on our previous findings, we sought to identify potential therapeutic compounds for GOs. To this end, we selected the 3 compounds with the highest sensitivity score in our computational screening (Fig. 8A). One of them, alectinib, is effective in tumors with high expression of the ALK protein [50], a common phenomenon in gliomas [51, 52]. We confirmed a higher RNA-seq expression of *ALK* in our cohort than in healthy brain tissue (Fig. S8B). Moreover, ALK could also be detected via IHC in GOs (Fig. S8A), confirming target relevance.

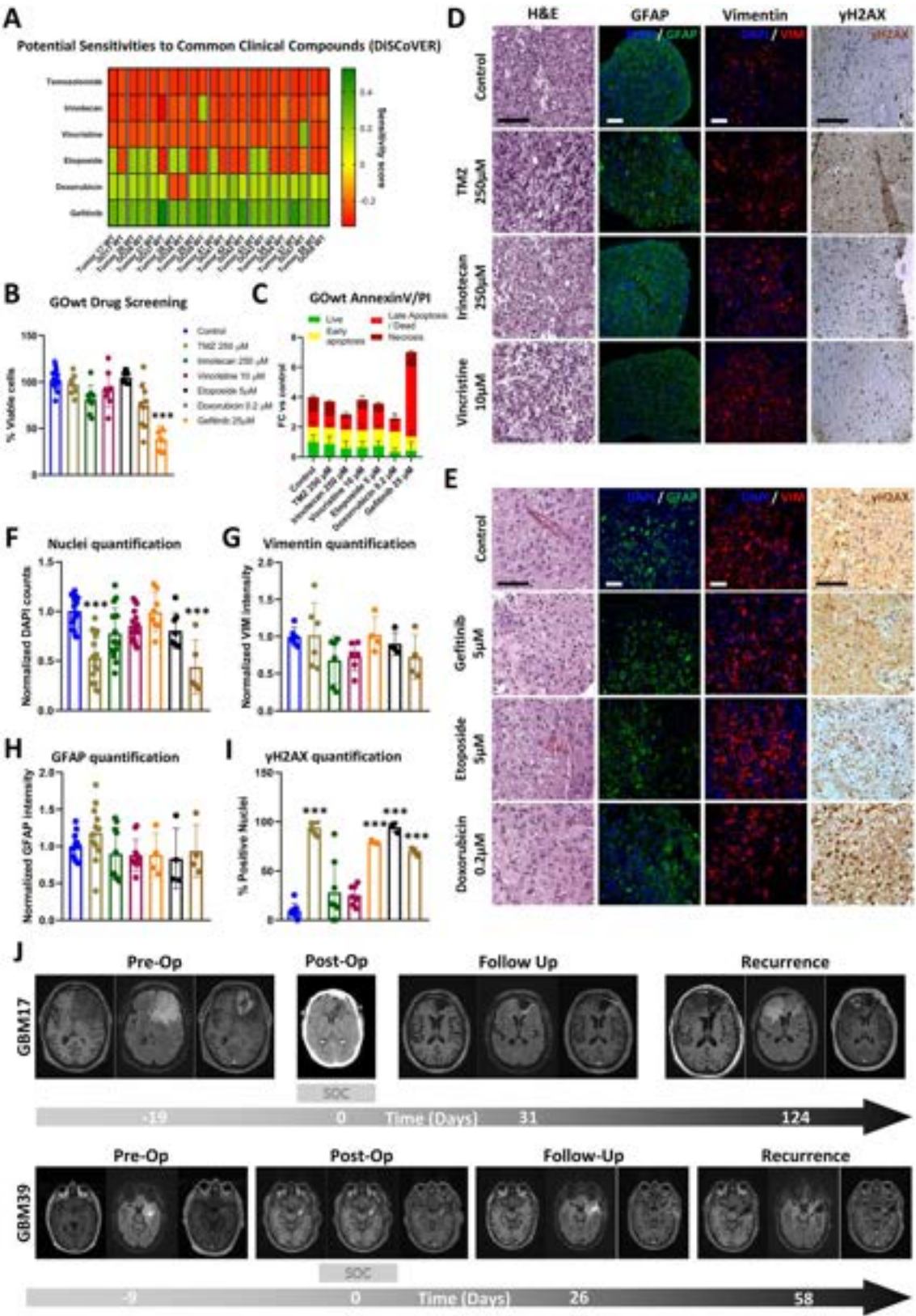


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Fig. 7 GOwts are resistant to standard-of-care chemotherapies: **(A)** Heatmap of predicted drug sensitivities to SOC therapeutic agents. Drugs are listed on the left, and each pair of rows represents a parental tumor and derived GO. Color corresponds to sensitivity score, with dark green representing the potential best responders, and dark red the most resistant samples. **(B)** MTS viability assay after 48 h of treatment. Data is normalized to control. Bars represent mean % of viable cells, with SD. $N = 3$ different GOs per treatment. **(C)** Stacked bar graph of annexin V/PI apoptosis assay on representative GOs after 48 h with the indicated drug concentrations of manually selected compounds. Green represents live cells, yellow for early apoptotic cells, red for late apoptotic/dead cells and brown for necrotic cells. Bar height represents Fold change normalized to control, with error bars indicating SD. **(D and E)** Representative H&E, GFAP (green), VIM (red) and γ H2AX staining images after 48 hours of treatment. Scale bar is 100 μ m. **(F–I)** Quantification of number of nuclei **(F)**, VIM **(G)**, GFAP **(H)**, and γ H2AX **(I)** from the images. Data are normalized to respective controls, with bars representing mean values and error bars indicating SD. Statistical significance is indicated as follows: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. **(J)** Representative MRI images depicting the clinical progression of patients GBM17 (top) and GBM39 (bottom) following SOC therapy. Sequential scans illustrate tumor evolution across key clinical stages: pre-operative baseline, post-operative status, follow-up period, and recurrence. Timepoints are annotated

We validated the *in silico* predictions in a prospective GO cohort, where we observed a significant reduction of cell viability (Fig. 8B and D) by all 3 compounds, together with a considerable reduction in cells per mL (Fig. S9B). Longer treatment times further decreased cell viability (Fig. S9C). Alectinib exhibited the lowest IC₅₀ value at around 25 μ M (Fig. S9D and Supplementary table 5). H&E staining confirmed these observations with a drastic cellularity reduction for alectinib and ruxolitinib (Fig. 8C). These antitumoral effects were reproduced in several other glioma models, such as the patient-derived GSC line GBM38, and 3 immortalized glioma lines (U87, U373, LN229) (Fig. 8E), all cell lines except GBM38, which was the most resistant, had a very similar IC₅₀ compared to GOs at around 20–23 μ M, comparable values of ruxolitinib, but GOs and U373 had the highest dabrafenib IC₅₀ values at 112 and 137 μ M, respectively (Fig. S9E–G and Supplementary Table 5).

Next, we assessed the impact of these compounds on cellular migration of our GOs using a transwell assay (Fig. 8F), which showed that all compounds produced a significant reduction in migration capabilities (Fig. 8G). These strong antimigratory effects of all compounds were corroborated via wound healing assays, except for dabrafenib in U373 (Fig. 8H and I).

Alectinib concentration was reduced from 50 μ M to 10 and 25 μ M for this experiment, as 24 h with this higher concentration was lethal (Fig. S10A).

To better understand how these compounds affected our GOs, we performed RNA-seq following treatment. GSEA analysis showed that all treatments were affecting key oncogenic pathways. Alectinib downregulated genes targeted by the transcription factor *MYC*, as well as key pathways for cell division, like the G2/M checkpoint and genes involved in the formation of the mitotic spindle, while simultaneously upregulating apoptosis via caspases, the TNF α and hypoxia response pathways. Ruxolitinib produced a significant downregulation of the PI3K signaling pathway, as well as TGF β 1 signaling and DNA repair mechanisms. On the other hand, we identified a single significantly upregulated pathway in the form of angiogenesis. Finally, dabrafenib affected key replication

and growth pathways, while also altering the metabolism of the tumor (Fig. 8J and S10B).

Moreover, we included an *in vivo* validation with heterotopic models of both GOs and U87 cells. Our data showed that alectinib and ruxolitinib led to a significant reduction in tumor progression (Fig. 8K) as well as end-point size (Fig. 8L and M). These results highlight the effectiveness of these 2 compounds in an *in vivo* model.

In short, we identified three compounds which showed great antitumoral effects *in vitro* in both GOs and cell lines, reducing viability, affecting migration capacity and altering key tumoral pathways, while alectinib and ruxolitinib also reduced tumor growth *in vivo*.

Discussion

The development of reliable preclinical models that faithfully replicate the cellular and molecular complexity of gliomas remains a critical unmet need in neuro-oncology. Current methods show low maintenance of tumor stromal cells and spatial heterogeneity and have low success rates for IDH-Mutgliomas, making them inadequate platforms for studying clinically relevant tumor biology [53].

In this study, we demonstrate that GOs preserve key histological features, cellular heterogeneity, and microenvironmental interactions of their parental tumors, including clinically relevant mutations and immune profiles. By integrating this model with transcriptomic-guided drug prediction using the DiSCoVER platform, we uncover novel therapeutic candidates with confirmed antitumor efficacy *in vitro* and *in vivo*. This approach contributes to glioma modeling and could support the future design of personalized clinical trials aimed at improving outcomes in these highly resistant and heterogeneous tumors.

Our overall success rate in GO establishment aligns with previous reports [20, 42]. We were able to successfully generate GOs from IDH-Mut gliomas, something challenging given their lower proliferation rate and less aggressive nature compared to GBM [1], with a much higher establishment rate than traditional glioma cell lines [54]. This efficiency positions GOs as a superior platform for modeling both GBM and IDH-Mut gliomas *in vitro*. Notably, our results suggest that a defined establishment time, seeding density, and GO size should be

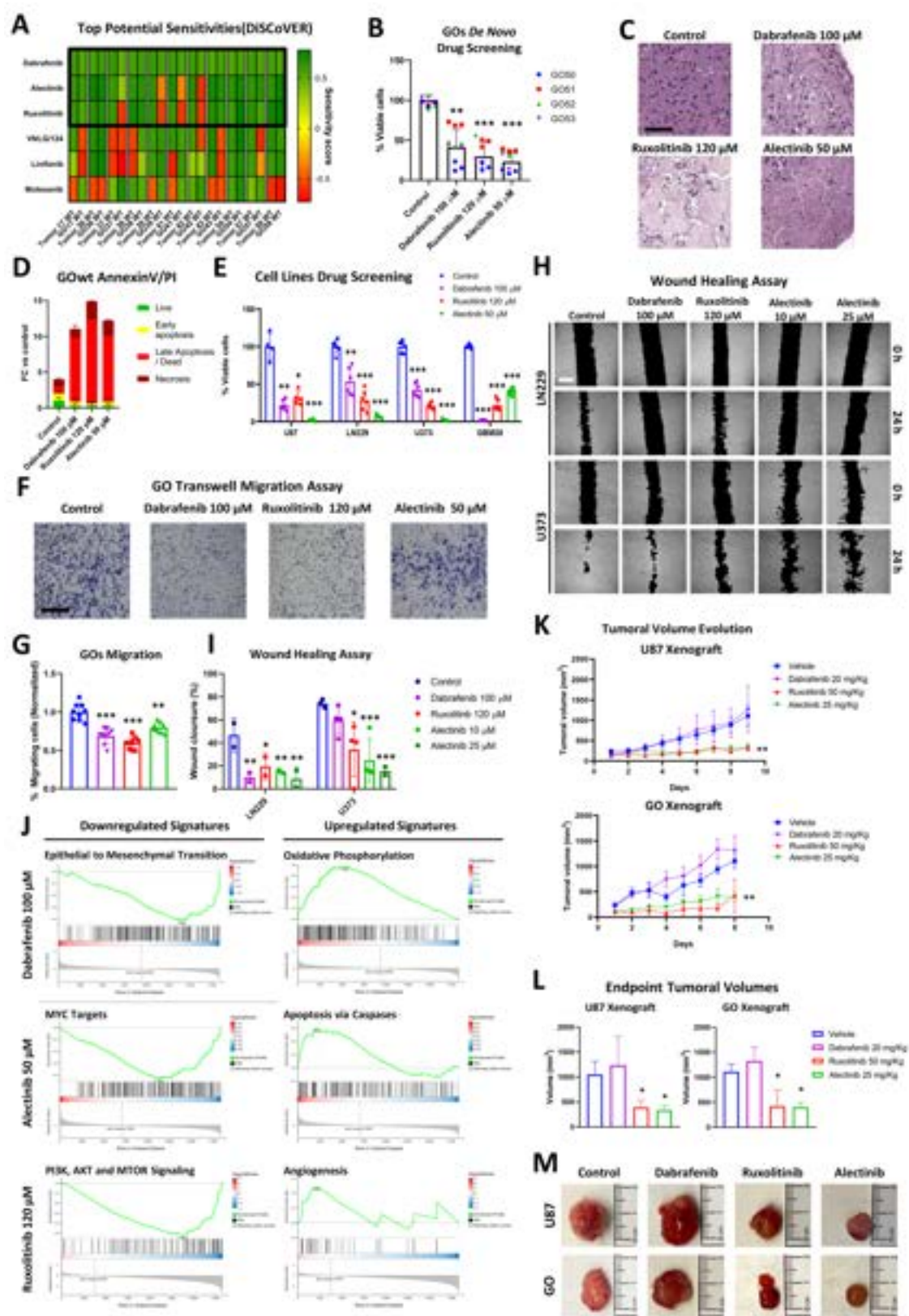


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Fig. 8 *De novo* screening identifies Alectinib, Ruxolitinib and Dabrafenib as potential glioma therapies: **(A)** Heatmap showing predicted drug sensitivities for the top six most promising compounds identified by DiSCoVER. Compounds are listed on the left, with each pair of rows representing a matched tumor tissue and its derived GO. Sensitivity scores are color-coded, with dark green indicating higher predicted sensitivity and dark red representing lower predicted sensitivity. $N = 11$ paired samples. **(B)** MTS viability assay assessing the effects of alectinib, ruxolitinib, and dabrafenib on a prospective cohort of freshly derived GOs ($N = 4$) after 48 hours of treatment. Each GO was tested in triplicate, and all values were normalized to their respective untreated control. Mean viability values and SD are represented, as well as triplicates from patients, which are color coded. **(C)** Representative H&E images of GOs treated with dabrafenib, ruxolitinib and alectinib. Scale bar is 100 μm . **(D)** Stacked bar graph of annexin V/PI apoptosis assay on representative GOs after 48 h with the indicated drug concentrations of compounds selected via DiSCoVER. Green represents live cells, yellow for early apoptotic cells, red for late apoptotic/dead cells and brown for necrotic cells. Bar height represents Fold change normalized to control, with error bars indicating SD. **(E)** MTS viability assay of alectinib, ruxolitinib and dabrafenib in established glioma cell lines (U87, LN229 and U373) and GSCs (GBM38). All values are normalized to their respective control. Each replicate is shown individually. **(F)** Representative brightfield images of GO transwell migration assay. GOs were dissociated into single-cell suspensions, seeded into transwell inserts, and treated with the corresponding drug for 6 hours. Scale bar is 200 μm . **(G)** Quantification of migrated cells following treatment ($N = 3$ independent patients GOs). Migration values are normalized to their respective untreated control. **(H)** Representative brightfield images from the wound healing assay at baseline and 24 hours post-treatment in LN229 (top) and U373 (bottom) cells scale bar represents 500 μm . **(I)** Quantification of wound closure in LN229 and U373 cells after 24 hours of treatment with ruxolitinib (red), alectinib (green), and dabrafenib (purple) **(J)** GSEA plots of hallmark signatures significantly downregulated (left) or upregulated (right) following GO treatment with alectinib (top), ruxolitinib (middle), and dabrafenib (bottom). Green lines show enrichment profiles; black bars indicate gene signature hits; grey curves represent gene ranking scores; blue to red scale shows signal-to-noise ratio of hits. **(K)** Tumor growth curves for in vivo xenografts. Each point represents the relative mean tumoral volume each day with SEM. Mice were treated daily with vehicle (blue), ruxolitinib 50 mg/kg (red), alectinib 25 mg/kg (green) and dabrafenib 20 mg/kg (purple). $N = 3$ mice per group. Samples were subjected to two-way ANOVA with daily mean comparison to determine statistical significance. **(L)** Bar graph of tumor volumes at experimental endpoint. Volumes (mm^3) are shown for vehicle (blue), ruxolitinib 50 mg/kg (red), alectinib 25 mg/kg (green), and dabrafenib 20 mg/kg (purple). Bars represent mean $\pm$ SEM. **(M)** Representative images of extracted tumors at endpoint, ruler is in cm. Statistical significance is indicated as follows: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

determined to ensure their suitability as a drug screening platform.

Although Jacob et al. [18, 19] highlighted the potential for biobanking patient-derived GOs, our results demonstrate that, while biobanked GOs preserve the histological characteristics of their parental tumors and freshly established GOs, their proliferation is significantly diminished when recovered in vitro. This observation aligns with reports from other studies utilizing this model [55, 56], where growth rates post-biobanking recovery are not explicitly described. This limitation may have implications for the utility of biobanked GOs in longitudinal studies. However, we observed that subcutaneous implantation of biobank recovered undissociated GOs in athymic mice is a viable way of expanding the model in vivo for downstream applications like drug testing.

To our knowledge, this is the first study to perform TEM on this model of GOs, revealing ultrastructural features of cellular microarchitecture within GOs. TEM analysis revealed a diverse array of cell populations within the GOs, including neurons and other glial cells, as well as intact organelles and preserved cell-cell interactions, mirroring physiological conditions [57], emphasizing the complexity of this model compared to traditional cell lines, which lack such interactions [58].

Given the potential of immunotherapy to revolutionize malignant glioma treatment, we examined the immune landscape of our models and their parental tumors. Both our patient tumors and their matched GOs showed an immune system dominated by myeloid cells, with both M1 and M2 microglia subpopulations present, and almost no lymphoid ($\text{CD4}^+/\text{CD8}^+$) infiltration, exactly as reported for GBM in vivo [59, 60]. Classical

immune-checkpoint molecules (PD-1, PD-L1, CTLA-4, CD80, CD86) were barely detectable in either sample type, matching the poor clinical performance of checkpoint blockade in glioma [61, 62], as well as other studies where these markers are analyzed in gliomas [63, 64].

However, there are two limitations to our immune profile. First, we did not pre-enrich CD45^+ cells like other studies [65, 66], so the scarce T-cell populations may be under-represented. Second, GOs were generated from densely cellular tumor cores; infiltrative margins (where lymphoid cells are relatively richer and more active) [67, 68] were not sampled.

Next, we assessed GOs responses to SOC therapies. As anticipated and coinciding with our *in silico* results, the standard chemotherapy treatments did not produce significant reductions in cellular viability, mirroring previously described clinical outcomes [69, 70], as well as the evolution of patients included in the screening, and previous studies using the same model or simpler GSC models [33, 71]. This resistance underscores the urgent need for alternative therapeutic strategies. Additionally, when screening for lesser used compounds like gefitinib we observed a marked reduction in cellular viability, which also coincided with our *in silico* results. An explanation for this evasion of DNA damage could be that tumoral cells in GOs undergo a transformation into senescent cells after being challenged by DNA damaging agents like TMZ, as some authors have pointed out [72, 73].

To explore alternative therapeutic options, we performed *de novo* drug screening using the DiSCoVER platform, integrating RNA-seq data from paired tumor and organoid samples. This analysis identified three promising candidates: the ALK1 inhibitor alectinib, the JAK

inhibitor ruxolitinib, and the BRAF inhibitor dabrafenib. Among these, alectinib exhibited the strongest cytotoxic effect, followed closely by ruxolitinib, which was the most effective at inhibiting migration, with dabrafenib ranking second in this regard. These compounds also affected critical pathways for cancer development in vitro. When assessed in vivo, alectinib and ruxolitinib significantly affected tumoral growth of both GOs and U87 cells. These are proof-of-concept therapeutic prioritizations, not yet validated as patient outcome predictors.

Interestingly, a recent clinical trial has demonstrated ruxolitinib safety in a small cohort of 60 patients (NCT03514069) highlighting its potential to be integrated into clinical practice [74]. Similarly, alectinib has shown encouraging results in other tumors, such as neuroblastoma [75], and is currently being investigated in a phase I/II trial for treatment-resistant CNS tumors in children and adolescents [76], with the added benefit of being fully permeable to the blood brain barrier. Finally, dabrafenib has been reported as an effective therapeutic agent restricted to patients with altered *BRAF*, limiting its applicability as a broader treatment option [77, 78].

Conclusions

In conclusion, GOs present limitations in proliferative capacities and biobanking efficiency that must be addressed to enable clinical implementation. Nonetheless, GOs represent an invaluable preclinical tool. Our findings further support GOs as a robust model that recapitulates the cellular, molecular, and histological complexity of gliomas. By integrating this model with bioinformatic drug prediction, we demonstrated its utility in personalized drug screening and uncovered novel therapeutic opportunities, in a short timeframe relevant to clinical practice. These findings support the potential of GOs to inform clinical decision-making and accelerate the development of more effective, individualized therapies for glioma patients. Based on our findings, we propose alectinib and ruxolitinib as candidate therapeutics warranting further clinical investigation in gliomas.

Abbreviations

IDH	Isocitrate dehydrogenase
GBM	IDH-wild type glioblastoma
mOS	Median overall survival
SOC	Standard of care therapy
TMZ	Temozolomide
PDX	Patient-derived xenograft
GO	Malignant glioma organoid
GOMut	IDH-mutant organoids
GOwt	GBM organoids
GSCs	Glioma stem-like cells
PFA	Paraformaldehyde
H&E	Hematoxylin and eosin
IF	Immunofluorescence
IHC	Immunohistochemistry
BrdU	Bromodeoxyuridine
qRT-PCR	Quantitative real time PCR

ddPCR	Droplet digital PCR
FC	Flow cytometry
TEM	Transmission electron microscopy
RNA-seq	RNA sequencing
scRNA-seq	Single cell RNA sequencing
ECM	Extracellular matrix
PI	Propidium iodide
D-2HG	D-2-Hydroxyglutarate
PAS	Periodic acid-Schiff

Supplementary information

The online version contains supplementary material available at <https://doi.org/10.1186/s40364-026-00894-3>.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 1
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7

Acknowledgements

We would like to acknowledge the expertise and assistance of the Histology Unit at San Pablo CEU (Madrid, Spain) in all histological preparations. We also thank the Electron Microscopy Unit at Centro de Biología Molecular Severo Ochoa (Madrid, Spain) for their support in interpreting electron microscopy images. The authors wish to thank the donors, and the Hospital Universitario Puerta de Hierro Majadahonda (HUPHM)/Instituto de Investigación Sanitaria Puerta de Hierro-Segovia de Arana (IDIPHISA) Biobank (Carlos III Health Institute Biomodels and Biobanks Platform – PT23/00015) for the human specimens used in this study.

Author contributions

AAS and NGR conceived the research concepts and designed the original experiments. AMS, LMM, JCN, CMR, MCP and PSG conducted experiments, while AMS and NGR performed data analysis. AHM performed RNA-seq and scRNA-seq analysis. Clinical samples, patient diagnoses and MRI images were provided by RGG, NL, RPA, SGRM, CAG, APN and AJSL. FPR confirmed diagnoses and contributed to the blind analysis of histological preparations. BMJ performed electron microscopy sample preparation and image acquisition. MVFP performed RNA-seq deconvolution. AMS and NGR wrote the manuscript, and all authors participated in manuscript review and editing.

Funding

This study has been funded by Instituto de Salud Carlos III (ISCIII) and co-funded by the European Union through the projects “PI21/01353” and PT23/00015 (ISCIII Platform for Biomodels and Biobanks-Organoids Hub) to NGR and AAS, and PI21CIII/00002 to PSG. AMS was supported by the Ministry for Universities, Spain (FPU21/02281). AHM is supported by a predoctoral fellowship from Universidad Francisco de Vitoria. MCP was supported by the Young Employment Initiative (Comunidad de Madrid). The publication is part of the CPP2023-010432 project funded by MICIU/AEI/10.13039/501100011033 and the EU’s FEDER.

Data availability

RNA-seq datasets are publicly available at Gene Expression Omnibus (GEO) under accession code GSE303683.

Declarations

Ethics approval

Ethical approval for the use of these samples was granted by the institutional review board of HM Hospitals (CEIm No: 23.06.2206-GHM) and Hospital 12 de Octubre (CEI 21/480). All patients provided informed written consent

prior to enrollment, and all procedures were conducted in accordance with the principles of the Declaration of Helsinki. All animal experiments were performed under PROEX 142.8/24 in compliance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, as well as other applicable national and institutional regulations, ensuring the highest standards of animal welfare and ethical research practices.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 26 August 2025 / Accepted: 11 January 2026

Published online: 06 February 2026

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