# Establishing Microglia-DMG Co-Cultures to Study GD2-CAR-T Cell Sensitivity



Major Research Project of the MSc in Cancer, Stem Cells, and Developmental Biology.

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# Contents

# Plain Language Summary

Diffuse midline glioma (DMG) is a highly aggressive type of brain tumor that affects children. The tumor cells spread throughout the brain stem, making surgical removal impossible. Since the brain is such a vital organ, it is protected by a barrier called the blood-brain barrier (BBB), which prevents many substances from entering the brain from the bloodstream. In DMGs, the BBB is particularly impermeable, making it difficult for drugs to reach and attack the tumor cells.

Immune cells called T-cells normally protect our bodies from infections and tumors, but they are found in very low numbers in DMGs. This combination of factors makes DMGs extremely difficult to treat.

In recent years, new immunotherapies like CAR-T cell therapy have been developed. This therapy involves modifying T-cells to specifically recognize and kill cancer cells. However, CAR-T cell therapy has shown low effectiveness in treating DMGs. Despite the low number of T-cells, the brain contains a high percentage of another type of immune cell called microglia, which are found around the tumor. It appears that the tumor cells manipulate these microglia to promote tumor growth and prevent immunotherapy from working. These altered microglia are known as tumor-associated microglia.

Currently, there are no laboratory models that allow scientists to study the interactions between DMG and microglia cells or to see how microglia affect the effectiveness of CAR-T cell therapy. To address this, we developed a co-culture system that allows DMG, microglia, and CAR-T cells to grow together in a controlled environment. This system will help us study how microglia influence tumor growth and reduce the effectiveness of CAR-T cell therapy. Additionally, it can be used to test drugs that specifically target tumor-associated microglia to see if this can improve the outcomes of CAR-T cell therapy.

Generative AI statement: This work was completed without the use of Generative AI-based tools. All research, analysis, and writing were conducted by the author manually.

# Abstract

Diffuse midline glioma (DMG) is one of the most deadly pediatric–type diffuse high-grade gliomas and there is currently no treatment for it. All DMGs are hallmarked by an alteration on Histone 3 Lysine 27 (H3K27), mostly resulting from a H3K27M mutation. This histone modification causes a widespread disbalance in the tumor cell expression signature, making it difficult to pinpoint the responsible genes for tumor onset, and thus, drug targeting. DMG is located in the brainstem and characterized by a diffuse growth profile, which impedes surgical resection. Furthermore, DMG harbour an intact blood-brain barrier, impeding the delivery of many therapeutic agents. Additionally, DMG conceal a 'cold' immune-microenvironment, highlighted by a low infiltration of T-cells and NK cells. On the contrary, myeloid cells make up at least a quarter of the tumor mass, suggesting that they play an important role in tumor growth.

Microglia are the resident myeloid cells of the central nervous system and play an important role in the surveillance, development, and maintenance of the brain. Recent studies support the hypothesis

that pediatric brain tumors use microglia for their benefit, creating an anti-inflammatory and immune-cold environment by mechanisms still unknown.

The recent revolution and evolution withing the immunotherapuy field has created new therapeutic strategies that might change prognosis of brain tumor patients dramatically. One of the most successful immunotherapeutic strategies includes CAR-T cell therapy, which has rendered impressive preclinical results in brain tumor models. However, the far majority does not contain a fully functional immune system, which could potentially explain the limited efficacy of clinical CAR-T therapies in pediatric brain tumor patients. Because of this, there is a need fo in vitro models that mimic the microglia-tumor interaction.

We analyzed the tumor microenvironment of immune-competent DMG mice models via CITE-Seq and identified tumor-associated microglia-specific markers that can be used as potential drug target in DMG. To efficiently test such drug targets, we have set-up a microglia-DMG co-culture system. Addiotionally, these co-cultures were used to assess the GD2-CAR-T cell efficacy against DMG cells with and without tumor-associated microglia cells.

This project allowed us enhance our comprehension of the role of microglia on tumor aggressiveness and progression. Moreover, in this study we addressed various inquiries concerning the efficacy of immunotherapy against DMG patients. Eventually, this project may contrubute to find a promising and efficient therapy for DMG.

# Introduction

## Diffuse midline glioma (DMG)

Diffuse midline gliomas (DMGs) stand as one of the most devastating forms of pediatric cancers, comprising approximately 20% of all tumors affecting the central nervous system (CNS) in children [1]. DMGs typically manifest with a median age at diagnosis of 7 years, and the median overall survival is reported to be 9–12 months. The established standard of care for patients is fractionated radiation therapy. However, DMGs are incurable tumors and this treatment approach provides only temporary relief of neurological symptoms and offers modest extensions in progression-free and overall survival, typically spanning a few months at best [2].

DMGs are highly aggressive, and, as the name implies, these tumors tend to disperse within the brain. DMGs are typically located in the brainstem, thalamus, spinal cord, or cerebellum. These midline structures are gateway between the spinal chord and the rest of the brain. Furthermore, the most primitive and old mechanistics of the brain, like breathing, reside there. [2]. The diffuse nature and delicate location of these tumors impedes surgical resection, limiting the range of possible interventions to needle biopsies for diagnostic purposes. Moreover, it is commonly known from treatment efficacy, biopsies, pathology stainings and the lack of contrast enhancement on magnetic resonance imaging (MRI), that DMG harbour a very intact blood–brain barrier (BBB), unfortunately, hindering over 98% of therapeutic agents [3].

About 80% of DMGs harbor a lysine-to-methionine substitution at position 27 in Histone 3 genes, specifically at Histone 3.3 (H3F3A), and to a lesser extent at Histone 3.1 (HIST1H3B). The H3K27M mutation has been demonstrated to inhibit the function of the histone-lysine N-methyltransferase Enhancer of zest homolog 2 (EZH2) within the polycomb repressive complex 2 (PRC2). This multiprotein

complex plays a role in maintaining transcriptional silencing through the trimethylation of lysine 27 on histone H3 (H3K27me3). Another subset of DMGs exists without the H3K27M mutation. Strikingly, these tumors display a widespread loss of tri-methylation. This phenomenon is potentially mediated by the overexpression of the EZH inhibitory protein (EZHIP), which functions as a K27M-like inhibitor of PRC2 [4].

Despite the global H3K27 hypomethylation observed in DMG, PRC2 activity is still detectable in those cells, and H3K27me3 peaks persist at hundreds of gene loci. This paradoxical phenomenon, marked by a global decrease in H3K27me3 but present in specific loci, may reshape DMG cell epigenetics and gene expression, ultimately promoting tumor formation. [5, 6].

DMG are hallmarked by a substantial infiltration of myeloid cells, but there is a notable absence of lymphoid cells. This lymphocitic absense is attributed to the fact that DMGs do not express the required chemokines or cytokines to attract these immune cells, such as T-cells and NK cells. Furthermore, DMGs do not produce significant amounts of immunosuppressive factors, leading to an immunologically inert or immune-cold microenvironment. This inert environment may pose a limitation on the efficacy of immunotherapeutic compounds in the treatment of DMG [7].

## Microglia

The high infiltration of myeloid cells in DMG puts the spotlight on microglia cells. Microglia serve as the primary resident myeloid cells within the central nervous system (CNS) and play a crucial role in the maintenance, development, and surveillance of the brain[7]. In response to CNS injury, microglia take on the responsibility of phagocytosing and eliminating microbes. Moreover, dead cells, protein aggregates, as well as other debris and soluble antigens that pose a threat to the CNS are typically phagocytosed by microglia. Throughout development, microglia play a pivotal role in shaping neural circuits by modulating synaptic transmission strength and refining neuronal synapses. Additionally, microglia release various soluble factors, including chemoattractants, cytokines, and neurotropic factors, contributing to diverse aspects of immune responses and tissue repair within the CNS [8]. Microglia act as key mediators in the recruitment and modulation of adaptive immune cells within the CNS. Their ability to present antigens, release signaling molecules, and interact with T cells contributes to the orchestration of a precise and regulated immune response in the brain.

Although the healthy brain has a high infiltration of microglia, macrophages are typically not present in the brain and are only found in damaged CNS tissues. However, the differentiation between microglia and macrophages remains a subject of ongoing debate. Macrophages and microglia are both types of immune cells, but they differ in their origin, distribution, and specific functions. Macrophages derive from blood monocytes, which originate in the bone marrow, while the origin of microglia has now been identified and linked to the initial colonization of the central nervous system (CNS) by mesodermal progenitors originating from the yolk sac [7]. Regarding location, macrophages are found throughout the body in various tissues and organs, including the lungs, liver, spleen, and skin, while microglia are exclusive to the central nervous system, residing in the brain and spinal cord. Finally, macrophages solely serve as immune cells and they are activated in response to infections, inflammation, or tissue damage and they are involved in a wide range of diseases. Contrarily, microglia are constantly involved in the cleaning of neurotransmitters in synaptic shafts and even beyond cleaning, microglia are involved in shaping the neuronal networs, by actively killing neurons that are not often used and stimulating neuronal growth within pathways that are often active. Additionally, microglia even push neuronal networks towards more efficient connections between other neurons. Microglia monitor the brain environment, remove dead cells, and contribute to the immune response

in neurological conditions, playing a crucial role in neuroinflammation, neurodegenerative diseases, and CNS disorders. While both macrophages and microglia share some functional similarities as immune cells, their distinct origins, locations, and roles highlight their specialization in responding to different aspects of the immune challenges faced by the body and the central nervous system, respectively [10].

Microglia exist in either resting or activated states, and their status depends on the inflammatory milieu, which varies between the healthy CNS and various disease states [9]. However, microglial activity is not binary, characterized by a simple on or off switch; instead, it exists along a spectrum of diverse functional states that are dependent on the microenvironment. Microglia can assume a variety of phenotypes, each linked to the expression of distinct markers, secretion of specific factors, and varying degrees of phagocytic activity [7]. Mature microglia exhibit extensively ramified processes, while in the developmental stage and when they are highly activated, they acquire an 'amoeboid' morphology characterized by larger, round cell bodies and shorter, thicker branches. In response to immune challenges or the context of brain diseases, microglia often undergo morphological alterations, which may be accompanied by the release of cytokines, chemokines, and/or trophic factors [10]. The ramified morphology is generally considered the resting state, whereas the amoeboid morphology is indicative of being in a developmental and activated state.

In the non-inflammatory or dampened immune phenotypic state of DMGs, tumor-associated myeloid cells undergo a pro-tumoral transformation through epigenetic changes [20]. Specifically, DMG cells induce a loss of histone 3 lysine 27 trimethylation in microglia, steering them toward a stem cell-like, tumor-promoting state [21]. Furthermore, preliminary results obtained in our laboratory performing cytokine array assays show a reduction in the secretion of pro-inflammatory cytokines, such as IL-6 or TNF-alpha, by microglia in the DMG environment. Additionally, microglia in DMG display distinct morphological features that differ from ramified (resting) microglia observed in the healthy brain [7]. The epigenetic and morphological changes support that microglia activation occurs despite establishing an immuno-cold environment in diffuse midline gliomas (DMG). Interestingly, DMG cells possess the capability to manipulate or hijack microglia to establish an anti-inflammatory microenvironment. This strategic modulation facilitates tumor cells in evading the immune system and contributes to the tumor's ability to escape immune surveillance.

Considering the impact that tumor-associated microglia seem to have on tumor growth, it is crucial to identify tumor-associated microglia markers in order to specifically target this cell population as a possible therapeutic strategy in DMG. Although still under debate, there are several markers that can differentiate microglia from blood-derived macrophages in both human and mouse systems. However, both microglia and macrophages under the pressure of external influences, tend to alter the expression of these markers in specific situations, adding complexity to the distinction between the two cell types. Moreover, there is no tumor-associated microglia marler signature that could be used to properly identify and target this cellular type. Because of this, specific markers should be identified to differentiate between macrophages and microglia and to specifically target tumor-ssociated microglia.

Overall, targeting tumor-associated myeloid cells emerges as a promising strategy to attack the non-inflammatory tumor immune microenvironment [7]. Thus, combining this approach with the current immunotherapeutic strategies holds potential as a valuable therapeutic strategy against DMG.

## CAR-T cell therapy

Immunotherapy has emerged as a groundbreaking approach in cancer treatment, offering advantages over conventional methods like surgery, radiation, and chemotherapy due to its specificity and reduced toxicity. Among these innovative therapies, chimeric antigen receptor (CAR)-engineered T cell therapy stands out as highly promising, demonstrating exceptional efficacy in combating various tumor types, particularly B cell malignancies. CAR-T cells are custom-engineered by modifying patient-derived T cells to express chimeric antigen receptors (CARs) on their surface. These CARs enable T cells to recognize and bind to specific proteins, or antigens, present on cancer cells [25].

Cell surface antigen screening studies and stainings performed in patient tumor tissues found surface markers in tumor cells that could be used as targets for CAR-T cells. The disialiganglioside GD2 was discovered as a potent CAR-T target in many tumor types such as retinoblastoma and neuroblastoma [22,23]. Furthermore, research has shown that anti-GD2 CAR T-cells, incorporating a 4-1BBz costimulatory domain, exhibit robust antigen-dependent cytokine production and effective killing of tumor cells in vitro [13].

High expression of the disialoganglioside GD2 in patient-derived H3K27M-mutant glioma cell cultures suggests its potential as a target for CAR T-cell immunotherapy in diffuse midline gliomas (DMG) [12,13].

Monje et al. (2022) did an extensive clinical study with GD2-CAR-T cells in DMG patients. Although they observed some efficacy and even a few long term survivors, the efficacy of GD2-CAR-T cell therapy in patients was limited [13]. This lack of succesful translation to DMG patients might be due to the fact that immunocompetent models have hardly been tested preclinically. GD2-CAR-T cell therapy shows promising outcomes in *in vitro* studies with DMG cells and also *in vivo* in immunodeficient mice. However, none of these setups holds a complete immune system. Based on these findings, we hypothesize that the tumor microenvironment plays a critical role in determining the effectiveness of CAR-T cell therapy. Previous studies demonstrate that anti-inflammatory macrophages are related to immune suppressive immune responses, contributing to tumor growth [24]. To asses the role of microglia in DMG tumors we developed DMG-microglia co-cultures that accurately mimic the in vivo microenvironment and evaluate the efficacy of GD2-CAR-T cell therapy within these co-cultures.

## Use of co-culture system to study microglia-DMG crosstalk

Unravelig the tumor microenvironment (TME) and its influence on the onset and development of tumors has garnered increasing interest. Moreover, a growing number of studies support the development of therapeutic strategies that target the the tumor microenvironment as a potential anti-cancer therapeutic strategy. Given the significant role of the tumor microenvironment (TME) in diffuse midline glioma (DMG) tumors and its potential contribution to the limited efficacy of immunotherapy in this disease, there is a critical need to establish a system that can unravel the underlying reasons for this phenomenon.

We cannot only rely on mouse studies, which require investment of economic resources, intense work and face ethical problems, as well as classic cell cultures that cannot mimic the tumor environment that can be found in patients. Thus, the creation of experimental models that reflect the microenvironmental conditions of DMG tumors becomes essential.

Co-culture systems serve as valuable tools to explore the involvement of immune cells in tumor progression. These models represent a robust experimental platform for investigating the crosstalk between tumor cells and diverse immune cells [11]. As such, in this project we will develop a DMG-microglia co-culture system that mimics the tumor microenvironment in DMG.

These co-cultures will be used to study the effect of microglia on CAR-T cell effectivity. Additionally, such co-cultures could be also used to test candidate drugs that target tumor-associated microglia. The combination of immunotherapeutic strategies such as CAR-T cell therapy with targeted tumor-associated microglia drug interventions will contribute significantly to the development of more effective and tailored treatment approaches for diffuse midline gliomas (DMG).

# Materials and methods

## CITE-Seq data analysis

We isolated the myeloid cell population from the brain of a control group of healthy mice and two groups of syngeneic immunocompetent DMG mice, one group with a H3.1 mutation and the other with a H3.3 mutation. The different myeloid populations of these mice groups were clustered regarding gene and protein expression using Loupe Browser 7 (10X Genomics).

## Establishing a DMG-microglia co-culture

### Effector:Target ratio determination

We wanted to find the Effector:Target ratio of GD2-CAR-T cells and SU-DIPG-13 cells that cause around 80% killing to the tumor cells. To measure it, we used GFP+ SU-DIPG-13 DMG cells, GD2-CAR-T cells and the matching untransduced CAR-T cells. We seeded 20.000 SU-DIPG-13 cells and we added GD2-CAR-T cells at different ratios (1:20, 1:10, 1:5, 1:1). The same was performed with the Untransduced CAR-T cells. After 48h in co-culture, cells were stained with the dead/alive eBioscience Fixable Viability Dye eFluor™ 780 (Thermofisher), and the CAR-T cell killing percentage was measured using Cytoflex LX for flow cytometry.

### Choosing the culture media

Based on previous experiments, we stated that all the cell lines involved must be able to survive in the same culture media for at least 48 hours. SU-DIPG-13 was used as DMG model, and as microglia model, we used the established microglia cell line HMC3. The tested culture media were: TSM, a DMG-specific media; ITMG, a microglia-specific media, and a mixture of both in 1:1 ratio. Media compositions can be found in Table S1. For each cell type, we seeded 10.000 cells in a well of 96 well-plate in each of the culture media in triplicate. After 48h in their respective media, luminescence was measured with Spectramax iD3 following the CellTiter-Glo Luminescent Cell Viability Assay protocol.

## Choosing the microglia model: HMC3 vs Monocyte-derived microglia.

HMC3 is an established microglia cell line coming from the SV40-dependent immortalization of human embryonic microglial cells. Although it is an easy-to-maintain cell line, there is another potential microglia model: Monocyte-derived microglia (MDM) cells. To test which cell type is more reliable and consequently, the one that will be used in the co-culture, GFP+ SU-DIPG-13 cells were cultured for 48h with HMC3 and with MDM, and images were taken using Leica Thunder Life to see how both cells behave together.

## PBMC isolation from whole blood and differentiation to Monocyte-derived microglia (MDM) cells

For the PBMC isolation from whole blood, 10 mL of Lymphoprep (StemCell technologies) was added to a 50 mL tube. Blood was diluted 1:1 with PBS (-Ca2+, -Mg2+) and carefully added on top of the Lymphoprep layer. After centrifugation for 20 minutes at 2400 rpm, acc3, brake 0 at room temperature, different layers can be detected in the tube (top layer=serum, white ring=PBMC, third layer=Lymphoprep, bottom layer=erythrocytes). The PBMC fraction was collected using a Pasteur pipet. PBMC were washed twice in PBS for 5 minutes at 1800 rpm at room temperature and counted with Tripan Blue in an automated cell counter. Finally, PBMCs were resuspended in 5 mL full ITMG medium and seeded in a T25 flask. After three days, ITMG was renewed. Based on previous experiments, we know that at day 6 PBMC have differentiated into MDM and are ready to be used for further experiments.

## Triple co-culture: SU-DIPG-13 with MDM and GD2-CAR-T cells

The MDM were detached on day 6 from the T25 flask. To do so, cells were first washed for 5 minutes in a mixture of PBS 1X with 1% of Tripsin, 1% of Accutase, and 1mM EDTA. Then, cells were treated with Trypsin for 20 minutes and then, detached from the bottom of the flask gently using a scraper. We seeded 10.000 MDM cells in each well of a 96-well plate and let them adhere to the well for 3-4 hours. Then, 20.000 GFP+ SU-DIPG-13 cells and 2.000 GD2-CAR-T cells were seeded on top of each well. Images of the triple co-culture were taken after 48h using Leica Thunder Life to asses the GD2-CAR-T cell efficacy.

## PBMC and HMC3 infiltration in SU-DIPG-13 spheres

PBMC isolated from blood can survive for 2 weeks in culture without replication taking place. We wanted to test if cell surveillance is increased when co-culturing PBMC directly isolated from blood with GFP+ SU-DIPG-13 cells. After PBMC isolation, the cells were stained with CellTrace FarRed following the manufacturer's protocol. Then, 20.000 GFP+ SU-DIPG-13 cells and 20.000 pre-stained PBMC were seeded in a well of an Opaque 96 well-plate. Images were taken after one and three weeks with the Leica Thunder Life microscope.

The same experiment was performed but using HMC3 microglia cell line instead of the PBMC.

# Results

## Myeloid clustering and drug selection to target tumor-associated microglia

Looking at the expression of different markers available in the literature, we identified and clustered the different myeloid populations in all three groups of samples (Figure 1A). It can be seen that there are two myeloid populations that are only present in the H3.1 and H3.3z: tumor-associated microglia and tumor-associated macrophages. Additionally, in the H3.1 UMAP we can see a clear separation of the myeloid and macrophage population. This separation is not complete in the H3.3 UMAP but still, the microglia populations gather together. Additionally, we can see a bigger population of healthy macrophages in the tumor compared to the control group, as in a healthy state, there is no macrophage infiltration in the brain. Moreover, we developed a marker signature for each of the myeloid populations that could be used to differentiate each of them (Figure 1B).

Finally, we identified a group of highly up-regulated specific tumor-associated microglia markers associated with immune regulation.

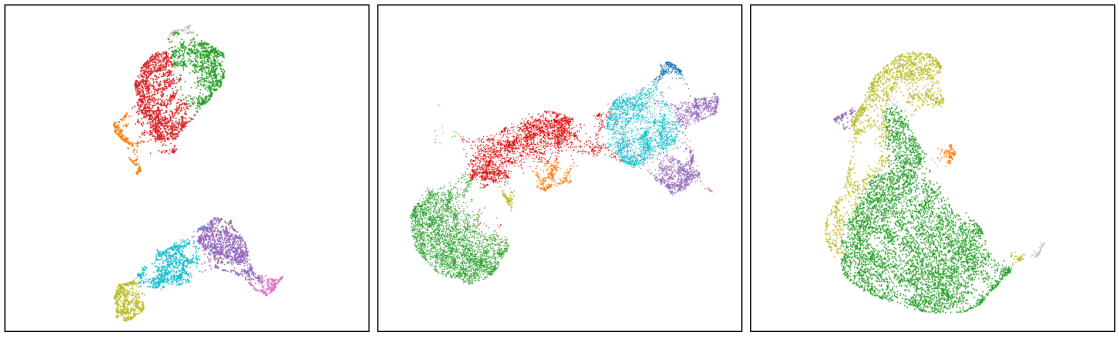
- Cystatin F encodes a glycosylated cysteine protease inhibitor, known as Cystatin F, with a putative role in immune regulation through inhibition of a unique target in the hematopoietic system. Expression of the protein has been observed in various human cancer cell lines established from malignant tumors as well as in disease-associated microglia (DAM). Recent studies have shown that cystatin F is a mediator of immune suppression in glioblastoma. Increased cystatin F mRNA and protein levels in immune, glioblastoma and glioblastoma stem-like cells or trans internalized cystatin F may have an impact on decreased susceptibility of glioblastoma cells to NK cytotoxicity [14].
- Spp1 encodes for Osteopontin (OPN) protein. It is a highly expressed gene in microglia of the early postnatal brain and in adults after injury. Moreover, it is a pro-inflammatory secreted protein upregulated in microglia in the mouse model of oxygen-induced retinopathy (OIR) and a member of a highly expressed gene signature of so-called neurodegenerative microglia that includes ApoE. In multiple sclerosis and Alzheimer's disease (AD) models and humans, OPN/SPP1 is elevated in the plasma and CSF in those with advanced disease [15]. Additionally, recent studies have found that the lower *SPP1* was related to a better response rate to immunotherapy [16]
- Glycoprotein nonmetastatic melanoma protein B (GPNMB) is part of a microglia activation state that is only present under neurodegenerative conditions and that is characterized by the up-regulation of a subset of genes including *TREM2*, *APOE* and *CST7*. Increasing evidence supports the role of GPNMB in driving immunosuppression in different contexts, ranging from autoimmune disorders to malignancy, notably in melanoma. GPNMB promotes immunosuppression by downregulating innate and adaptive immunity via M2 MΦ skewing, as well as the suppression of T cell responses via inhibition of T cell function and infiltration. [17].

Those tumor-associated microglia markers could be used to investigate potential drugs that specifically target tumor-associated microglia:

- Up-regulated Cst7, which encodes an endosomal/lysosomal cathepsin inhibitor known as cystatin F, is a biomarker for DAM at advanced stages of AD and is promoted by activation of RIPK1. DNL747 and GSK2982772 are RIPK1 inhibitors that could be used to target Cst7 highly expressing cells [18].

- Docking and dynamic studies revealed that Entrectinib showed excellent binding affinity against OPN/SPP1 protein [19].
- The soluble form of GPNMB arises after the cleavage of ADAM10 [17], so maybe ADAM10 inhibitors could be used to target tumor-associated microglia. Aderbasib (INCB007839) is a potent, orally active, and target specific low nanomolar hydroxamate-based inhibitor of ADAM10 and ADAM17.

A)

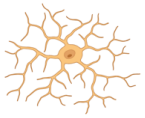


H3.1 H3.3 Control

Tumour Microglia
Microglia
Tumor-dividing microglia
Macrophages
Immunosuppressive Macrophages
Microglia derived from CD206+ Macrophages
Tumour Macrophages
Low motility Macrophages

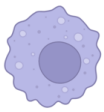
B)

Microglia



| | |
|---|---|
| Healthy | Tmem119, P2RY12, Sparc |
| Tumor-associated | ApoE, Cst7, CD45, Lgals3, AXL, TREM2, Serpine 2 |
| Tumor Dividing | Mki67, ApoE, Cst7, Melk, Top2A, Bib1, Cdk1, Serpine 2 |
| Derived from CD206+ Macrophages | Mrc1, Dab2, ApoE, CD163<br>No: Lgals3, Itgax, S100a6<br>Less: S100a11, CD45, Crip1 |

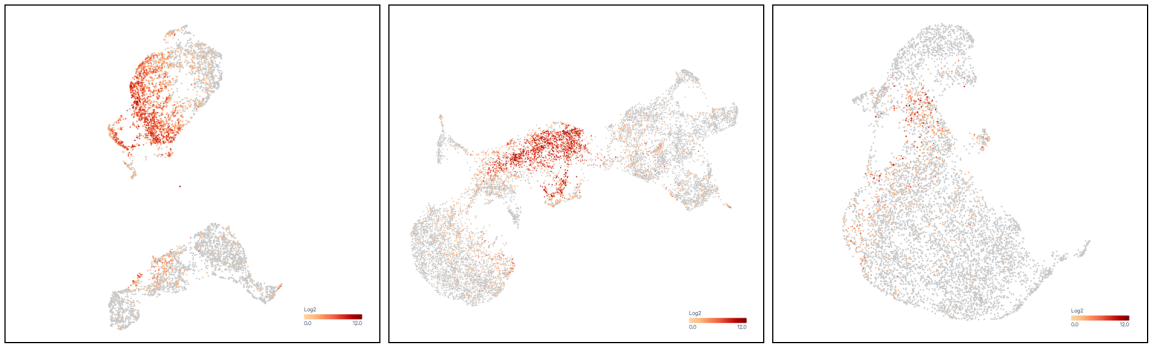
Macrophages



| | |
|---|---|
| Healthy | CD45, Lgals3, S100a6, S100a11, Crip1, Plac8, Ly6c2 |
| Tumor-associated | ApoE, TREM2, Lgals3<br>Less: Crip1 |
| Immunosuppressive | Ccl5, Ccl22, CD279, CD274 |
| Low motility | Less: Plac8, S100a4, Rps7 |

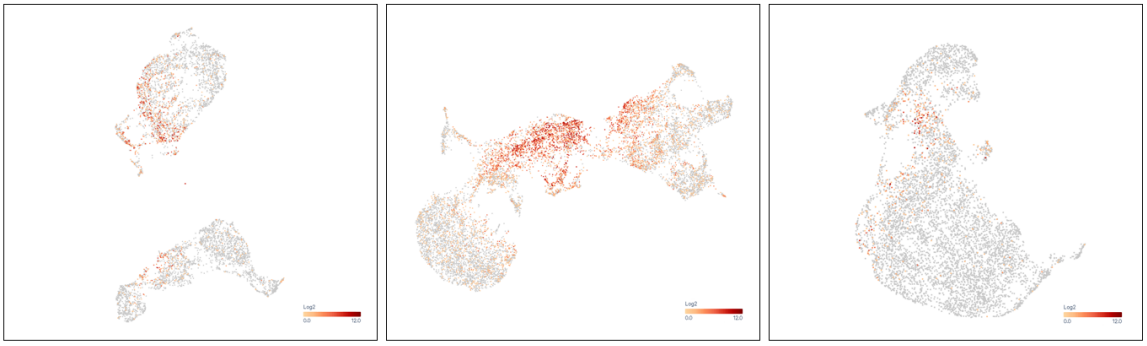
C)

Cystatin F



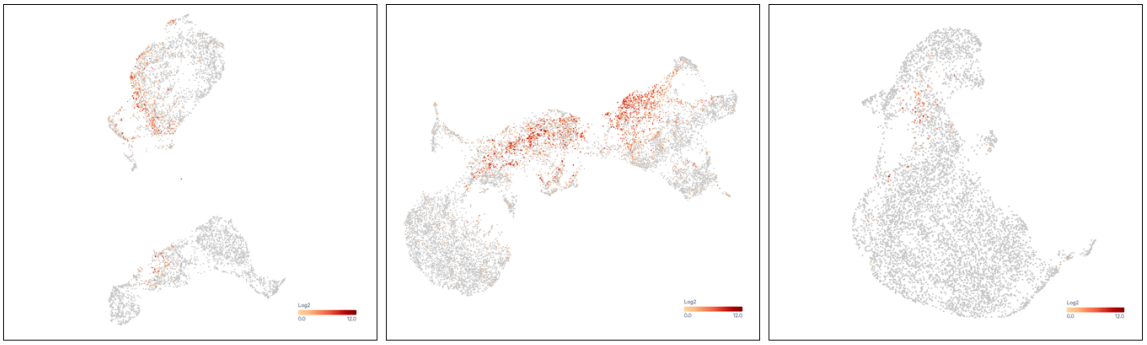
H3.1 H3.3 Control

Spp1



H3.1 H3.3 Control

GPNMB



H3.1 H3.3 Control

**Figure 1.** A) Clustering of the myeloid populations from the three different groups of syngeneic immunocompetent mice: H3.1, H3.3 and control group. B) Marker signature of each of the identified myeloid populations across the samples. C) Gene expression of Cystatin F, Spp1 and GPNMB across the three mice groups. Those genes are highly up-regulated genes in tumor-associated microglia.

## Effector:Target Ratio

Our objective was to select a E:T ratio with an 80% of killing of tumor cells. Thus, we could see a variation in the killing when adding microglia to the culture.

We tested the killing of GD2-CAR-T cells as well as the matching untransduced CAR-T to show that the killing of the CAR-T is due to its specificity towards GD2 expression in SU-DIPG13 cells instead of cytotoxicity of the CAR-T. In Figure 2 we can see that the 1:10 ratio, meaning one GD2-CAR-T cell per ten SU-DIPG-13 cells, shows around 80% SU-DIPG-13 killing and almost no untransduced CAR-T cell toxicity. For these reasons, the 1:10 E:T ratio was choosed for the co-cultures.

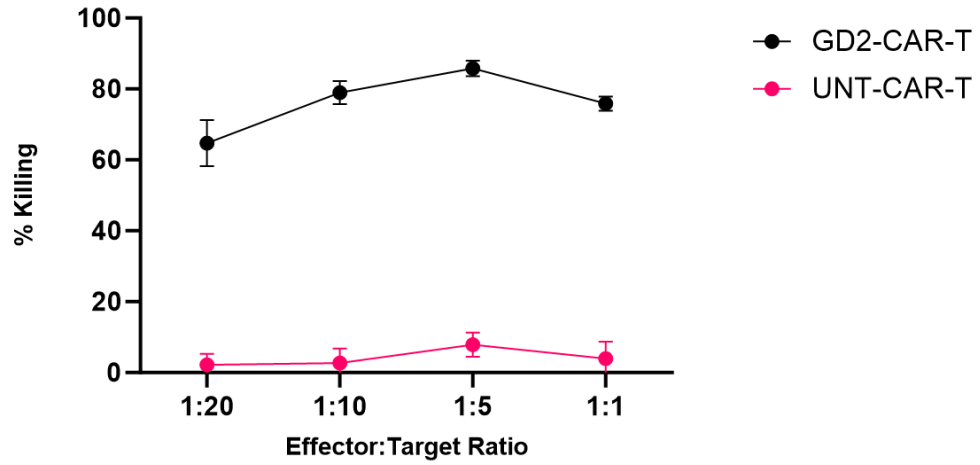



**Figure 2.** Graphical representation of the killing percentage of the different E:T ratios of GD2-CAR-T cells (black) and the matching untransduced CAR-T (pink) with SU-DIPG-13 cells.

## Culture media

CellTiter-Glo Luminescent Cell Viability Assay is a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. The generated luminescent signal is proportional to the amount of ATP present, which at the same time is directly proportional to the number of cells present in the culture. In Figure 3 we can see the luminescence measured in absorbance units plotted against the three different media that were tested: TSM, ITMG and a 50:50 mixture (Table S1). The left graph shows that the viability of HMC3 cells does not vary when the culture media is changed. However, the right graph shows that the viability of SU-DIPG-13 cells significantly varies when these are cultured in ITMG media, which is specific for microglia cells. According to these results, the ITMG media will be the one used in the co-cultures.

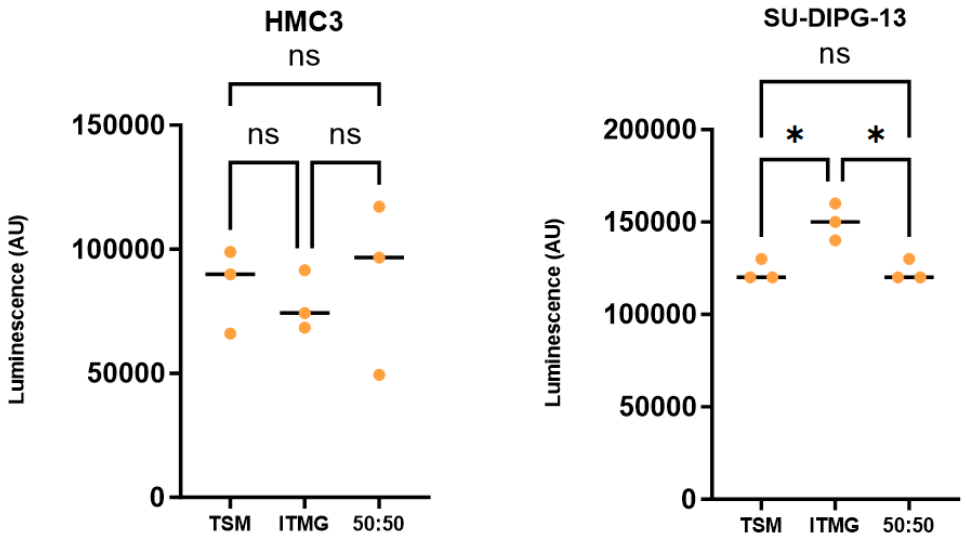


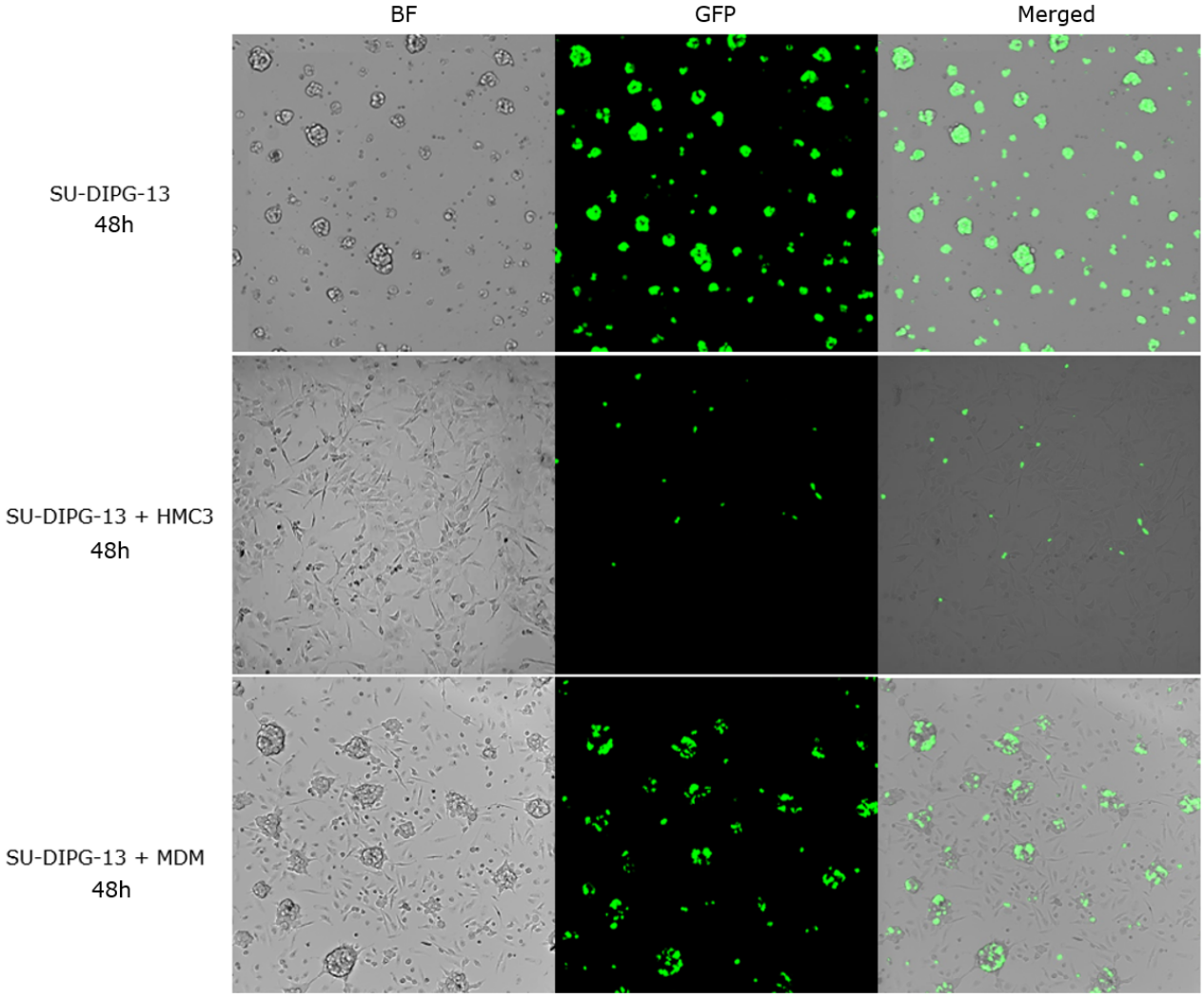

**Figure 3.** Graphical representation created with GraphPad Prism of the luminescence (Absorbance Units) against the three different media tested (TSM, ITMG, and a 50:50 mix) for the HMC3 (microglia cell line) and SU-DIPG-13 (DMG cell line) cells. The statistical analysis was performed using One-way ANOVA.

## Monocyte-derived microglia as the optimal microglia model

GFP+ SU-DIPG-13 cells were co-cultured separatedly with HMC3 and Monocyte-derived microglia (MDM). After 48h in co-culture, images of the cells were taken (Figure 4. A). In the first row, SU-DIPG-13 cells are cultured alone, GFP+ SU-DIPG-13 spheres can be identified in the culture. The second row shows the SU-DIPG-13 cells co-cultured for 48h with the microglia cell line HMC3. In the Bright Field (BF) picture, we can see the adherent microglia cells but no SU-DIPG-13 spheres can be seen. When looking at the GFP channel, some green spots can be seen. These results show that after 48h, the HMC3 cells can "eat up" the tumor spheres. The third row shows the co-culture of SU-DIPG-13 cells with MDM for 48h. In the BF image, we can see some adherent microglia cells together with some SU-DIPG-13 spheres, which can be confirmed when in the merged picture we see GFP-positive tumor spheres as well as adherent GFP-negative microglia cells. These results show that MDM are the best model for the co-cultures, as they can mimic the tumor microenvironment that happens in patients where the tumor can grow in spheres when microglia is present.

After PBMC isolation from blood, these cells are seeded in ITMG and after six days it can be seen (Figure 4.B) that almost all the cells have acquired a microglia-like morphology and are ready to be used for the following experiments.

A)



B)

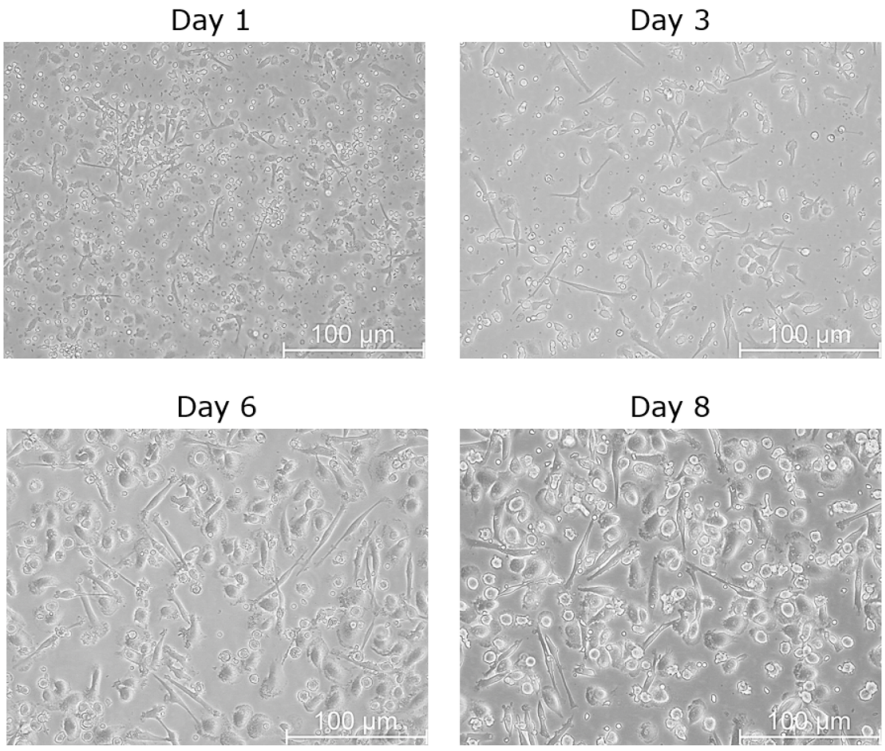



**Figure 4.** A) Bright field (BF), 488 nm (GFP) and merged images taken with the Leica Thunder Life after 48h of SU-DIPG-13 alone, SU-DIPG-13 co-cultured with HMC3 and SU-DIPG-13 co-cultured with MDM. B) Differentiation process of Peripheral Blood Mononuclear Cells (PBMC) towards Monocyte-Derived Microglia (MDM) after 8 days in ITMG media.

## Triple co-culture

Having determined the optimal conditions, we were able to set the triple co-cultures (Figure 5). In the first image, SU-DIPG-13, we can see the tumor cells growing in spheres in culture. In the second image, we can see SU-DIPG-13 cells with GD2-CAR-T cells in a 1:10 E:T ratio. Almost all tumor spheres are completely disrupted, indicating that they are being attacked by the GD2-CAR-T cells. The third picture shows the triple co-culture where MDM cells are also present. We can see some disrupted tumor spheres but also some of intact spheres, meaning that they haven't been attacked by the GD2-CAR-T cells. These results suggest that when microglia is present, the GD2-CAR-T cell effectivity is reduced as they cannot attack all the tumor cells in the culture. This results demonstrate that our triple co-culture system mimicks the patient's situation and could be used for further studies to improve DMG treatment.

As controls, SU-DIPG-13 cells were treated with 5 µM of Panobinostat and we can see that the tumor spheres are disrupted, the same as happens with the SU-DIPG-13 cells when co-cultured with the GD2-CAR-T cells. Also, we have the MDM with GD2-CAR-T cells, to show that the latter don't have any effect over the microglia cells. Finally, we have the tumor cells co-cultured with the microglia cells. Here we can see that the tumor spheres are bigger when microglia are present compared to the situation where SU-DIPG-13 cells are growing alone in culture. This confirms our hypothesis that microglia cells contribute to tumor growth.

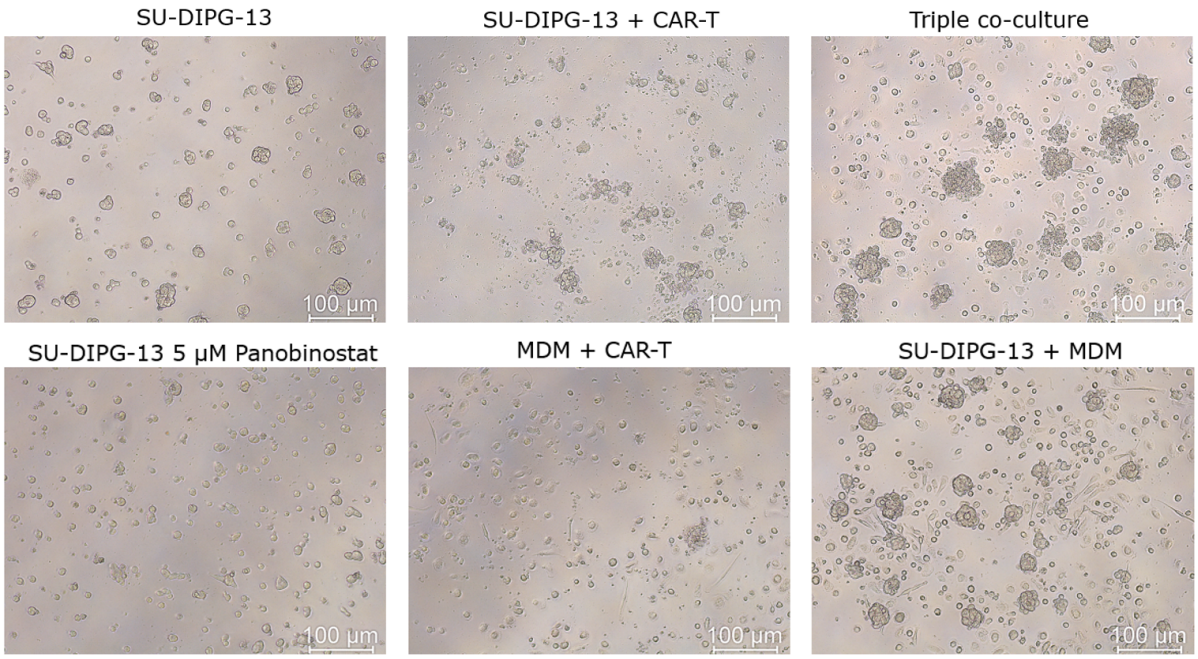
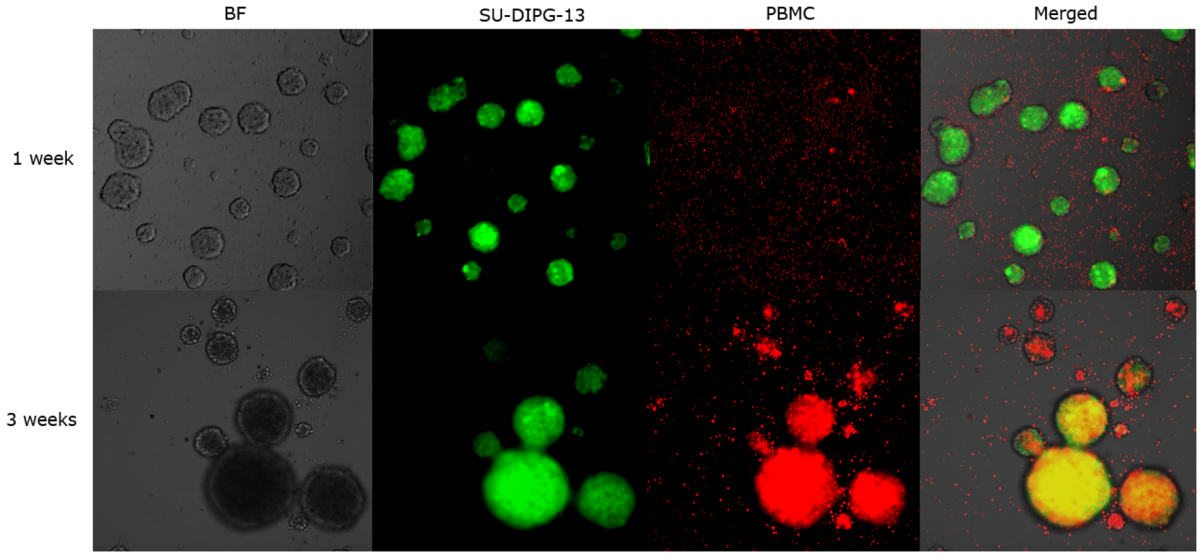



**Figure 5**. Bright-field images of SU-DIPG-13, SU-DIPG-13 with GD2-CAR-T, SU-DIPG-13 with GD2-CAR-T and MDM, SU-DIPG-13 treated with 5µM of Panobinostat, MDM with GD2-CAR-T and lastly, SU-DIPG-13 with MDM.

## PBMC and HMC3 infiltration in SU-DIPG-13 spheres

Microglia derived from the blood can survive in culture for two weeks. Recent studies have shown that Kupfer cells, which are the macrophages of the liver, can be maintained in culture for a few days, but when co-cultured with hepatocyte spheroids, the Kupfer cells can infiltrate the spheres and survive longer in the culture. As microglia are considered the macrophages of the brain, we decided to test if by culturing the PBMC directly isolated from blood with SU-DIPG-13 spheres we could maintain the microglia population for more than two weeks. To do so, images of the co-cultures were taken after one and three weeks (Figure 6. A). After one week in co-culture, we can see that the PBMCs (red) are mostly outside the tumor spheres (GFP positive). Some of the PBMC seem to start infiltrating the spheres but not to a big extent. However, after three weeks in culture, we can see that almost all the PBMC cells are inside the SU-DIPG-13 sphere. Taking into account that the CellTrace FarRed staining disappears when the cells fate, we can say that these results suggest that SU-DIPG-13 spheres seem to serve as a bioscaffold for the microglia derived from blood to survive in the culture for more than two weeks. The same set-up was established to test if the same occurs with the HMC3, the microglia cell line. It can be seen (Figure 6.B) that even after 24h, the HMC3 seem to infiltrate the tumor spheres.

A)




B)

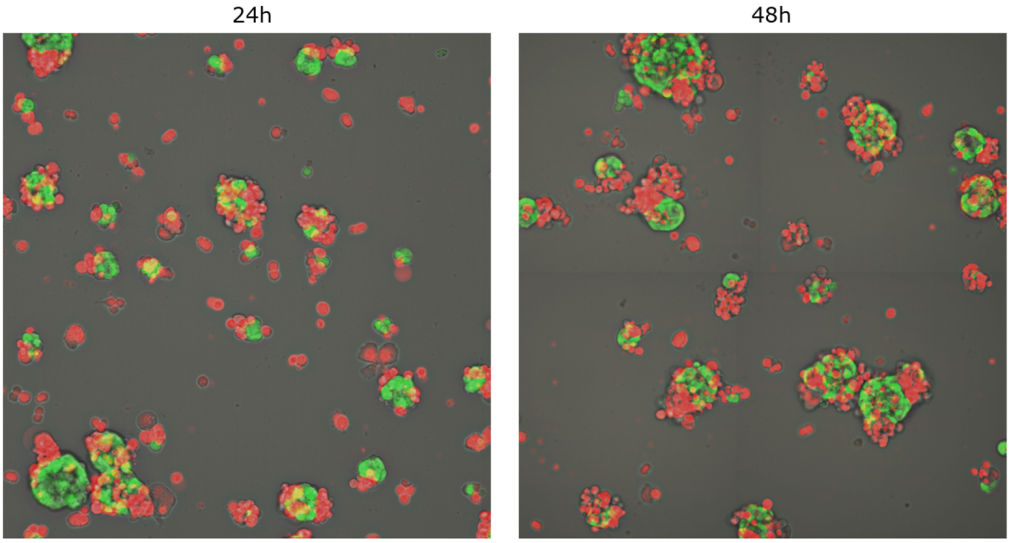



**Figure 6**. A) Bright-field, 488 nm (GFP) and 630 nm (Far Red) images after one and three weeks of the SU-DIPG-13 cells co-cultures with PBMC directly isolated from blood. B) Merged images of the co-cultures with SU-DIPG-13 (green) and HMC3 (red) cells after 24h and 48h.

## Limitations of the study

One of the limitations of the study was the staining of the different cell types used in the co-culture. We tried to set-up the triple co-culture by staining each of the cells with a different color, using different Cell Tracker dyes for the GD2-CAR-T and the MDM, and using our GFP+ SU-DIPG-13 model. However, this experiment failed and only bright-field pictures were able to be taken.

Another thing to take into account is that the cell tracker dyes may affect the viability of the PBMC and the HMC3 cells. Because of this, the infiltration experiment would need to be repeated without the use of the tracker dyes.

## Conclusions

From this project, we can conclude that:

- It is possible to cluster different myeloid populations in DMG and identify specific tumor-associated microglia markers that could be used as potential therapeutic targets.
- The SU-DIPG-13 and MDM co-cultures are a promising set-up to study the DMG microenvironment.
- When SU-DIPG-13 cells are co-cultured with MDM, bigger SU-DIPG-13 spheres are formed, and GD2-CAR-T cell efficacy is reduced, mimicking patient situation.
- The DMG tumoroids act as a biological scaffold for microglia co-cultures.

## Future perspectives

After the selection of drugs that specifically target tumor-associated microglia markers, we aim to use these drugs in our triple co-culture set-up to see if by attacking the tumor-associated microglia, the GD2-CAR-T cell sensitivity is increased (Figure 8). This will confirm that the tumor-associated microglia are the main responsible of the lower efficacy of this immunotherapy in DMG patients.

On the other hand, the infiltration experiment will be repeated without the use of tracker dyes for the microglia cells. Then, the spheres will be dissociated and we will use flow cytometry sorting with specific microglia and SU-DIPG-13 surface markers to separate both cell types. Once the microglia cells have been isolated from the spheres, we aim to check the expression of several markers (e.g ki67 as proliferation marker) to check the identity and characteristics of these cells after three weeks inside the tumor spheres (Figure 8). This will allow us to know if the tumor spheres allow microglia not only to survive longer in culture but also to replicate, which normally doesn't happen after they have been isolated from blood.

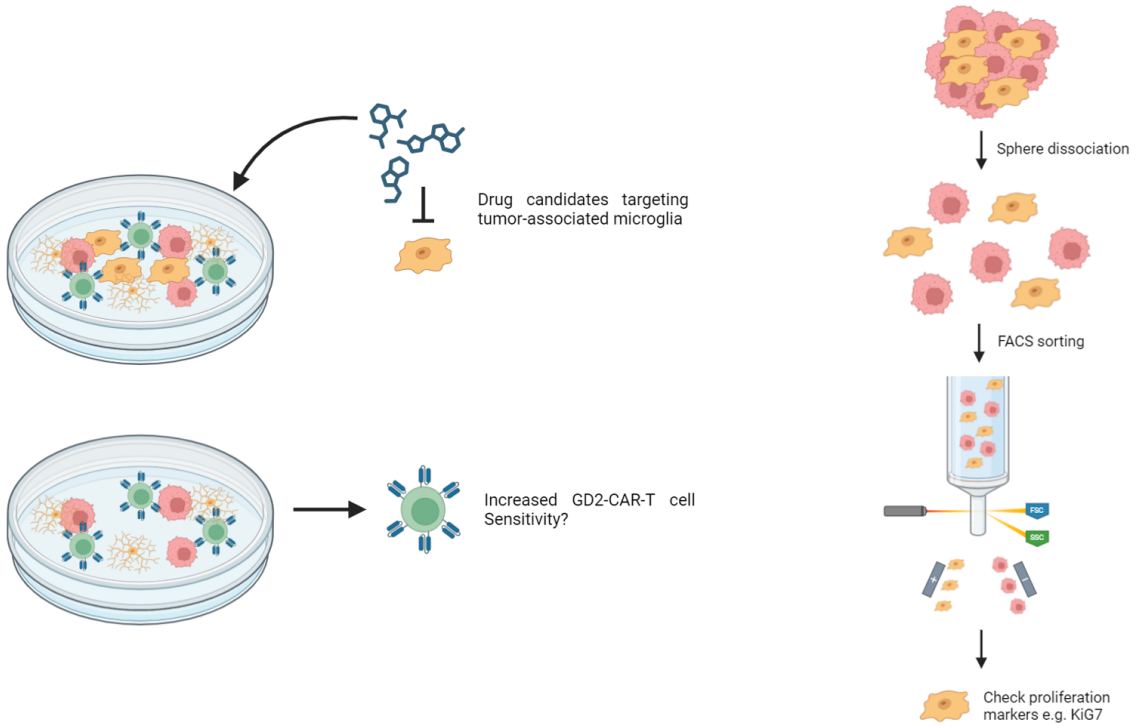



**Figure 8**. Graphical abstract created with Biorender of the future perspectives of this study. On the left, we have the representation of the addition of drugs that target tumor-associated microglia to see if after attacking this myeloid population, GD2-CAR-T cell sensitivity is increased. On the right, we can see the schematic representation of the next experiment related to the infiltration of microglia into the SU-DIPG-13 spheres: sphere dissociation, FACS sorting, and checking the expression of proliferation markers, e.g ki67, in microglia cells.

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Finally, I would like to thank my family and friends for their unconditional support.

# Supplementary material

**Table S1**. Components of the TSM and ITMG media.

| Media name | Reagents | Base media | Working media |
|---|---|---|---|
| TSM | DMEM/F12 with Phenol Red without glutamine (Thermo Fisher #12634-010)<br>Neurobasal-A medium (Thermo Fisher #10888-022)<br>HEPES 1M (Thermo Fisher #15630-080)<br>MEM Non-essential amino acid solution (Thermo Fisher #11140-035 )<br>GlutaMAX Supplement (Thermo Fisher #35050-061)<br>Sodium Pyruvate 100mM (Thermo Fisher #11360-070)<br>B27 Supplement without vitamin A (Thermo Fisher #12587-010)<br>N-2 Supplement 100X (Thermo Fisher #17502001)<br>Basic Fibroblast Growth Factor (Peprotech #100-18B)<br>Epidermal Growth Factor (Peprotech #AF-100-15)<br>Platelet-derived Growth Factor AA (Peprotech #100-13A)<br>Platelet-derived Growth Factor BB (Peprotech #100-14B)<br>Heparin 5000IE/ml (Local Pharmacy)<br>Antibiotic/Antimycotic (Merck A5955-20ML) | 1:1 mix Neurobasal-A medium and DMEM/F12 supplemented with:<br>- 1x Glutamax<br>- 1x HEPES 1M<br>- 1x MEM-Non-essential Amino Acid solution<br>- 1x Sodium Pyruvate<br>- 1x Antibiotic/Antimycotic | For 50 mL media:<br>- 49mL TSM Base<br>- 1mL B27 supplement without vitamin A<br>- 0,5mL N2 supplement<br>- 20ng/mL bFGF<br>- 20ng/mL EGF<br>- 10ng/mL PDGF-AA<br>- 10ng/mL PDGF-BB<br>- 5 IE/mL Heparin |
| ITMG | Advanced Dulbecco's Modified Eagle Medium (ADMEM)<br>GlutaMAX (35050061, Gibco)<br>IL-34 (130-105-780, Miltenyi Biotec)<br>TGF-β1 (130-095-066, Miltenyi Biotec)<br>M-CSF (130-096-491, Miltenyi Biotec)<br>GM-CSF (130-095-372, Miltenyi Biotec) | | ADMEM supplemented with:<br>- 2 mM GlutaMAX<br>- 100 ng/mL IL-34<br>- 50 ng/mL TGF-β1<br>- 25 ng/mL M-CSF<br>- 10 ng/mL GM-CSF |

Figure S1 shows the gating settings selected for the flow cytometry assay to determine the E:T ratio. Three different conditions are shown: SU-DIPG-13 alone, SU-DIPG-13 cells exposed for 48h to 5 µM of Panobinostat, and SU-DIPG-13 with GD2-CAR-T cells in a 1:1 E:T ratio. The first column in each raw shows the gating of the dead and also the alive cells. From it, we obtained the single-cell graphs in the second column. The third columnd shows the FITC (or GFP) positive cells, which corresponds to the tumor cells. When SU-DIPG-13 are treated with Panobinostat, we can see two different populations of GFP-positive cells. This is because when cells are dying, they still express some GFP but at a lower intensity compared to the living cells. The last column shows the live cells , which are negative for the dead/alive eBioscience Fixable Viability Dye eFluor™ 780. The positive cells are the dead tumor cells. The highest proportion of dead tumor cells can be seen in the 1:1 E:T ratio due to the effect of the GD2-CAR-T cells.

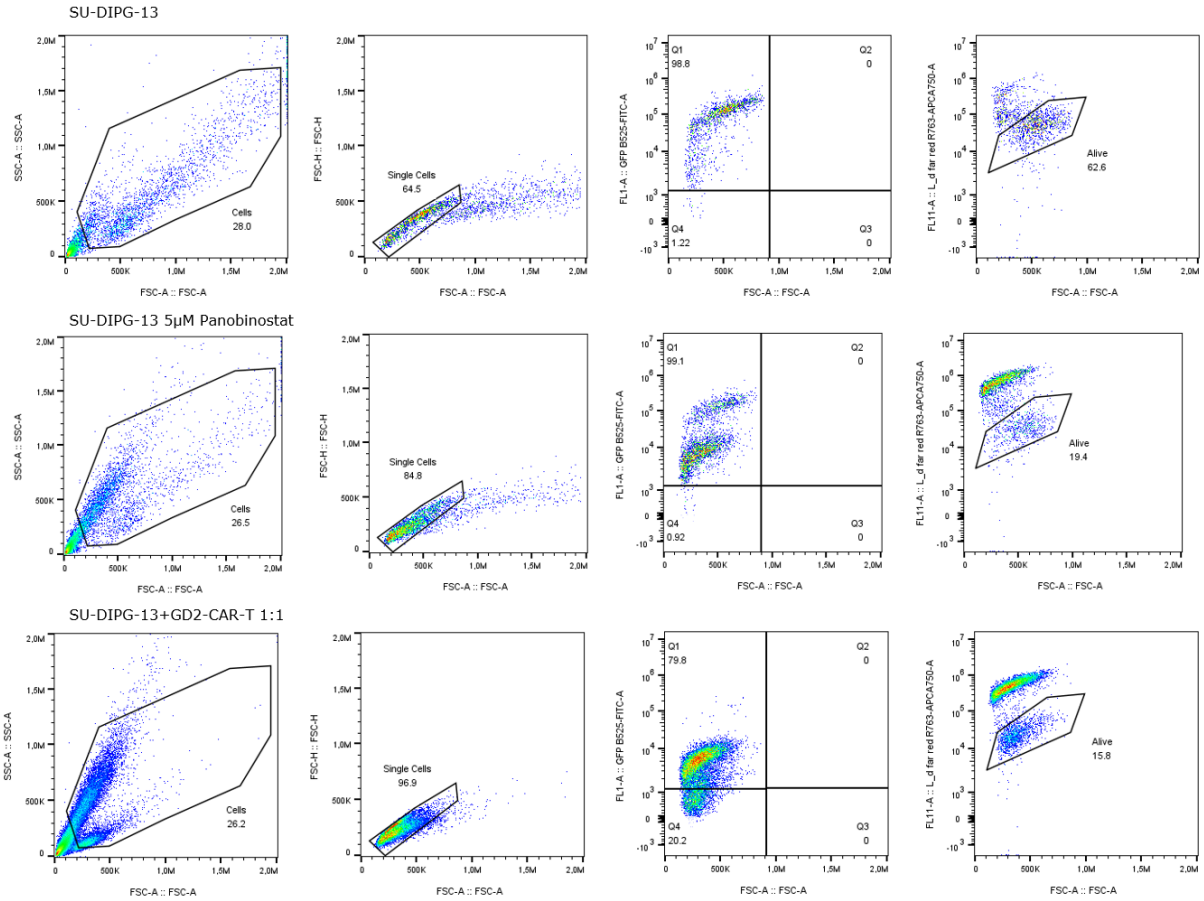


**Figure S1**. Gating settings in the flow cytometry experiment of the E:T ratio using GFP positive SU-DIPG-13 cells and GD2-CAR-T cells. Three different conditions are shown: SU-DIPG-13 cells alone, SU-DIPG-13 cells treated with 5µM of Panobinostat for 48h, and 1:1 E:T ratio.

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