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PhD thesis

CROSSTALK BETWEEN GLIOMA CELLS AND TUMOR-ASSOCIATED-MACROPHAGES: MECHANISMS AND THERAPEUTIC STRATEGIES

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Abstract

Macrophages are highly plastic cells, able to respond to several microenvironment signals acquiring distinct functional phenotypes. In Glioblastoma (GBM), the most common malignant brain tumor, tumor-associated-macrophages (TAMs) represent the major infiltrating immune cell populations and are key drivers of the local immunosuppression supporting tumor progression and the resistance to immunomodulating treatments. Mutations in enzymes isocitrate dehydrogenases (IDH) occur in GBM subsets, characterized by more favorable prognosis. Also, other molecular alterations and metabolic features, such as accumulation of the oncometabolite 2-hydroxyglutarate, are associated with IDH-mutations. Despite many therapeutic strategies, GBM recur in almost all patients and prognosis remain poor, the development of new therapeutic strategies is strongly needed. In the last decades, innovative engineered nanovectors bears a promise for GBM treatment: these multifunctionalized liposomes encapsulate drugs and are able to cross the blood brain barrier that will guarantee the localized release of candidate drugs to control neuroinflammation. The aim of the present study is to investigate the relevance of the cross-talk between macrophages and glioma cells, needed to overcome limitations of the current therapeutic approaches. Firstly, we analysed the interplay between macrophages and U87 glioma cells lines, harbouring wild-type or mutant IDH genes, through direct and indirect cocultures. Then we investigated the mechanisms underlying the macrophages-glioma cells' crosstalk in hypoxia, a key aspect of the tumor microenvironment. In addition, in the context of nanovectors' strategy, we set coculture models of macrophages and patient-derived GSCs to explore the potential of macrophages as target cells of encapsulated drugs for the rebalance of the glioma microenvironment. In our results, we showed that glioma-derived factors, released by U87 IDH1-WT and IDH-MUT, are able to polarize macrophages toward a pro-tumoral M2-like phenotype with the increase of the CD206 expression. Moreover, the effect of TCM collected from U87 IDH1-MUT is different compared with TCM from U87 IDH1-WT on macrophages' differentiation and polarization. In addition, we demonstrated that hypoxic condition induces its target genes in a cell-specific manner, with significant differences between Wt and IDH-mutant U87 cultures. Similarly, in cocultures macrophages shape tumor cells responses to hypoxia. In the nanovector experiments we

evaluated that the vitality of macrophages treated with free drugs is different based on polarization conditions. Moreover, macrophage in coculture with GSC cells are less sensible to the drug treatment compare to macrophages alone.

Disclosure for research integrity

I state that this scientific research was conducted following the principles of good research practice of the European Code of Conduct for Research Integrity, based on the principles of reliability, rigor, honesty, respect and accountability.

List of Abbreviations

AMP- activated protein kinase (AMPK)

Angiopoietin like 4 (ANGPTL4)

Arginase (ARG1)

Argininosuccinate synthase 1 (ASS1)

ATP-dependent helicase (ATRX)

Blood Brain Barrier (BBB)

Bone-marrow derived macrophages (BMDMs),

C-C chemokine receptor type 7 (CCR7)

Central nervous system (CNS)

Colony stimulating factor (CSF-1)

Dendritic cells (DCs)

Glioblastoma (GBM)

Glioblastoma stem cells (GSCs)

Glioma-associated microglia/macrophages (GAMs)

Glucose transporter1 (GLUT1)

Heme oxygenase 1 gene (HMOX)

Hypoxia-inducible factor 1- α (HIF-1-α)

Infiltrating lymphocytes (TILs)

Insulin-like Growth Factor Binding Protein 6 (IGFBP6)

Interleukin-1β (IL-1β)

Interleukin-10 (IL-10)

Interleukin-6 (IL-6)

Interleukin-8(IL-8)

Isocitrate dehydrogenases (IDH)

Kynurenine (Kyn)

Lactate Dehydrogenase A (LDHA)

L-tryptophan (L-Trp)

Malate Dehydrogenase (MDH1/2)

Mean fluorescence intensity (MFI)

Mechanistic target of rapamycin (mTOR)

Migration Inhibitory Factor (MIF)

Myeloid-derived suppressor cells (MDSCs)

Neutrophils (PMNs)

Nitric oxide synthase (NOS)

O6-methylguanine-DNA methyl transferase (MGMT)

Peripheral blood monocytes (PBMC)

Phosphoglycerate kinase (PGK1)

Programmed death-ligand 1 (PD-L1)

Regulatory T cells (Treg)

TDO (tryptophan-2-3dioxygenase)

Transforming growth factor β (TGFβ)

Transmembrane protein 119 (TMEM119),

Tricarboxylic acid cycle (TCA)

Tumor microenvironment (TME)

Tumor necrosis factor alpha (TNFα)

Tumor-associated macrophages (TAMs)

Vascular endothelial factor (VEGF)

α-ketoglutarate (α-KG)

2-Hydroxyglutarate (2-HG)

Introduction

1.Glioblastoma

1.1 Classification

Gliomas represent the most common malignant tumors of the central nervous system (CNS) that derived from progenitor glial cells and neuronal stem cells (1). It is possible to discriminate different types of glioma based on the cell of origin: oligodendrogliomas, ependymomas and astrocytomas, among which the last is the most common subtype(2). Before 2016, gliomas were classified only by their histology. A more accurate classification of different glioma subtypes was achieved in 2016 by the World Health Organization (WHO), which integrated the classification system with molecular criteria, in order to predict the efficacy of treatments and to enhance personalized therapeutic strategies(3). WHO included IDH mutations as markers for the classification of gliomas separating tumors in IDH-wild type (IDH-wt) and IDH-mutated (4,5). It has been reported, that in low grade gliomas the prevalence of mutations in isocitrate dehydrogenases (IDH)(6,7) 1 and 2, two NAD(P)+ - dependent enzymes that convert isocitrate to α-ketoglutarate (α-KG)(8). Primary glioblastoma (IDH-WT tumors), correspond to 90% of gliomas and predominate in adults over 55 years of age; whereas tumor IDH-mut involve younger patients (9). IDH-mut tumors are frequent in younger patients, whereas IDH-wt tumors, also called primary or de novo glioblastoma, represent about 90% of gliomas and predominate in patients over 55 years of age. Of note, other mutations occurring in glioma influence diagnosis, such as oligodendrogliomas that carry 1p/19q codeletion. The point mutation in codon 132 is the predominant alterations, which correspond in the substitution of arginine with histidine (R132H) (8). This point mutation induces a neomorphic activity on IDH1: in this condition the enzyme catalyzes the aberrantly reduction of α-KG into 2-Hydroxyglutarate (2-HG) (10). 2HG is characterized by two stereoisomers : D-2HG and L-2HG, that are produced from α-ketoglutarate by distinct enzymes. L-2HG is produced in hypoxic and acidic conditions by Malate Dehydrogenase (MDH1/2) and Lactate Dehydrogenase A (LDHA) (11). L-2HG is involved in differentiation and proliferation of T cells and is recently described to stimulate LPS-induce expression of the cytokine IL-1β in macrophages. The oncometabolite D2-HG plays a role in the process of glioma development and progression, in fact increased levels of D2-HG results in elevated levels of Hypoxia-inducible factor 1- α (HIF-1-α) and of its target

genes, such as VEGF, GLUT1, PGK1(12). While D-2HG has been shown to influence tumor growth, this oncometabolite has been shown to modulate anti tumor immunity (1). Moreover, another prognostic marker is the hypermethilation of the O6-methylguanine-DNA methyl transferase (MGMT) promoter. MGMT encodes for a ubiquitously expressed DNA damage repair enzyme and its inactivation by methylation is related to an increased sensitivity to temozolamide and to an higher survival of glioma patients (13). The revised 2021 WHO classification further integrates molecular mutations of CNS tumors in the categorization. Primary gliomas of the adult may be classified in 3 main groups: GBM IDH1-WT(14). Astrocytoma IDH1-mutant; Oligodendroglioma IDH1-mutant and 1p/19q-codeleted. Firstly, Glioblastoma is characterized by rapid progression and poor prognosis and a median survival about 15 months. Astrocytoma IDH1-mutant is also characterized by the mutation of ATP-dependent helicase (ATRX) encoding gene and of TP 53, in addition based on histology the grade II, III or IV could be assigned. Finally, oligodendrogliomas are grade II or III and are characterized by a better response to radio chemotherapy and longer survival compared to other gliomas.

1.2 The Immune Landscape of Glioblastoma

The tumor microenvironment (TME) includes a proportion of non-neoplastic cells, such as infiltrative inflammatory cells and tissue resident cell types, as astrocytes, neurons, microglia and brain vasculature (15). Moreover, there is a subset of cells with stem like features known as glioblastoma stem cells (GSCs), characterized by increased resistance to chemotherapy and radiotherapy, that usually follow surgical removal of tumor mass. Residual resistant GSCs lead to glioma recurrence and progression(16). Over the last years, glioma microenvironment has been studied in order to discover new insight in the interactions between tumor cells and the immune system(4). Today it has been known that the CNS is considered an immune-privileged tissue in which the inflammation and adaptive immunity are controlled(17)(18). In human and rodent glioma studies, histopathological, flow cytometry, mass spectrometry and single cell RNA sequencing analysis demonstrated a significant heterogeneity of immune cells in the TME (19–22)(Figure 1). Infiltrating lymphocytes (TILs) and other immune effector cells (23) are rare both in lower and higher-grade gliomas, in this scenario tumors are generally defined “cold” and the immune therapies such as immune check-point blockade are poorly effective. Meanwhile, the myeloid cells are the most predominant immune cell population in glioma patients (24), this

lineage are constituted by resident microglia and macrophage populations (as perivascular macrophages), bone-marrow derived macrophages (BMDMs), neutrophils (PMNs), myeloid-derived suppressor cells (MDSCs) and dendritic cells.

The neutrophils are the major circulating myeloid population in human and they act as first defence line of immune systems against pathogens. In GBM patients, the number and the activation state, measured as the integrin CD11b expression, of circulating PMNs have been defined as early predictors of tumor progression and disease outcome (25–27). Moreover, several researchers evaluate those neutrophils play an important role in stimulating tumor angiogenesis (28): through in vivo experiments they demonstrated that neutrophils promote neovascularization(29). Indeed, within tumor tissue the number of PMNs and their activation status correlates with glioma grade, in particular, reduced neutrophil infiltration has been seen in low grade glioma that have a better prognosis compared to high grade glioma(30).

MDSCs are a heterogeneous population of myeloid immature cells with a peculiar immunosuppressive activity(1). MDSCs are constituted by two main subsets: polymorphonuclear (PMN-MDSCs) and monocytic (M-MDSCs), with phenotypic features of neutrophils and monocytes, respectively. In glioma patients, several studies report that the intratumoral and the systemic frequencies of MDSCs correlate with tumor grading and prognosis (31). In particular, low-grade glioma patients show better survival and low number of MDSCs, while the accumulation of these myeloid cells correlates with a poor prognosis in GBM patients.

Dendritic cells (DCs) are professional antigen-presenting cells with a central role in stimulating and shaping immune responses(32). In homeostatic conditions, DCs are not present in the brain tissue, thus in pathological conditions as GBM can infiltrate the brain parenchyma through endothelial venules and afferent lymphatic vessels (33). Several researchers using recent single-cell transcriptomic techniques on human GBM samples confirm the presence of glioma infiltrating DCs, but as a minor component compared with macrophages and microglia (respectively in the range of 5% vs 95%)(34,35). The activation of dendritic cells correlated to an anti-tumor immunity, they increased the expression of MHC class II, the costimulatory molecules and C-C chemokine receptor type 7 (CCR7), and displayed high ability to produce cytokines such as interleukin-6 (IL-6) and IL-12 (36). Interestingly, recent studies investigate the role of DCs in glioma progression focusing on Nrf, a redox-sensitive transcription factor that contrasting the effects of reactive oxygen

species, that regulates the DC homeostatic functions. However, the tumor microenvironment of GBM has been demonstrated to induce overexpression of Nrf in DCs, which results in the suppression of DC maturation and the consequent decrease in effector T cell activation(37).

Finally, glioma-associated microglia/macrophages (GAMs) represent the major population present in the tumor microenvironment, 30% of the immune cells, with a central role in the tumor progression and resistance to immunotherapies. Several researchers have investigated the importance of the IDH mutation status as a modulator of the infiltration of immune cells in glioblastoma. According to preclinical studies, lower content of GAMS has been found in IDH-mut compared to IDH-wt human tumors,(38) suggesting a down-regulation of the leukocyte chemotaxis on tumors with IDH mutation (30). This myeloid population will be discussed with more details in the following sections.

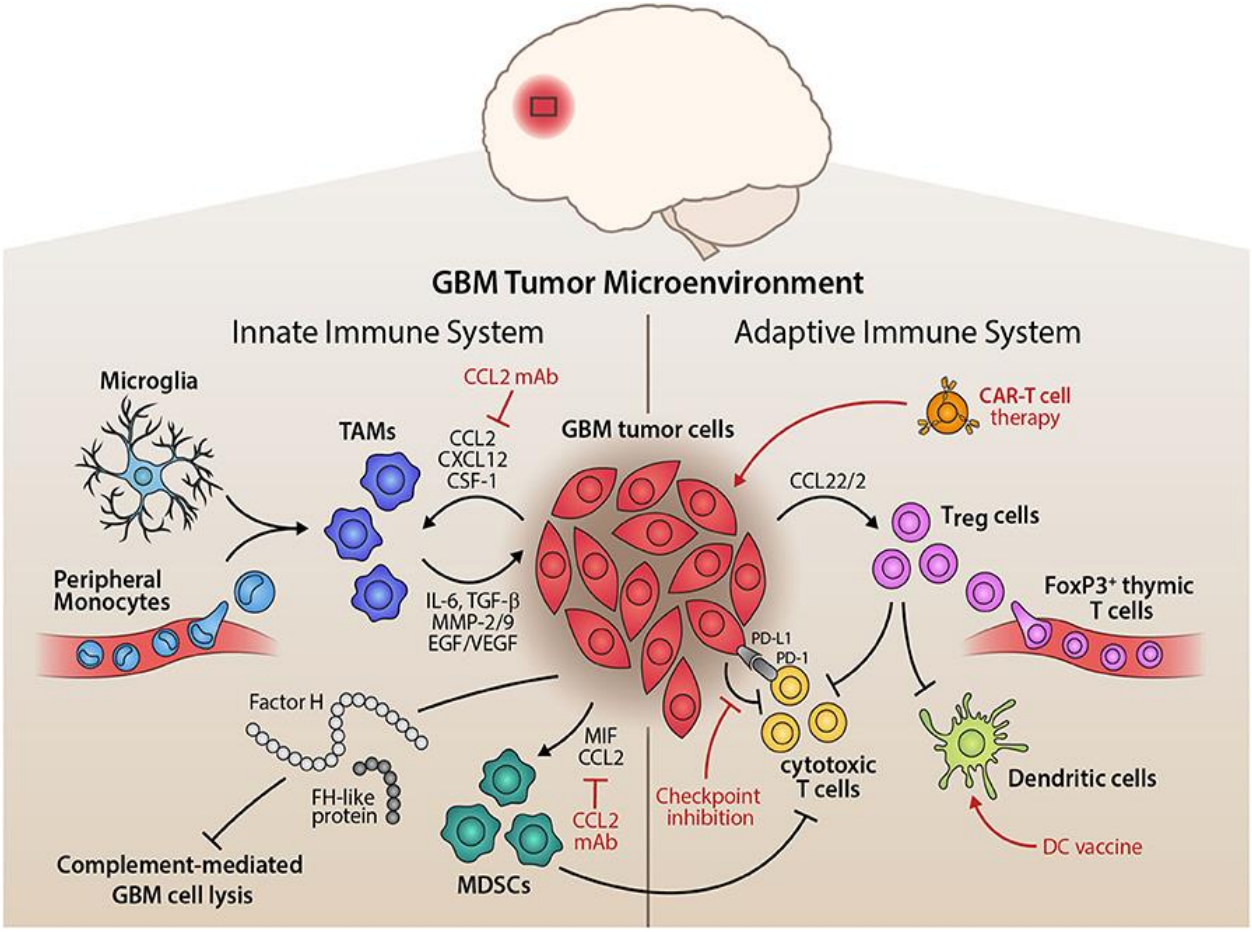


Figure 1: The GBM Microenvironment. This figure describes the crosstalk of innate and adaptive immune factors in the glioma microenvironment. On the right: the adaptive immune system is composed by cytotoxic T cells, that are functionally suppressed through the recruitment and activity of the regulatory T cells (Tregs) On the left, is represented the innate

immune system: tumor-associated macrophages (TAMs), mainly composed by microglia and peripheral monocytes-derived macrophages. Moreover the therapeutic strategies identified are characterized by inhibiting chemoattractants , such as anti-CCL2 monoclonal antibody, by inhibition of immune checkpoint , and approaches of dendritic cell vaccination and finally with chimeric antigen receptor (CAR) T cells that target the tumor cells (39) (Mod. From DeCordova. et al, Front. Immunol., 2020).

2 .1 Macrophages

Macrophages are highly plastic cells, able to undergo distinct polarized activation in response to microenvironment signals (40,41). Also in tumor, macrophages, called Tumor associated macrophages (TAM), are affected by TME's stimuli. *In vitro* studies implemented the classification of activated macrophages according to a binary system: pro-inflammatory cytokines stimulation, such as IFNγ, induced a "classical" pro- inflammatory activation state (M1 phenotype), whereas anti-inflammatory cytokines, like IL-4, polarized macrophages into an "alternative" M2 phenotype (42). In tumors, this nomenclature has been used for years to discriminate M1-like anti-tumor versus M2-like pro-tumor macrophages, assumed to be the majority of TAMs (43). Indeed, in mouse and human cancers, TAMs largely expressed molecules associated with M2-like phenotype, such as arginase (ARG1), IL-10 and transforming growth factor β (TGFβ) that cause immune suppression and fibrosis in tumor microenvironment. In some specific human cancers (44), the prevalence of macrophages with classical activation phenotype the up regulation of IL-12, tumor necrosis factor alpha (TNFα) and nitric oxide synthase (NOS) has been reported to correlate with favorable clinical outcome. Whereas, in brain tumors has been reported that M2-like phenotype macrophages, with a putative M2c state associated to immune regulation, matrix deposition and tissue remodeling (45). However, the binary and simplistic classification described *in vitro* can't be applied to the in vivo scenario: in tissue macrophages are highly heterogeneous with dynamic and extremely plastic phenotypes and functions, which are continuously shaped by tumor microenvironment. Single-cell RNA-sequencing of glioma associated macrophages (GAMs) has shown a peculiar gene expression profile, characterized by the frequent co expression of M1- and M2- signature genes (46) in

individual cells. In addition, profiling of human GAMS revealed that *ex vivo* macrophages from GBM patients exhibit a distinct phenotype of activation, closer to non-polarized (M0) undifferentiated phenotype (27). For this reason, other works have been made to increase number of surface markers for distinguish different subsets of macrophages : for example M1-like macrophages have been associated with the expression of CD40, CD74, MHC-II and phosphorylated STAT1, whereas M2-like macrophages express CD163, CD204, arginase-1 (ARG1) and phosphorylated STAT3. However, these markers have also failed to provide a robust separation, thus full understanding of their relative contribution to disease pathogenesis is still unclear. In conclusion, to understand the GAMS phenotype is necessary to provide a classification that summarize distinct states of GAMs in human glioma and in general in tumor biology (24).

2.2 Glioblastoma's associated macrophages

Glioblastoma associated macrophages and microglia constitute the 30% of the tumor mass and representing the majority of tumor invading myeloid cells (47). Within CNS parenchyma, microglia are unique cells specialized cells that populate brain since early embryogenesis; in the adult brain microglia perform a broad spectrum of functions, including neurons support, synaptic pruning, phagocytosis of apoptotic cells as well as immune surveillance. Microglia are characterized with a self-renewal, without contribution of bone-marrow derived progenitors, thus making microglia the only immune cell population residing in the brain. However, under some pathological conditions, like glioblastoma, local inflammation can altered the integrity of blood- brain barrier that induce from the circulation, the infiltration of inflammatory monocytes, which subsequently differentiate into monocyte-derived macrophages(4). Moreover, in glioblastoma it is possible to distinguish: resident parenchymal microglia, perivascular macrophages and peripheral monocytes-derived cells (48). In GBM, TAMs are recruited to tumour site by different mediators, such as CCL2, CXCL1, GM-CSF released by neoplastic cells (Figure 2). After accumulation, TAMs functions are progressively overturned towards a pro-tumorigenic phenotype. Recently, it has been reported in GBM specific roles of microglia and monocyte-derived macrophages in response to differently types of CNS insults. For example, it has been recently shown that the immune suppressive microenvironment in GBM patients depends on the accumulation of monocyte-derived macrophages (49). The ability to distinguish between these two cell populations has been problematic because of the absence of a unique defining marker. It has been reported that in recent works to distinguish microglia from other myeloid cells used

the markers CD45 in which evaluated microglia with higher expression of CD11b and low expression of CD45 and BMDM are defined with higher expression of CD45 and low expression of CD11b(50).However, the definition of microglia and BMDMs by their CD45 levels is inadequate, since the activation of microglia can rapidly increase the expression levels of this marker, thus limiting the effective discrimination of both cell populations(51). Recently, another marker to define the microglia was proposed, which is the transmembrane protein 119 (TMEM119), applicable for mouse and human(24). Further investigations will be crucial in identifying lineage-specific markers to discriminate the two-cell type in glioma patients. Single cell RNA sequencing (scRNA-seq) studies discovered that bone marrow derived macrophages (BMDM) and microglia (MG) have different phenotypes as well as spatial distribution in gliomas. Pinton et al.(49) showed that BMDMs exhibit characteristic of pro-tumoral cells, exerting strong immunosuppression while MG showed very limited suppression. Interestingly, they demonstrated in the study that, BMDMs presented an iron metabolism that is related to sustain tumor growth and metastasis and phagocytosis. In addition, they demonstrated that infiltration of BMDMs reached the highest percentage in grade IV gliomas, correlating with significant alterations in circulating monocytes in the same subset of patients. Contrary to microglia, in gliomas the presence of BMDMs and the elevated expression of BMDMs-related genes significantly correlated with the clinical outcome of glioma patients (51). Poon et al.(38)showed that the number of TAMs is significantly lower in untreated IDH-mutant GBM, but they are more pro-inflammatory thus suggesting that this feature may contribute to the better prognosis of these tumors (20). The frequencies of BMDMs observed in the IDH1 WT glioma tumor microenvironment (TME) was higher than BMDMs frequency in the TME of IDH1-mutant gliomas, which is associated with better clinical outcome of patients.

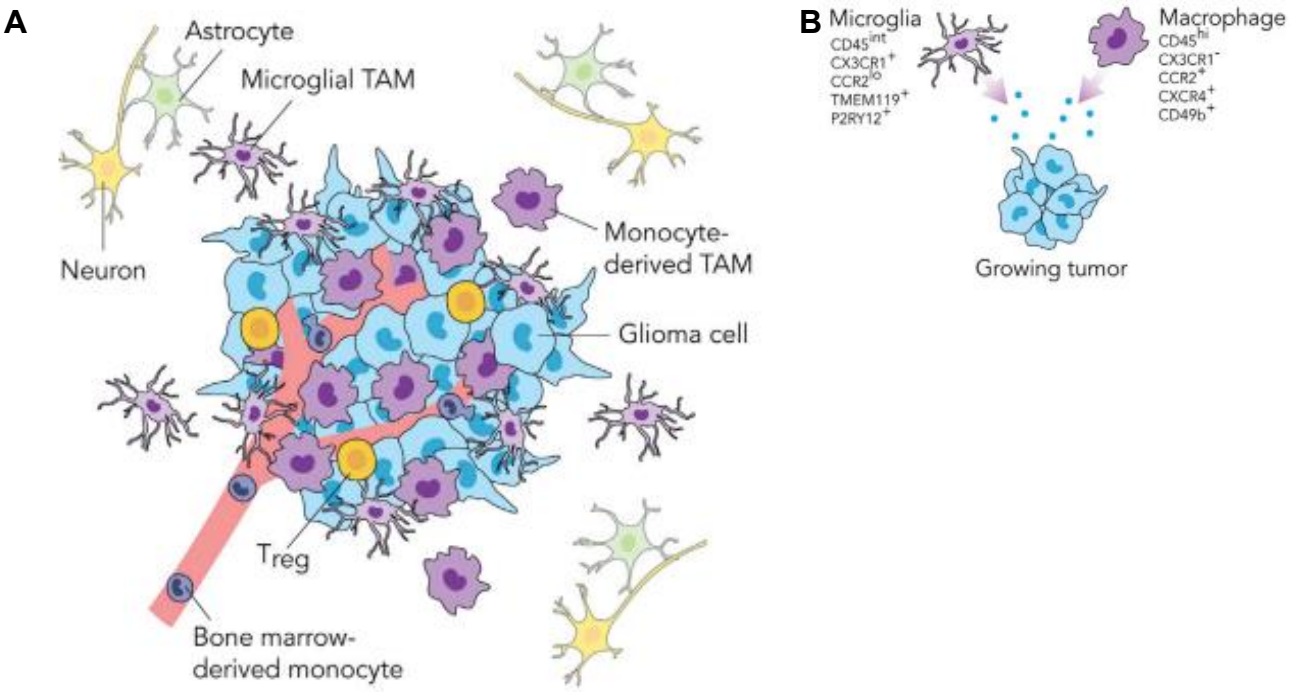


Figure 2: Glioblastoma's associated macrophages (GAMS). (A) GAMs are the most abundant non-neoplastic cells in gliomas and comprises microglial TAMs and blood-derived infiltrating monocytes/macrophages that localize in the perivascular and necrotic regions. Whereas, microglial GAMs are more abundant in the peritumoral regions. (B) The surface markers of micrgolia are CD45, CX3CR1, CCR2, TMEM1198 and P2RY12, whereas macrophages cold be discriminate from microglia using CXCR4 and CD49b markers. Both myeloid cells contributed to tumor growth producing survival factors, such as VEGF-A.(Modified from Akins et al., iScience,2020) (2)

2.2 Role of GAMs in tumor maintenance and progression

In glioma progression, inflammatory cells can infiltrate and subsequent compromise the integrity of blood brain barrier that induce the infiltration of monocytes from circulation and the subsequent differentiation in macrophages (4,48). Today, it is known that GAMs represent the majority of immune infiltrate and are recruited by a lot of chemokines, including CCL5 (35,52), CCL7, CXCL12 (53), and CX3CL1 (54). Moreover, tumor cells lead to GAMs invasion with the expression of colony stimulating factor (CSF-1) (55), which regulate macrophage proliferation, differentiation and survival, and guide the polarization of macrophages towards the M2-like, pro-tumorigenic phenotype into the tumor. Moreover, a significant production of the chemokine CCL2 from patient-derived GSCs and from GAMs has also been reported. CCL2 is part of a larger gene expression signature that determines the presence of tumor-infiltrating lymphocyte (TIL)/MDSCs/Treg infiltration in glioblastoma. In particular, monocytes and macrophages recruited by CCL2 contribute to the immunosuppressive microenvironment together with Tregs trafficking and MDSCs in response to CCL2 (56–58). Recently, Pinton et al, evaluate significant alterations in circulating monocytes that were present in glioma patients, correlating with accumulation of tumor macrophages. In particular, they evaluated in detail the composition of blood monocytes by FACS analysis to discriminate the three main subsets that are classical monocytes (CD14+/CD16−), intermediate (CD14+/CD16+) and non-classical subsets (CD14−/CD16+) and analyzed the expression of CCR2 on their surface (49) . Their results indicate a decrease of intermediate monocytes and, in the same subset, a significant increase of CCR2+ cells, thus suggesting that this population is actively recruited at tumor site.

After accumulation and differentiation, macrophages functions are progressively overturned towards a pro-tumorigenic phenotype. Functionally, microglia and monocyte-derived macrophages react differently to various types of CNS insults (59) and the specific roles for these distinct cell populations are now starting to emerge in GBM. It has been recently shown that BMDMs contribute to the immune suppressive microenvironment in GBM patients. In GBM, TAMs are recruited in the surroundings of tumour site by the release of different mediators. The infiltration of BMDMs increases from the margin area to the core of the glioma lesion and is almost absent in the marginal area (49) . In this respect, Muller et al. found out that BMDMs are enriched in perivascular and necrotic regions of tumor, while microglia are enriched in the leading edge of tumor infiltration (51). In particular, microglia

were diffuse throughout gliomas, while different subsets of BMDMs were found in close proximity to blood vessels. Klemm et al. also showed a significant enrichment of MG and BMDMs in the perivascular niche (20). However, compared with microglia, BMDMs were distributed to a much closer proximity to vascular structures.

2.3 Metabolic Profile of GAMS

The Tumor microenvironment is characterized by low pH, limited oxygen, high nutrient competition and accumulation of metabolites. A lot of difficult conditions cause metabolic changes to respond energy demands of immune and tumoral cells. Myeloid cells are characterized by extensive metabolic plasticity, that allows these cells to rapidly fulfill and adapt to metabolic cues from the surrounding environments. In fact, microenvironment of the tissue can induce profound shift in the preferred metabolic pathways that regulate macrophage plasticity: for example, pro-inflammatory conditions strongly upregulate glycolytic pathways, while anti-inflammatory phenotype preferably relies on OXPHOS and FAO. In gliomas, it has been shown, in human gliomas, that in BMDM there is a decreased glycolytic metabolism compared to microglia and is associated with increased immunosuppression in the TME and poor patient survival (51) (Figure 3) Moreover, the enrichment of oncometabolites in the tumor microenvironment act on GAMs and other immune cells to support tumor growth. For example, the accumulation of lactate produced by tumor cells induces a pro-tumorigenic phenotype of macrophages through a mechanism mediated by HIF1α (60).Whereas, GAMs potentiate the metabolic shift of GBM cells to aerobic glycolysis (61) . The accumulation of D-HG caused by the mutation of IDH1/2 genes supports tumor progression. D-2HG down-regulate anti-tumor- T cell activity by interfering with TCR signaling in a mouse glioma model (62). Therefore, this activity of immune suppression is founded in microglia, in which D-2HG inhibits activation by AMP- activated protein kinase (AMPK)-mediated downregulation of m-TOR and down regulates nuclear factor kb (NF-kb) – induced inflammatory responses (63). Moreover, several studies evaluated that the metabolism of tryptophan induces a state of immunosuppression (64). Friedrich et al., demonstrated that D-2-HG, which increases the activity of TDO (tryptophan-2-3dioxygenase) in myeloid cells, induces the metabolism of L-tryptophan (L-Trp) to the AhR ligand kynurenine (Kyn). Kyn dries the translocation of AhR to the nucleus, where it up regulates the production of IL-10 and decreases expression of the co-stimulatory molecules

CD86 and CD80 (64) . Arginine in healthy conditions promotes a series of metabolic reactions to regulate cell growth and proliferation including the synthesis of nitric oxide, polyamines, glutamine, and proline (65). In glioma there is the inhibition of argininosuccinate synthase (ASS1), so gliomas are unable to synthesize arginine. Indeed, tumor cells are highly dependent on the availability of arginine in the extracellular environment. Therefore, the increased uptake of arginine and high expression of arginase by tumor cells results in an immunosuppressive phenotype (65). However, microglia and BMDMs demonstrate a distinct metabolic profile, even if share the same microenvironment. These metabolic differences emphasize a unique transcriptional and epigenetic changes that may reveal the different functions of various populations of GAMs in GBM.

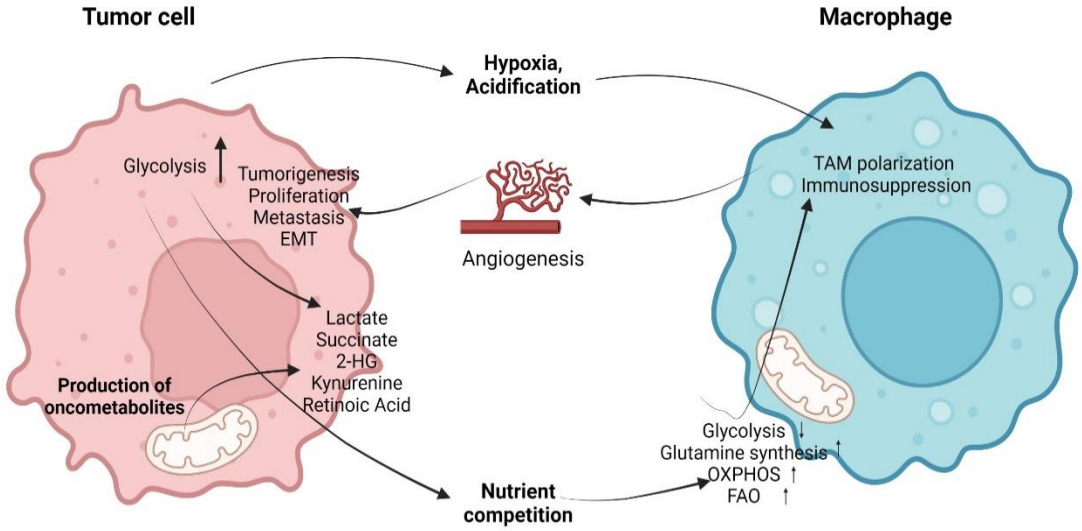


Figure 3: Metabolism and crosstalk between macrophages and tumor cells in the tumor microenvironment (TME). The TME is characterized with hypoxic regions and together with Warburg metabolism, promotes the accumulation of lactate in cancer cells by the upregulation of the glycolysis. Then, lactate is released by tumor cells and lead to the acidification of the Tumor microenvironment and impacts the phenotype of tumor-associated

macrophages . Moreover, mutations in the enzymes involved in the tricarboxylic acid (TCA), as well as changes in metabolism, support the accumulation of oncometabolites such as succinate, 2-hydroxyglutarate (2HG), kynurenine. The accumulation of these oncometabolites induce a pro-tumoral TAM phenotype and function. (66) (Modified from Goede et.al.,Biology,2020).

3.1 Hypoxia in Glioblastoma

Hypoxia is a key feature in solid tumors and describes a condition in which an organism is deprived of adequate oxygenation. The effect of hypoxia in glioblastoma can be observed by magnetic resonance imaging (MRI) where is identified a significant oxygen restriction with reduced or damaged blood flow (67,68). Histological analysis of Glioblastoma describes distinctive pathological features include necrotic foci with pseudopalisades and microvascular hyperplasia, which are suggested to induce growth and invasion in GBM. These pseudopalisades are created by tumor cells migrating away from a central necrotic region and forming an invasive front (69,70) . The hyperplasia is a form of angiogenesis that is induced in response to the up regulation of proangiogenic factors such as vascular endothelial factors (VEGFs), interleukin-8(IL-8) by the cells that forms pseudopalisades. Molecular markers of hypoxia such as VEGF and the transcription factor HIF-1 and tumor vascularity can be identified on dynamic contrast enhanced MRI and correlate with worse survival of patients (71). HIF is a transcription factor that senses low cellular oxygen levels and regulates the expression of genes involved in angiogenesis, glucose metabolism such as glucose transporter1, (GLUT1), VEGF and phosphoglycerate kinase (PGK1)(12). HIF is characterized of a constitutively expressed β subunit and at least three regulated α subunits (HIF1α, HIF2 α and HIF3 α). In hypoxic conditions the α subunit translocate to the nucleus and dimerize with the subunit β and induce the transcription of hypoxia responsive genes (70). In hypoxic conditions the tumor microenvironment of glioblastoma modulate immune effector molecules. For example, in hypoxic conditions glioma cells increase Il-6 and IL-8 (72,73)IL-6 induces upregulation of the programmed death-ligand 1 (PD-L1) on circulating

myeloid cells (74). Moreover, the activation of HIF-1α induce the increased level of lactate which is transported out of the cells by the monocarboxylate transporter (MCT4) (75) . In hypoxic areas, the lactate produced by tumor cells can be transported by MCT1 and used as fuels for the tricarboxylic acid cycle (TCA) by better oxygenated cancer cells (76). The accumulation of lactate lead to acidification of the tumor microenvironment, which can be recognized by macrophages and induce a pro tumoral profile in GAMs (77). Tumor derived lactate is taken up by GAMs through MCT, leading to VEGF and the 1 -arginine metabolizing enzyme arginase-1 (ARG1) genes and down regulate T-cell activation and proliferation (78). Moreover, MCT-mediated H+ efflux improves the extracellular acidification of microenvironment, in which tumor cells can outcompete normal cells and thus induce cancer progression. Tibullo et al., evaluated the potential crosstalk between lactate and Insulin-like Growth Factor Binding Protein 6 (IGFBP6) in microglial cells and demonstrated that lactate modulates microglia in anti-inflammatory response and remodels the environment in glioma through IGFBP6. (79)

4.1 Therapeutic Strategies

Standard of care treatment comprise surgery, radiotherapy and chemotherapy using Temozolamide (TMZ). Immunomodulating therapies have been proven successful in highly immunoreactive tumors. As glioblastoma develop for a long time in the immune privileged site provided by the blood brain barrier, which prevents the negative selection of antigen tumors by immunoediting, it is considered an ideal target for tumor antigen vaccines. Therefore, some clinical trials at early phase have shown limited efficacy, demonstrating at the same time potential and limitations of this approach(80). Temozolomide with its immunomodulatory effects has been used with direct anti-tumor effects (81). Several studies evaluate that lymphoablative doses of Temozolamide were shown to increase tumor antigen-specific immune response in mouse model and in patient with Glioblastoma (82,83). Moreover, in the context of GAMs, TMZ alone or in addiction with radiotherapy shown to inhibit a pro-tumorigenic phenotype in macrophages (84,85). Therefore, recently it has been studied a method to reduce and reprogram GAMs to be more inflammatory and immunogenic (86). For example, the colony stimulating factor (CSF-1), as driver of differentiation, polarization and recruitment of GAMs, has been studied and targeting the

CSF-1 receptor on GAMs. A specific inhibition of CSF-1R has been shown to prolong survival in animal model of GBM (87). Therefore, in pre-clinical model the inhibition of CSF1-R can block the radiotherapy – induced alternative activation in microglia and macrophages and the acquisition of recurrence-specific phenotypes in these cells; but not promising results were observed in a phase II study of a CSF1R inhibitor in patients with recurrent Glioblastoma (88). Recently, Larsen et al showed that the secretion of IL-33 from glioma cells induces the recruitment of monocytes with an M1-like phenotype from circulation and then stimulates the reprogramming of the GAMs to a pro-tumorigenic M2-like phenotype inducing the proliferation of tumor cells (89). They demonstrated that the inhibition of IL-33, using ST2 receptors induce the reduction of immunosuppression in the tumor microenvironment. A deeper investigation of molecular mechanisms supporting the immunosuppressive role of GAMs is a key to guide patient's selection and optimize success rate of immunomodulating therapies.

4.2 Engineered Nanovectors, a promise for GBM treatment

Advances in nanotechnology have led to the development of new therapy options for various and can be used to deliver chemotherapeutic agents specifically to the tumor tissues without causing systemic toxicity (90). However, the Blood Brain Barrier (BBB) represents an obstacle for the drug delivery (90). Recently, several clinical trials that using nano-therapies for GBM treatment have been published (91,92). Nanoparticles are small structures, with a size range between 1 and 100 nm, made by either inorganic, polymeric or lipid-based which can be loaded with drugs (93) to the CNS by penetrating the BBB and increasing the drug half-life. Lipid-base NP are made up of a simple lipid bilayer with an internal aqueous compartment, these NP can hold a large payload of drugs and their chemical versatility makes them ideal candidates for multi-functionalization. Moreover, a specially functionalized NP can stimulate, or alter the innate immune system, by inducing cytokine production, stimulating downregulation mechanisms or immunosuppressing immune cells (94). Most of the chemotherapy drugs are hydrophobic molecules, making difficult their systemic administration(93) Thus, their encapsulation in a nanomaterial would ensure better transport and controlled release to the targeted cells or tissues (95) and would help to cross the BBB and reach GBM by passive or active targeting processes (96) avoiding side effects in healthy tissues.

Aim

This thesis is focused on the tumor-associated macrophages, that are the major players in cancer growth and progression. The aim of this thesis was to understand the mechanisms of the crosstalk between glioma cells and TAMs in glioma microenvironment and to exploit this, different approaches based on in vitro models have been used:

1. We analysed the interplay between macrophages and U87 glioma cells lines, harbouring wild-type or mutant IDH genes, through direct and indirect cocultures. Then we investigated the mechanisms underlying the macrophages-glioma cells' crosstalk in hypoxia, a key aspect of the tumor microenvironment.

2. To test the efficacy of innovative nanovector-based strategies, we set coculture models of macrophages and patient-derived cells (GSCs) to explore the potential of macrophages as target cells of encapsulated drugs for the rebalance of the glioma microenvironment.

Material and Methods

1. Human macrophages isolation and polarization

Peripheral blood monocytes were isolated from healthy donors by different density gradient centrifugations. Lympholyte-H (Cederlane, USA) gradient was used to separate peripheral blood mononuclear cells (PBMCs) from granulocytes and red blood cells.

Then, a Percoll 46% gradient (Lonza, USA) was applied to isolate monocytes from lymphocytes. Isolated monocytes were plated in petri dish in RPMI 1640 medium (Lonza) without FBS for 20 min. Non-adherent cells were discarded, and cells were incubated in RPMI 1640 medium supplemented with 10% FCS overnight. Moreover, the differentiation of monocytes into macrophages was obtained by adding 100 ng/mL of human M-CSF (macrophage stimulating factor; Milteny) for 7 days.

After seven days of differentiation, medium was changed and macrophages were polarized toward pro-inflammatory M1 phenotype stimulating with LPS (E. coli 055:85, 100ng/ml, Sigma) and IFNγ (20ng/ml, R&D) or into alternative M2 phenotype with IL-4 (20ng/ml, Miltenyi) for 24h. Resting macrophage, M0, were unstimulated for the same period of time.

2. Isolation of Classical (CD14+CD16-) and Non Classical Monocytes (CD14-CD16+)

In order to separate Classical Monocytes (CD14+CD16-) cells from a buffycoat, we used the kit StraightFrom Buffycoat CD14 Microbead Kit (Miltenyi) following the data sheet. The magnetically labeled CD14+ cells are retained within the column and the unlabeled cells run through. Whereas, to isolate Non Classical Monocytes (CD16+ CD14-) cells from PBMC we used CD16+ Monocyte Isolation kit (Miltenyi), following the data sheet the magnetically labeled CD16+ monocytes are retained on the column and are eluted after removal of the column from the magnetic field.

3. Cell line cultures and tumor conditioned media preparation

U87 IDH1 WT (ATCC, HTB-14) is a cell line with epithelial morphology that was isolated from malignant gliomas. IDH1 mutant-U87 Isogenic Cell Line (ATCC, HTB-14IG) is a glioma IDH1R132H mutant isogenic line derived from the parental U87MG (ATCC HTB-14) cell line. Both glioma cell lines were cultivated in DMEM high-glucose (LONZA) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Penicillin/Streptomicyn (P/S).

To prepare TCM, the cell lines were plated in flasks until the 90% of confluence. Then, media were discarded,the flasks were rinsed two times with saline solutions and were added for 24h. The conditioned media was collected, filtered at 0.20 um and stored at -20°C .(97)

4. Stimulation of monocytes with Tumor Conditioned Medium

Isolated Monocytes were seeded in 6-well containing complete RPMI medium (supplemented with 10%FBS and 1% penicillin-streptomycin (PS)) plus 25 ng/mL of H-MCSF or 30% of the tumor conditioned medium fromU87 IDH1 WT or U87 IDH1 MUT. Cells were grown for 7 days in the incubator at 37°C and 5% CO_2.

5. Flow Cytometry staining and analysis of monocytes-derived macrophages

The phenotypic characterization of macrophages was performed using anti-human APC /CD14 (BD), PECY7/CD16 (BD), BV421/CD206 (BD), BV510/ HLADR (BD) following data sheets. All analysis were performed with BD LSR Fortessa and were carried out with FACS Diva software.

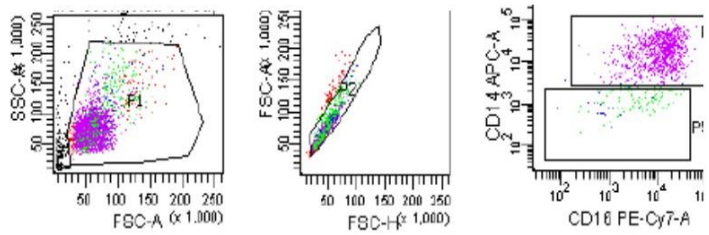


Figure 4. Gating strategy applied to monocytes-derived macrophages treated with Tumor conditioned media (TCM). Macrophages were identified through physical

parameters (FSC-A/SSC-A), the exclusion of doublets (FSC-A/FSC-H) and finally grouped into two macrophage populations: CD14+CD16+; CD14-CD16-.

6. Induction of hypoxia

Cells that were cultivated in normoxic condition were maintained at 37°C in humidified incubator at 20% O2, 5% CO2 in air; Hypoxic conditions were produced placing cells for 4 hours in theincubator Thermo Fisher Heto at 37°C with a mixture of 1% O2, 5% CO2 and 94% nitrogen. Cells coltivated in normoxic condition were maintained at 37°C in the normal incubator at 20% O2 and 5% CO2 in air.
Different treatments were performed simultaneously for the same time.

7. U87-macrophages coculture and FACS-sorting

For co-culture experiment, glioma cell lines (U87 IDH1 WT, IDH1 MUT) were plated in 6 well plates, then after 16h differentiated macrophages were added to tumor cells' wells. The cell ratio applied was 1:3 of U87 vs macrophage. After 24h of co-culture in normoxic conditions, co-culture were maintained in hypoxic incubator for 4 hours. Then, the cells were detached and single cell suspension is prepared for FACS-sorting. Fixable Viability Stain 700 (BD Horizon) was used to exclude dead cells. Next, cells were stained with anti-human CD45 FITC (BD) in order to separate macrophages (CD45+) from U87 cells (CD45-). FACS-sorting was performed on a FACS Melody cell sorter (BD Bioscience) using FACS Diva software.

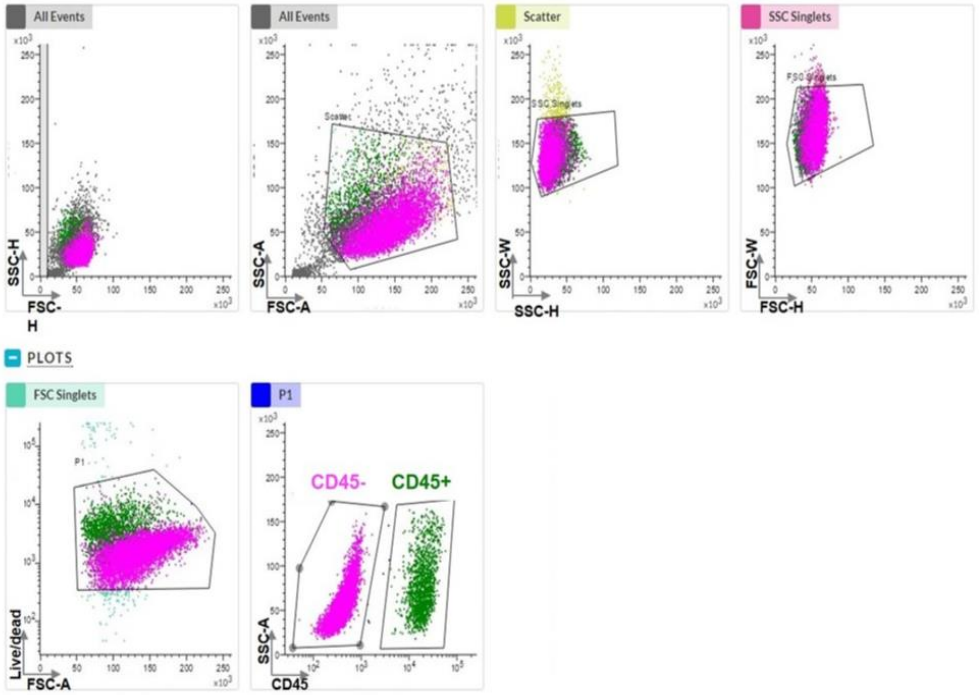


Figure 5. Gating strategy applied to macrophages and U87 cells FACS-sorting. U87-macrophages co-cultured cells were stained to identify live cells and to discriminate macrophages from U87 cells using anti-CD45 staining. Gating strategy identifies cells through physical parameters (FSC-H/SSC-H), excludes doublets (SSC-W/SSC-H and FSC-W/FSC-H), and viability staining (FSC-A vs Live/Dead). Last dot-plot discriminate CD45- cells (U87 cells) and CD45+ cells (macrophages).

8. RNA isolation and qRT-PCR

Cells were lysed with Quiazol reagent (Quiagen) and RNA was extracted using DirectZOL RNA Miniprep kit (ZymoResearch) according to the manufacturer's instruction. Total mRNA amount was quantified by NanoDrop 2000c (Thermo ScientificTM) and retro-transcribed into cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using Sybr Green Fast Advanced Master Mix (Applied Biosystems) and the sequences of primer pairs specific for each gene were designed with Primer3 (https://primer3.ut.ee/) and are reported in the table below:

GENE	FORWARD	REVERSE
VEGFA	TCGTTTAGGATGGGGCACTT	ATGTCAGGAGAATGGGCACA
ANGPTL4	GCCCTGCTGATCACTGATTG	AACACTACTACCTGGCACCC
HMOX	ATTCTCTTGGCTGGCTTCCT	CCCCTCTGAAGTTTAGGCCA
GLUT1	CTAGTCTGTGCCCTTGACCA	TAACTGAAGGGCTTGAGGGG
RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCT TC
CD206	GGGCAGTGAAAGCTTATGGA	CCTGTCAGGTATGTTTGCTCA
CD163	GAGCAGCACATGGGAGATTG	GTGGAAAACAGTGTGGCCAT

Table 1. List of primers for Sybr Green PCR

Reactions were performed on a VIIA-7 Real-Time PCR Detection System (applied Biosystems). The thermal cycling conditions were standard fast-cycling; relative expression values were calculated using ΔΔCT method normalized on RPLP0 (Ribosomal Protein Lateral Stalk Subunit P0) as housekeeping.

9. MTT Assay

In order to study the vitality of macrophages after drugs treatments, we performed the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. Cell viability studies were carried out on human macrophages M0 and polarized M1 (IFN γ 20 ng/mL plus 100ng/mL LPS) and M2 (IL-4 20ng/ml). The seeding density of macrophages was set before drugs treatments experiments. Optical readings of the cell-reduced MTT products were measured at 570 nm.

10. Co-culture of Glioma Stem Cells (GSC) and Macrophages

GSC were obtained in collaboration with Dr. Passoni e Dr. Matteoli from GBM patients after surgical removal of the tumor. GSC were cultured in Neurocult medium supplemented with heparin, primocin, fibroblast growth factor (FGF) and Epidermal Growth Factor (EGF). Then, GSC were added to 7 days differentiated macrophages at the ratio 1:3 in RPMI wihout serum and treated for 72 hours with the drugs: Trametinib 30nM (Apex-Bio) and Rapalink 10nM (Apex Bio).

After 72 hours, cells were stained with anti-human CD45 FITC (BD) in order to distinguish macrophages (CD45+) from GSC cells (CD45-) and the Annexin V APC/7-amino-actinomycin kit (BD) to discriminate apoptotic cells. Labeled annexin V stained phosphatidylserines (PS) residues on the plasma membranes of apoptotic cells. Late apoptotic cells, such asnecrotic cells, lose their cell membrane integrity and are permeable to the DNA intercalator 7-AAD (). Early apoptotic cells are Annexin V+/7-AAD-; late apoptotic cells are Annexin V+/7-AAD+.

11. Cytokines quantification by ELLA

ELLA (ProteinSimple, Bio-techne, Minneapolis, MN, USA) is an automated enzyme linked immunoassays based on a cartridge-based system with a microfluidic technology, it produces highly reproducible, rapid, and sensitive quantitation of analytes from small sample volumes.

Based on the manufacturer's instructions, the dilution of samples and the loading of diluted samples and the washing buffer into the cartridge need the operator handling. This technology avoids many limitations of current plate-based ELISAs such as poor sensitivity and lack of reproducibility.

The calibration curve for each cartridge is generated by the manufacturer and the ELLA system acquires calibration-related parameters through the reading of the cartridge's barcode. The fluorescent signals are read inside the ELLA instrument and used for quantification based on master calibration curves.

The cartridge is customized with a specific panel of antibodies, every cartridge has a specific barcode and could be run into the Simple Plex Runner GUI. The Ella platform requires up to 25 µL of sample, the dilution of samples is 1:2 and samples were automatically subdivided by the cartridge's microfluidic in three reservoirs called GNRs, eliminating the need to load technical replicates.

We used the supernatant of macrophages treated with conditioned media of U87 cells, of direct coculture of macrophages with U87 cells or GSC cells.

The customized cartridge could contain up to 16 samples for 4 analytes (**Figure3**): plate 1 was set to detect VEGF, IL-6,IL-1β, and CCL2; plate 2 for IL-10, EGF, B7-H1; plate 3 for Periostin (POSTN), Osteopontin (OSF2), Macrophage Migration Inhibitory Factor (MIF) and MMP-9 ; plate 4 detect TGF-β and this cartridge could contain up to 72 samples.

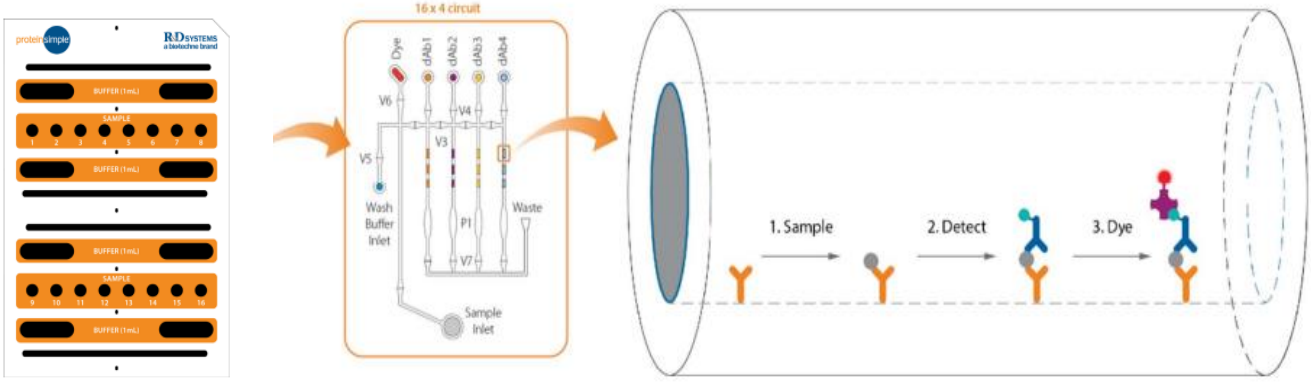


Figure 6: 16x4 Simple Plex Cartridge for 16 samples and 4 analytes. Each of the 16 samples is analyzed in an independent microfluidic circuit, and reagent delivery within each circuit is precisely controlled in automated mode by pneumatically actuated valves (V1–V7) and pistons (P1). In each circuit, the sample is split into four analyte-specific microfluidic channels arranged in parallel, and each channel has a dedicated well containing the detect antibody (dAb1–dAb4) corresponding to the triplicate glass nanoreactors (GNRs) in that

channel (color coded for the four analytes)(98) (Modified from Dysinger et al., Journal of immunological method, 2017).

At the end of the assay, the channels are scanned and the resulting data is processed to produce relative fluorescence units (RFU) and concentration values for each located GNR. The analysis of data is characterized with resulting set of GNRs that are used to produce a channel mean RFU and mean concentration.

12. Statistical analysis

Statistical analysis was performed using Prism version 7.03 (GraphPad software). Comparisons were calculated by One-way ANOVA test applying Tukey's multiple comparisons correction. The level of statistically significant difference was defined as $p \leq 0.05$.

Results

1: Reproducing a glioma's microenvironment in vitro, primary macrophages polarize toward a pro-tumoral M2-like phenotype in presence of glioma cells.

1.1 Culture of primary monocytes with Tumor Conditioned media (TCM) from U87 glioma cell lines leads to an increased expression of differentiation and M2 polarization markers.

Glioma microenvironment is constituted mainly by myeloid cells that support tumor growth, in turn tumor cells produced many factors to modulate immune cell activities. In order to define the impact of glioma derived soluble factors on primary human macrophages activation, tumor conditioned media was collected from two glioma cell lines that differ for the mutation of the IDH gene, U87 IDH WT and U87 IDH MUT, mimicking the aggressive glioblastoma with IDH wild-type and other diffuse gliomas IDH mutated with a favorable prognosis in clinic. Isolated human monocytes from healthy donor buffycoat were cultured in the presence or absence of conditioned media for 7 days or 24 hours after their complete differentiation toward macrophages. CD14 and CD16 markers expression were analyzed by FACS analysis on monocytes-derived macrophages to discriminate fully differentiated macrophages (CD14+/CD16+) from undifferentiated macrophages (CD14+/CD16- and CD14-/CD16-). Our results show that the treatment with conditioned media increased the percentage of CD14+CD16+ macrophages compared to macrophages treated only with MCSF (**Figure 7A**). Then we evaluated the polarization markers HLA-DR and CD206. Our results show that the treatment with conditioned media increased the mean fluorescence intensity (MFI) of CD206 compared to macrophages treated only with MCSF (**Figure 7B**). No differences were observed for HLA-DR expression. Moreover, TCM collected from U87 IDH1-MUT was significantly more potent as compared with TCM from U87 IDH1-WT in terms of fully differentiated macrophages' percentage CD14+/CD16+ and in terms of the polarization marker CD206 expression. Then, we performed by qRT-PCR a gene expression analysis of CD206 and CD163in macrophages treated with TCM from U87 cells (**Figure 7.C**). We found that there was an higher expression of CD206 in macrophages treated with TCM of U87 IDH MUT cells compared to TCM from U87 IDH WT treated macrophages, both at 7 days and at 7 days plus 24 hours treatment after differentiation. Moreover, the expression of CD163 increases in macrophages treated with the conditioned media from

U87 IDH MUT cells compared to media from U87 IDH WT cells, both at 7 days and at 7 days plus 24 hours treatment after differentiation.

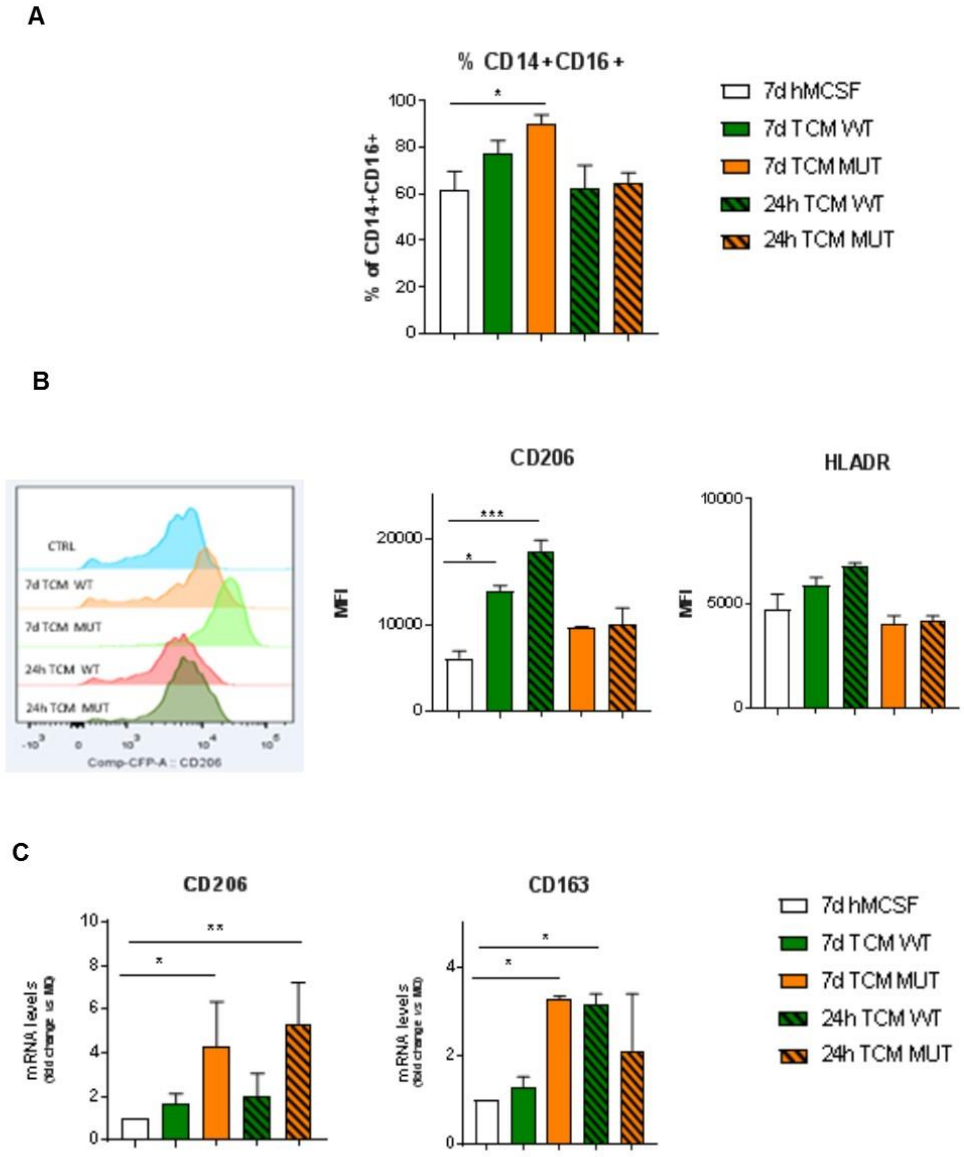


Figure 7: U87 (IDH WT/ IDH MUT) conditioned media induces M2 polarization of primary human macrophages. (A) Percentages of CD14+CD16+ macrophages calculated on total macrophages, in indicated sample conditions. **(B**) Representative histograms and quantification of CD206 expression in CD14+/CD16+ macrophages by FACS analysis. **(C)** Quantification of representative M2 markers (CD206, CD163) by qRT-PCR in macrophages. Data are expressed as fold change on untreated macrophages. In all graphs: Two-way ANOVA were applied followed by Tukey's multiple comparisons test. *P<0.05; **P < 0.01; ****P < .0001 (n=3)

1.2 Direct co-culture of primary macrophages with U87 glioma cell lines leads to an increased expression of M2 polarization markers

Tumor infiltrating macrophages play a critical role in tumor growth. Therefore, understanding cell comunication between myeloid cells and tumor cells is fundamental for tumor outcome. (99) For this reason, we performed a direct co-culture between monocyte-derived macrophages and U87 cells, IDH WT and IDH MUT. To study macrophages activation, after coculture macrophages (CD45+ cells) and U87 cells ($CD45^-$ cells) have been isolated by FACS sorting and we performed a gene expression profile of macrophages focusing on the M2-like polarization markers CD206 and CD163. In **Figure 8** we show that there was a significant increase in the expression of CD206 gene in macrophages that were in coculture with U87 cells,both IDH WT and IDH MUT, compared to macrophages alone. Moreover, there was a tendency of the CD163 gene to increase in macrophages in coculture with U87 cells (IDH WT/MUT) compared to macrophages alone.

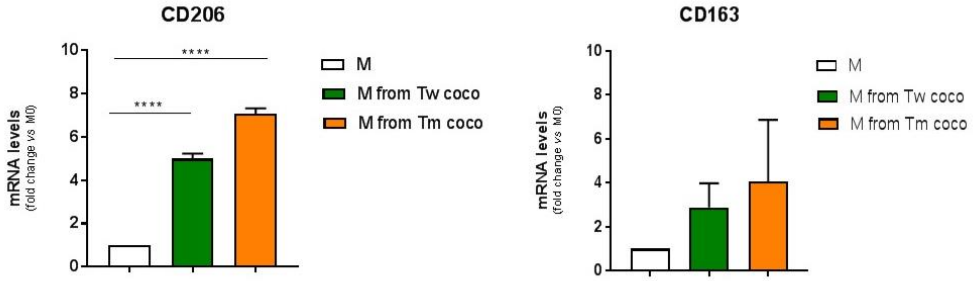
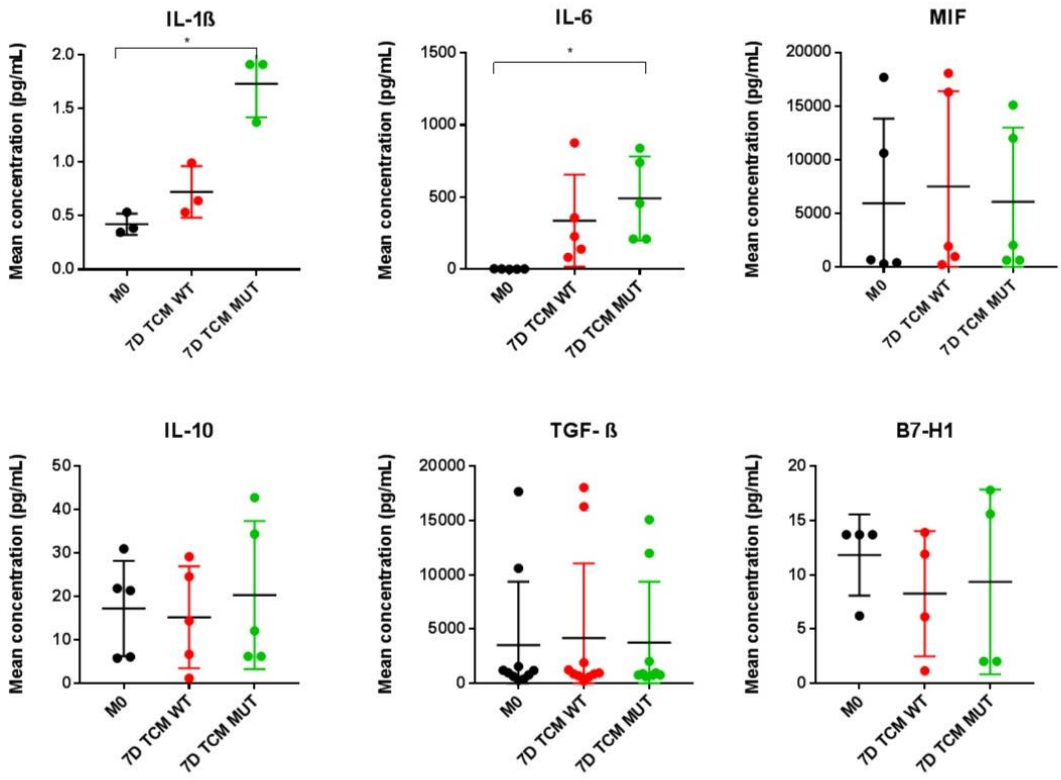


Figure 8: Quantification of representative M2 markers (CD206, CD163) by qRT-PCR in macrophages-U87cocultures. Data are expressed as fold change on not-treated macrophages. Two-way ANOVA were applied followed by Tukey's multiple comparisons test. (*P<0.05; **P < 0.01; ****P < .0001 (n=3)

1.3 Definition of the cytokines content in the glioma TME.

Tumor cells ad GAMs are able to secrete anti-inflammatory factors constituting an immunosuppressive microenvironment. These conditions support the suppression of NK cells and T cells activities, the polarization of macrophages in a M2-like phenotype and the inhibition of dendritic cell maturation. In order to study those factors produced by glioma cells and by macrophages, we assessed protein quantification of cultures' supernatants by ELLA, a fully automated cartridge-based system that measure biomarkers in multiple sample types. We show that the mean concentration of IL-6 and IL-1β \increased in macrophages treated with conditioned media of U87 IDH MUT cells for 7 days compared to not-treated macrophages (**Figure 9A**). However, levels of (Macrophage Migration Inhibitory Factor) MIF, IL-10, Transforming Growth Factor-B (TGFβ) and B7-H1 were not different between groups treated with TCM (**Figure 9A**). In the direct cocultures (**Figure 9B**), only U87 IDH1 WT cells affected IL-6 secretion by macrophages, while MIF, B7-H1 and IL-10 levels display a tendency to increase in coculture of macrophages with U87 IDH WT/MUT cells compared to macrophages alone.

A


IL-1ß
IL-6
MIF
IL-10
TGF- ß
B7-H1
Mean concentration (pg/mL)
M0
7D TCM WT
7D TCM MUT
*

B

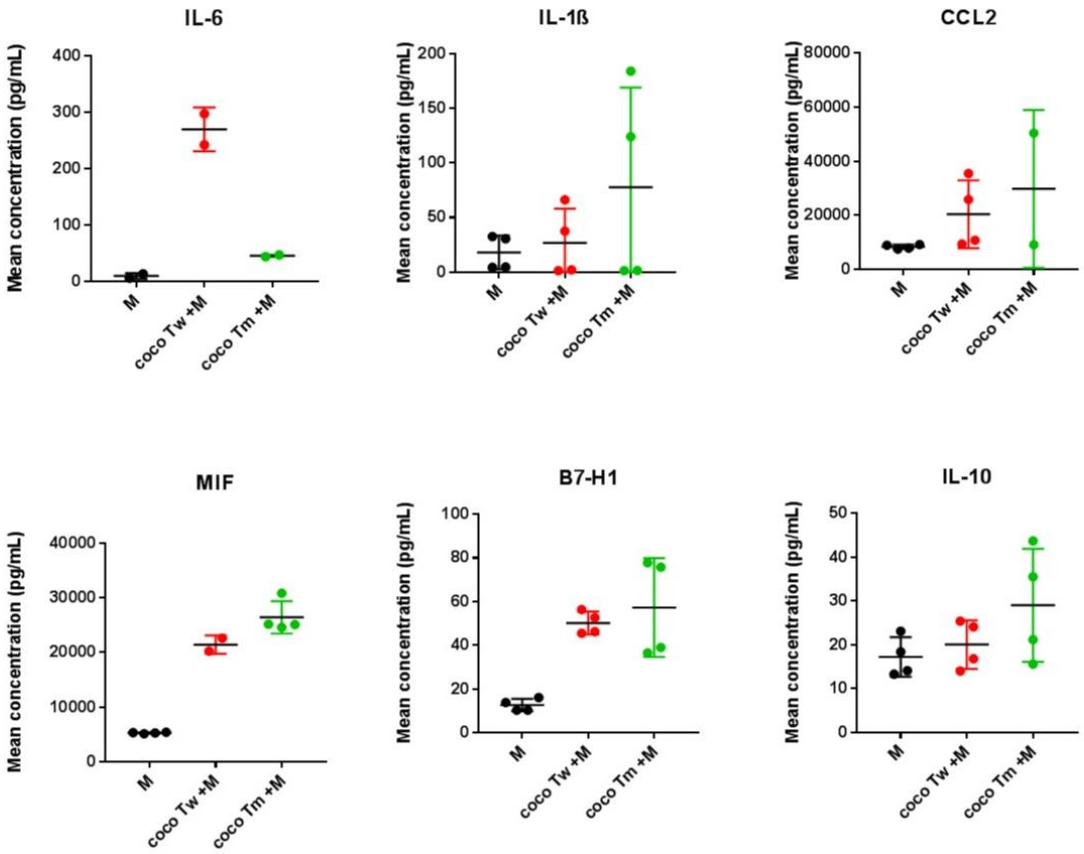


Figure 9: **Cytokines have been produced by U87 cells and macrophages in culture.** (A) Quantification of cytokines produced by macrophages treated with U87 (IDH WT/IDH MUT) TCM (A) and in coculture system by indicated cells (B). One-way ANOVA followed by Tukey's multiple comparisons test. *P<0.05; **P < 0.01; ****P < .0001

1.4. Definition of the cytokines content in the coculture of GSC cells and macrophages

To validate previous results obtained by glioma U87 cell lines and macrophages, we implemented our in vitro TME model with primary glioma stem cells. GSCs represent a subpopulation of GBM cells characterized by increased resistance to chemotherapy and radiotherapy, that usually follow surgical removal of tumor mass. Residual resistant GSCs lead to GBM recurrence and progression[17] and are therefore the preferential target for brain cancer therapeutics. We set coculture of monocytes-derived macrophages and GSCs, then

we analyzed supernatant using ELLA in search of soluble factors that contribute to the functional activities of myeloid cells **(Figure 10)**. Our results showed that CCL2 levels increased in the coculture of macrophages with GSC cells compared to macrophages alone. Moreover, there was a significant increase of the level of MIF, OPN, EGF and VEGF in single cultures of tumor cells and in macrophages-GSC cells' cocultures compared to macrophage alone, probably indicating that these cytokines were produced by GSCs. The levels of IL-1β were higher in macrophages alone and in coculture with GSC cells compared to single culture of GSCs, these data probably argued that IL-1β produced by macrophages was not influenced by the presence of tumor cells.

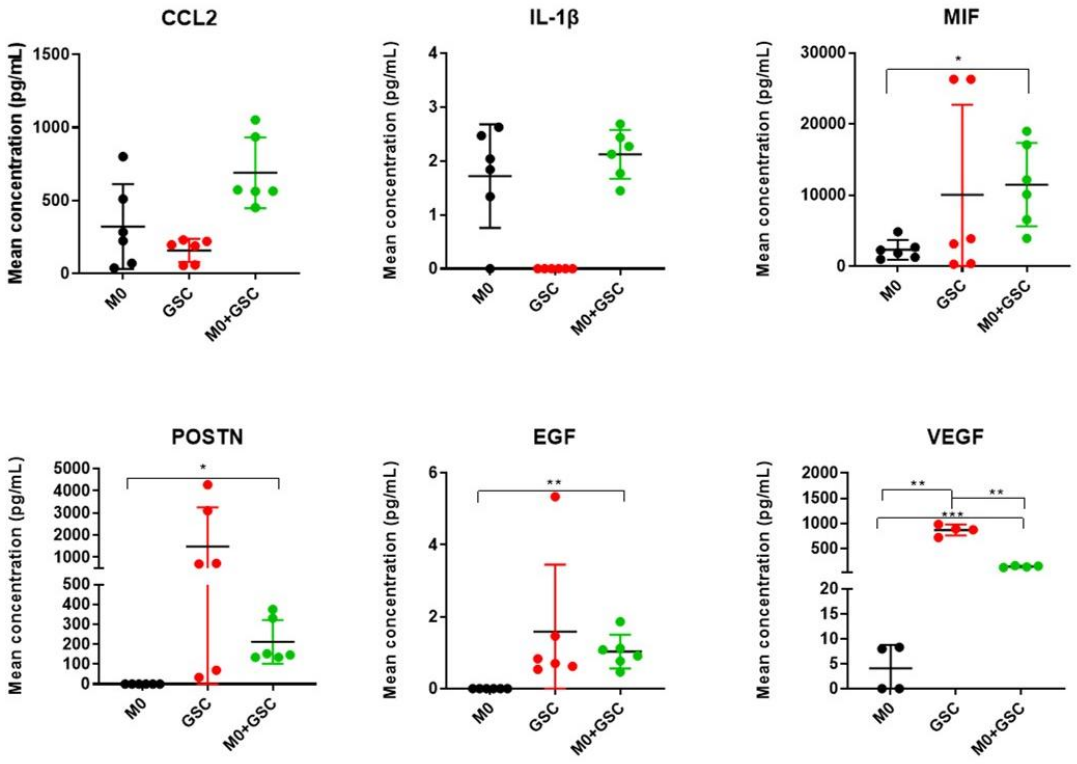


Figure 10: **Cytokines have been produced by GSCs cells and macrophages.** (A) Quantification of cytokines produced by macrophages and GSCs alone and in coculture (n=3). One-way ANOVA followed by Tukey's multiple comparisons test. *P<0.05; **P <0.01; ****P <0.0001

2 Effects of tumor oxygen levels on the interaction between macrophages and glioma cells

2.1 U87 IDH1 WT and IDH MUT cells respond differently to hypoxia

Hypoxia is a feature of TME which induces microvascular proliferation and the consequent tumor growth and could shape specific macrophage phenotypes. The tumor mass regions reflect different O_2 tension: the edge displays normoxic levels of O_2, while tumor core is hypoxic. Hypoxia induces cellular stress and changes in the metabolic profile of cells, in tumor cells hypoxia induces the activation of the hypoxia-inducible facor 1 α (HIF1α) pathway, which in turn upregulates the levels of the metabolite lactate. In the hypoxic regions of the tumor, the accumulation of lactate can be mediated by monocarboxylate transporter (MCT1) and used as fuel for tricarboxylic acid cycle (TCA) to restore oxygen levels. The metabolic profile of glioma cells has been demonstrated to be also affected by IDH mutations (100). Primary GBM IDH wild-type usually displays an aggressive behaviour compare to gliomas with the mutation of IDH (101). Mutations in the IDH enzymes disrupt the functionality of the proteins, causing the production of D-2-hydroxyglutarate, a competitive inhibitor of α-ketoglutarate, which in turn increase HIF levels (102). In order to evaluate the effects of oxygen tension on tumor cells, we applied 20% and 1% of O2 to U87 IDH WT and IDH MUT cells, and we further analyzed the expression of selected responsive genes: Glucose Transporter 1 (GLUT-1), Vascular Endothelial Growth Factor A VEGFA (), Angiopoietin like 4 (ANGPTL4) and heme oxygenase 1 gene (HMOX), that are known to be affected by oxygen deprivation. In fact, we observed a significant difference between U87 IDH WT and IDH mutant, the expression of these genes increased after 4 hours in glioma IDH WT cells under hypoxia as compared to U87 IDH mut (**Figure 11**).

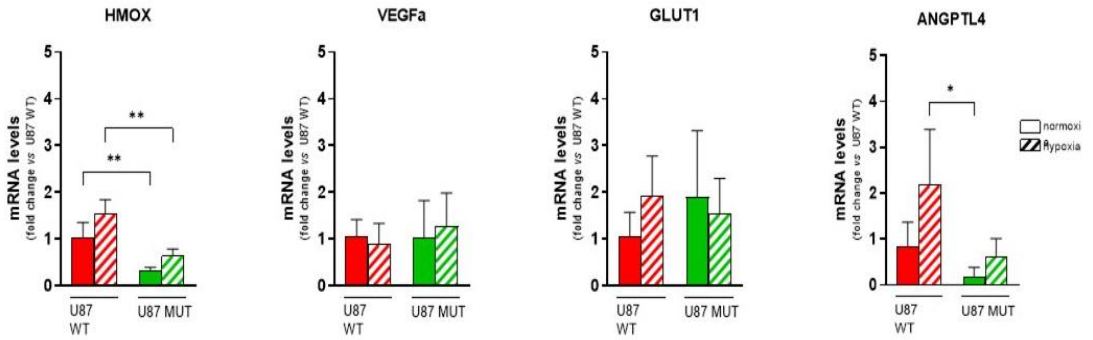


Figure 11: Hypoxia induces a specific genes expression in U87 cell lines.
Quantification of hypoxia-target genes by qRT-PCR in U87 cell lines (IDH WT/IDH MUT) under normoxic (full bars) or hypoxic (crossed bars) conditions (4 hours of treatment). One-way ANOVA, followed by Turkey's test; **P < .01, ***P < .001, ****P<.0001; *pvalue between normoxia and hypoxia.

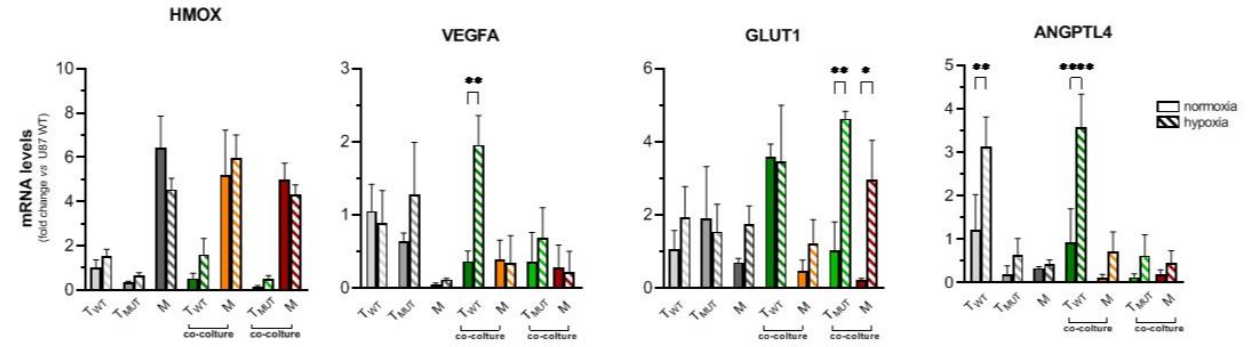
2.2 Macrophages shape U87 (IDH1 WT/MUT) cells' responses to hypoxia

In order to study the interaction between glioma cells and myeloid infiltrating cells under hypoxic conditions, direct cocultures of IDH1-WT or mutated U87 cells with monocyte-derived macrophages from healthy donors' buffy coats were performed applying different 0_2 tensions. We evaluated the gene expression profiles of FACS-sorted macrophages ($CD45^+$ cells) and U87 cells ($CD45^-$ cells). Hypoxia target genes, including GLUT-1, VEGFA, HMOX and ANGPTL4, were monitored in both cell types, tumor cells and macrophages. Interestingly, VEGFA is inducing in tumor cells but less in macrophages, whereas HMOX is induce in all the cell populations considered (**Figure 12A**). Finally, the addition of macrophages to tumor cells upregulates expression levels of HMOX, VEGF, GLUT1 and ANGPTL4 in tumor cells compared to single culture, demonstrating that macrophages shape tumor cells responses to hypoxia. At the other side, in accordance with the literature, macrophages in presence of U87 upregulated VEGFA and HMOX in hypoxic conditions compared to normal 0_2 tension. Infact it has been demonstrated that in hypoxic regions the activation of HIF1α in tumor cells lead to the release of high amount of lactate, inducing the

acidification of the microenvironment. This condition induces a pro tumoral profile of macrophages (103) stimulating the expression of VEGF and angiogenic functions to support vascularization and tumor growth.

Then we assessed the effect of hypoxia in coculture system of glioma cells with specific myeloid subsets: differentiated macrophages, classical and non-classical monocytes. Classical (CD14+ CD16-) and Non-Classical monocytes (CD16+CD14-) were isolated from healthy donors' buffycoat using specific kit of separation. The results show that there was a heterogenic modulation of hypoxic responsive genes between the three myeloid populations (**Figure 12B**). In the classical monocytes (CD14+CD16-) the expression of GLUT 1 was higher compared to macrophages in normoxic and hypoxic conditions. Also, the expression of VEGFA increase in hypoxic condition in classical monocytes. Whereas, no differences were observed for the expression of ANGPTL4 in hypoxic and normoxic condition in classical monocytes. Non-classical CD16+CD14- monocytes showed a higher expression of GLUT1 and ANGPTL4 compared to macrophages. Moreover, the expression of VEGFA increased in non-classical monocytes in hypoxic condition. Our results show that the gene expression of GLUT1, VEGFA and ANGPTL4 was higher in monocytes (classical and non-classical) compared to macrophages. No differences were observed for HMOX expression in normoxic and hypoxic conditions in the three myeloid populations.

A


HMOX
VEGFA
GLUT1
ANGPTL4
mRNA levels
(fold change vs U87 WT)
normoxia
hypoxia
co-colture

B

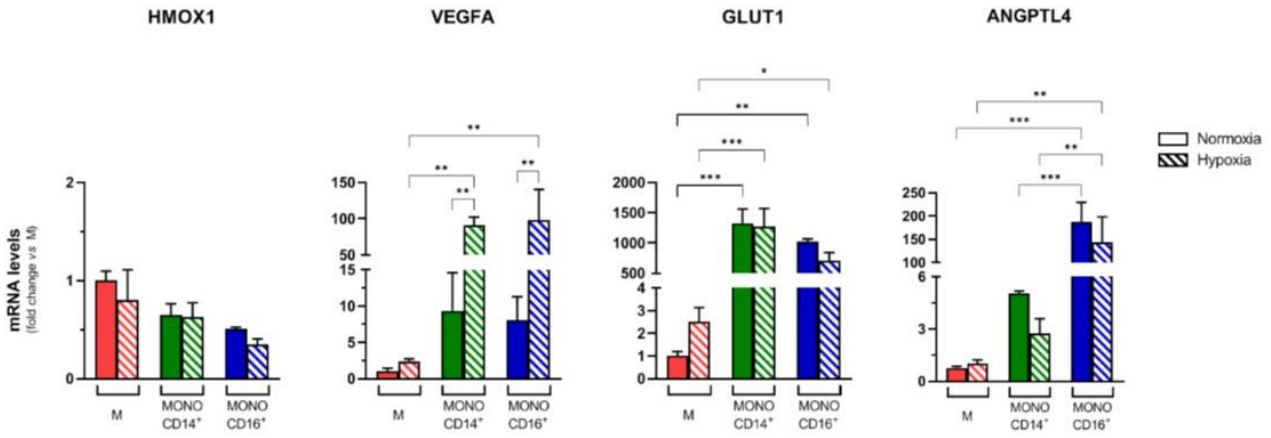

HMOX1
VEGFA
GLUT1
ANGPTL4
mRNA levels
(fold change vs M)
Normoxia
Hypoxia
M
MONO CD14+
MONO CD16+

Figure 12: Hypoxia induces its target genes in a cell-specific manner.
Quantification of hypoxia-target genes by qRT-PCR in macrophages-U87 cocultures **(A**) and in macrophages and monocytes single cultures (**B**) under normoxic (full bars) or hypoxic (crossed bars) conditions (4 h). One-way ANOVA, followed by Tukey's test; **P < .01, ***P < .001, ****P<.0001 vs U87 WT.

3: The role of Tumor Associated Macrophages (TAM) in response to drugs to control neuroinflammation in gliomas

3.1: Pimasertib and Trametinib affect the vitality of macrophages.

Trametinib and Pimasertib are small molecule inhibitors of MEK 1/2, signal trasducers of the MAPK pathway, which have been demonstrated to cause regression of pediatric low-grade gliomas in vitro and in vivo. [111].The MAPK pathway is hyper-activated in 85% of GBM and has been demonstrated to support proliferation and survival of glioma tumor cells. These two highly selective allosteric inhibitors of Mek1/2, Pimasertib and Trametinib, are currently tested in clinical trials for different solid tumors. Macrophages were polarized toward M1 or M2 phenotype for 24 hours and then were treated with Pimasertib or Trametinib at increasing concentrations. After 72 h, cell vitality was assessed using MTT assay. Our results show that the vitality of macrophages decreased by increasing the concentration of drugs and revealed a higher effect of Trametinib on macrophage vitality compared to Pimasertib, independently from the macrophage polarization status (**Figure 13**). We tested also a selective inhibitor of the mechanistic target of rapamycin (mTOR), Rapalink-1. The PI3K/Akt/mTOR pathway is involved in the maintenance of the subpopulations of Glioma Stem-Cells (GSCs)[108] because it is necessary to ensure the physiological processes of proliferation, metabolism, survival, differentiation and autophagy. As for MEK inhibitors, we polarized macrophages to obtain M1 or M2 phenotype and 24 hours after polarization cells were treated with Rapalink at increasing concentrations. After 72 h, cell vitality was assessed using MTT assay. Our results show that the vitality of macrophages decreased in presence of Rapalink (**Figure 13**).

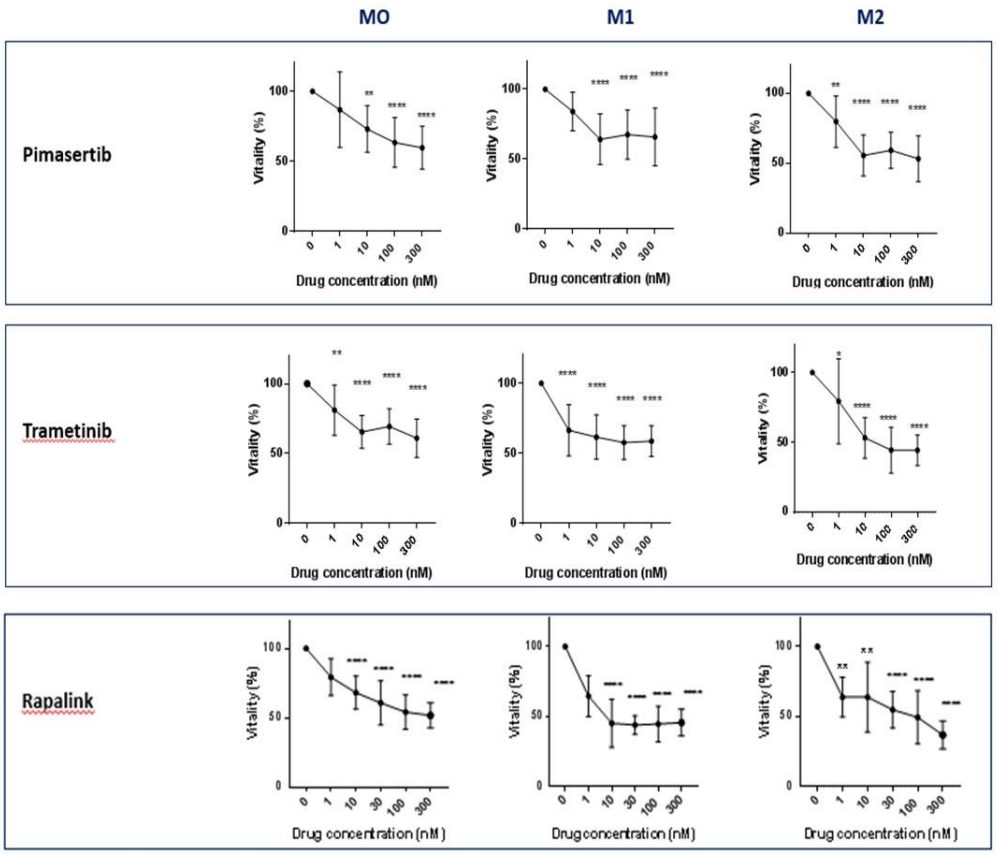


Figure 13: **Effect of MAPK and mTOR inhibitors' drugs on macrophage vitality.**
Macrophages (M0, M1, M2) were treated for 72 hours with the indicated drugs and the vitality of macrophages was evaluated with MTT assay measuring the absorbance at 450 and 630 nm.
One-way ANOVA, followed by Tukey's test; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs controls (n=3).

3.2 The vitality of GBM Stem cells (GSC) and macrophages were reduced after the treatment of Trametinib and Rapalink.

We then tested the drugs Trametinib and Rapalink in the direct cocultures of differentiated macrophages and patients-derived Glioma Stem cells. We analysed early and late apoptotic cells by FACS analysis using Annexin V/7AAD staining on the two populations, GSCs were identified as CD45- and macrophages as CD45+, and quantifying the absolute cell count in every wells we calculate the number of viable cells. The concentration of the drugs that we used was 30 nM for Trametinib and 5 nM for Rapalink that correspond to the previously calculated LOG EC50 for macrophages and tumor cells. In our results we obtained that the vitality of macrophages and GSCs decrease significantly after the treatment with drugs Trametinib and Rapalink in single cultures. In cocultures conditions the macrophages are less sensitive to the drugs compare to macrophages alone. However, GSCs in coculture with macrophages showed a decrease of vitality after the treatment with Trametinib and Rapalink (**Figure 14**).

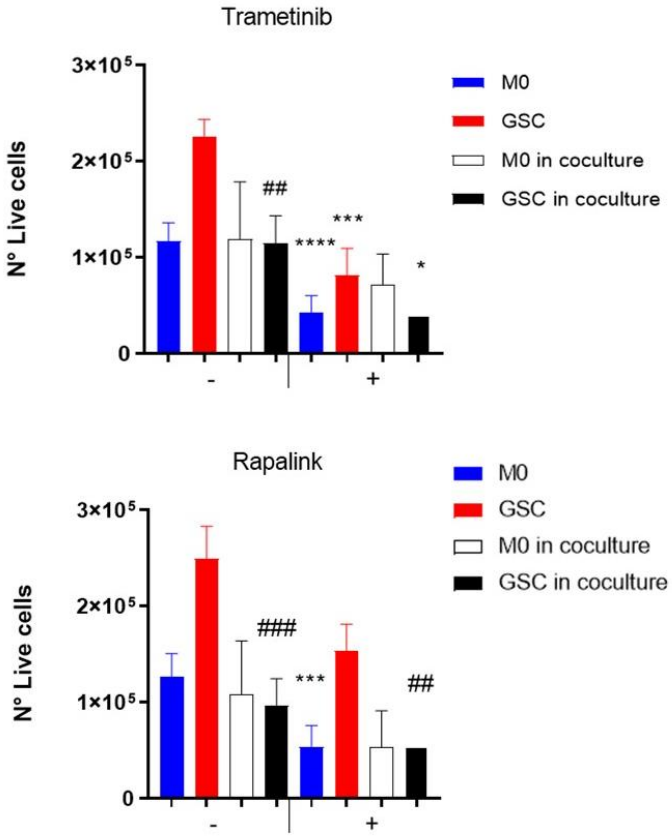


Figure 14: **Effect of MAPK and mTOR inhibitors on coculture of macrophage and Glioma stem cells (GSCs) vitality.**

The vitality of macrophages and GSC cells after indicated drugs treatments (- no treatment; + with treatment) in single and coculture was evaluated by FACS analysis using Annexin V/7AAD staining. Histograms represent the absolute numbers of live cells manually counted, then subtracted from the percentage of apoptotic cells. One-way ANOVA, followed by Turkey's test; *pvalue (treated vs not treated); #pvalue (single vs coculture)*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (n=3)

Discussion

The rationale of this thesis is related to the strong interest of my laboratory in understanding the macrophage biology in the tumor context. Macrophages are highly plastic cells undergoing to specific functional states in response to microenvironment signals. Macrophages infiltrating tumors are called tumor-associated macrophages or TAMs, this nomenclature reflects their key role in the tumor biology. In fact, TAMs are the major infiltrating immune cell populations and drive tumor progression supporting locally tumor growth, neovascularization, metastasis process and resistance to therapeutic agents (104,105). We focused this study on the human diffuse gliomas, the most aggressive brain tumors, which have been classified based on genetic alterations in enzymes isocitrate dehydrogenases (IDH): gliomas IDH WT and gliomas IDH mutated. IDH WT tumors are in general primary glioblastoma (GBM), counting 90% of gliomas and predominating in adults over 55 years of age; whereas tumor IDH mut are frequent in younger patients and are characterized by more favorable prognosis. In order to clarify the functional implication of microenvironmental conditions on the interplay between tumor cells and macrophages, we explored the impact of soluble factors released by tumor cells in conditioned medium on the modulation of macrophage's phenotype in culture. As surrogate of glioma we used the commercially available human GBM U87 cell lines, stably expressing mutational (R132H) or wild type IDH, generated by the CRISPR/Cas9 gene editing technology. Moreover, we isolated monocytes from fresh healthy donors buffycoats and differentiated them to macrophages. We showed that tumor factors from both U87 IDH WT and IDH MUT cells' cultures robustly induce the differentiation of monocytes in macrophages CD14+CD16+ cells compared to monocytes treated with the human macrophage growth factor MCSF. In addition, TCMs from both U87 IDH WT and IDH MUT cells are able to polarize macrophages toward a M2-like phenotype expressing the marker CD206. Interestingly, we evaluated that the effect on macrophage differentiation and activation was significantly increased in presence of the TCM collected from U87 IDH1 MUT compared to U87 IDH1 WT supernatants. Then, a direct coculture model using U87 cells and macrophages have been established and we confirmed that the expression of CD206 gene increased in macrophages that were in contact with U87 cells compared to untreated macrophages. Then, we investigated the cytokines content in our glioma TME models demonstrating an increase of IL-6 and IL-1β in macrophages treated with TCM of U87 IDH MUT compared to untreated macrophages. Moreover, also in direct coculture of U87 cells with macrophages the level of

IL-6 increased in the supernatants compared to macrophages alone. However, we didn't find statistically differences in other pro-tumoral molecules, such as MIF, B7-H1 and IL10. Furthermore, in another experimental setting we used glioma stem cells (GSCs) to validate previous results obtained by glioma U87 cell lines in primary cells derived from GBM. It has been well established the role of the GSCs in supporting tumor growth and in driving resistance to therapies. Moreover, within GBM niches, GSC cells have been found in contact with myeloid cells, implying a link between these two cell populations (106). In the cocultures experiments, we observed that MIF, OPN, EGF and VEGFA significantly increased in the supernatant of GSCs and of macrophages-GSCs cultures compared to macrophages alone, probably indicating that these cytokines were predominantly produced by GSCs. Moreover, we showed the induction of CCL2 in the coculture of macrophages with glioma stem cells compared to macrophages alone and not present in single cultures of tumor cells, demonstrating that the upregulation of CCL2 in macrophages was induced by GSCs. These results supported the knowledge that soluble mediators and direct glioma-TAMs interactions activated intracellular pathways in macrophages addressing them toward a pro-tumoral phenotype. Infact, it has been already reported that glioma derived factors are able to polarize macrophage into immunosuppressive M2-like macrophages by the increase of TGF-β1 and interleukin (IL)-10 expression (107,108). Then, Gattas et al performed a 3D-cultures system using spheroids of glioma U87 cell lines together with human primary CD14+ monocytes and observed that inside spheroids monocytes acquired an anti-inflammatory phenotype with high levels of CD206, CD163 and MERTK (109). Moreover they demonstrated that monocytes were able to induce tumor growth measured as increased dimensions of spheroyds. Moreover, Henrich et al. created a 3D-bioprinted GBM model using the murine glioma cells GL261 and the murine macrophage RAW264.7 line (110). The authors showed that in their mini-brain models RAW264.7 cells polarized in a M2-like phenotype with high expression of CD206 and arginase-1 (110). Finally, our results showed that our experimental setting, as a simplified tool to reproduce the tumor microenvironment, allows an easy manipulation of the cell interplay and an in-depth study of the underlying mechanisms. In addition, this model also reflects the metabolic cues of TME, in fact recently it has been described that IDH mutant glioma cells produce the oncometabolite 2 hydroxiglutarate (2HG) in vitro (111,112) and that this oncometabolite inhibits the inflammatory functions and induces a pro-tumoral phenotype of macrophages. Indeed, it has been demonstrated that 2HG suppresses macrophages' inflammatory response (113) and the LPS-induced expression of the cytokine IL-1β in macrophages

(114). Also, Zhen et al. reported that the conditioned media of murine GL261 cell lines activeted BV-2 microglia cells with the increased expression of pro-tumoral cytokines, such as IL-1β, TNF-α, IL-6 and CCL2 and that the pretreatment with a synthetic cell permeable 2HG suppressed these effects (63) . Concluding, in accordance with the knowledge that 2HG, produced by glioma cells bringing the IDH mutations, act as an immunosuppressive metabolite modulating macrophages' functional states, our results demonstrated that the conditioned media and the direct coculture of U87 IDH MUT cells were more efficient compared to U87 IDH WT cells to increase the differentiation of monocyte-derived macrophages and the acquisition of an anti-inflammatory phenotype in macrophages expressing CD206 and pro-tumoral cytokines, such as IL-6 and IL-1β. In coculture with glioma stem cells we also demonstrated that CCL2, another potent pro-tumoral factor, was induced in macrophages by tumor cells.

Then, I focused this thesis on the role of hypoxia in the glioma TME. It's known that hypoxia induces microvascular proliferation and the consequent tumor growth through the production of metabolic intermediates for biosynthetic pathways (70). In addition, these oxygen-deprived conditions have been shown to reduce activation levels of tumor-infiltrating lymphocytes (TILs), resulting in immunosuppression and evasion of immune detection and could shape specific macrophage phenotypes. We modelled the different interactions occurring at the tumor edge vs the tumor core by exposing cocultures of macrophages and U87 cells (IDH WT/MUT) to normoxic and hypoxic conditions, using a specific incubator. To evaluate gene expression profiles of both cell types, cells have been separated by FACS-sorting into $CD45^+$ cells corresponding to macrophages and into $CD45^-$ tumor cells. Our results demonstrated that this model reproduced the tumor microenviroment, since the selected genes (GLUT-1, VEGFA, HMOX and ANGPTL4) were modulated in tumor cells by hypoxic conditions. In addition, we showed significant differences between U87 IDH WT and IDH mutant cells, indeed the gene expression of hypoxia-responsive genes increased more in U87 IDH WT cells compared to IDH MUT counterpart. Focusing on macrophages in coculture with U87 cells, we observed an increase expression of VEGF and HMOX, driving the acquisition of a pro-angiogenic phenotype of macrophages. These results are in accordance with the literature, indeed, it has been shown that the angiopoietin (ANG) receptors TIE2 is up regulated in macrophages in hypoxic conditions supporting their pro-tumoral functions (115,116). The expression of ANG-2 by tumor cells acts as a chemoattractant for macrophages and induces in those cells the release of pro-angiogenic

factors, including MMP9, COX2, and PDGFB (115,117). Furthermore, Chen et al., reported that in presence of hypoxic conditions the activation of G protein-coupled receptors (GCPRs) induces the expression of inducible cyclic AMP early repressor, which in turn supports the pro-tumor transitions of GAMs by NFKB signalling inhibitions(118) . We further studied the effects of hypoxia on monocytes, that give rise to tumor-associated macrophages in the TME in response to tumor signals. We isolated from healthy donors buffycoats classical (CD14++CD16−) and non-classical (CD14lowCD16high) monocytes. We observed that the expression of GLUT1, VEGFA and ANGPTL4 was higher in both monocytic populations compared to macrophages in normoxic and hypoxic conditions. Then, we demonstrated that VEGFA expression increased under hypoxia in monocytes compared to monoctyes in normoxic conditions and to macrophages. These results supported the knowledge the monocytes are the first cells recruited in the TME and they support tumor growth and angiogenesis. Interestingly, these experimental settings, coculture of monocytes/macrophages and U87 WT/MUT cells in normoxic and hypoxic conditions, will be further analyzed thought a transcriptomic approach (bulk-RNA sequencing) by an external group to establish an integrate mathematical models to predict tumor dynamics (119).

In last part of my PhD project, I focused my studies on the role of GAMs as target cells of innovative immunotherapies based on nanovector (NVs), which is a part of the project “New frontiers of engineered nanovectors to improve treatment efficacy and safety in neurological disorders” funded by Fondazione Regionale per la Ricerca Biomedica. Today, immunomodulating therapies have been proven successful in highly immunoreactive tumors. Because gliomas are cold tumors with low numbers of CTL, they are considered ideal targets for tumor antigen vaccines; however, many early phase clinical trials have shown limited efficacy (80). Recently, advances in nanotechnology led to the development of new nanovectors, which enable effective drug delivery to the brain bypassing the BBB. These nanovectors are liposomes characterized by a simple bilayer of lipids with an internal aqueous compartment and their chemical versatility makes them ideal candidates for multi functionalization. The selected drugs of the project mentioned above were the selective allosteric inhibitors of Mek1/2 Pimasertib and Trametinib, these drugs are now present in many clinical trials for different solid tumors where the MAPK pathway is hyper-activated, as in the 85% of GBM. In addition to MEK inhibitors we also analyzed the effect of RapaLink-1, a selective inhibitor of mTOR pathway. It has been reported that Rapalink showed

improved in vivo potency for GBM treatment and inhibition of cell growth, and proliferation(120). The effects of encapsulated drugs have been studied on glioma stem cells by a collaborator and in my experimental setting, which consists in coculture of GSCs and monocytes-derived macrophages, to highlight the role of GAMs as therapeutic target cells and to disclose the molecular pathways supporting drug resistance. At the moment, the progression of the project is limited to the application of the free drugs, without NVs. First, we studied the effect of drugs on single macrophage cultures, macrophages were polarized toward M1 and M2 and treated with Pimasertib and Trametinib. Our results showed that the vitality of macrophages are affected by the two drugs independently to the macrophage polarization status. We obtained the same results with the drug Rapalink. Then, we tested the drugs in direct cocultures of macrophages and GSCs and we observed that the macrophages in presence of GSCs are less sensitive to the drugs compare to macrophages alone. However, GSCs in coculture with macrophages showed a decrease of vitality after the treatment with Trametinib and Rapalink. The possible explanation of these results is related to the nature of macrophages and to the experimental limits of the TME model because macrophages in coculture with GSCs display plasticity and acquire drug resistance, however this effect on macrophages is not sufficient to sustain tumor growth. In the future, testing encapsulated drugs in nanovectors will give more informations about the cross-talk between macrophages and GSCs in response to this innovative strategy with an high potential therapeutic efficacy.

Conclusions

To conclude, we investigated the role of macrophages in the context of GBM biology through an in vitro tumor microenvironment model. Our results confirmed the relevance of the interplay between glioma cells and macrophages, and this will allow the progression of the study on glioma biology to improve therapeutic strategies. Moreover, results from the in vitro hypoxia-related model will enable to establish and integrate mathematical models with the aim of drive the clinical practice and the personalized medicine. Finally, we explored the potential of macrophages as target cells of NVs-encapsulated drugs for the rebalance of the glioma microenvironment. These engineered nanoparticles are promising therapeutic approaches and this study will enhance the knowledge on the molecular mechanisms underlying the use of NVs.

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List of figures and tables

Dissemination of results

Glioblastoma is the most aggressive tumor of brain cancer in adults. Clinically, most patients with this disease die in less than a year. Standard of care relies on surgery, even if its highly infiltrative nature frequently prevents an optimal resection. Patients are also treated with adjuvant therapies, but acquired resistance mechanisms limit the outcomes. Several studies have reported a significant infiltration of immune cells in the tumor microenvironment (TME). Among them, glioma-associated macrophages (GAMs) are the most abundant, representing up to 30% of tumor mass, and their high number has been shown to correlate with clinical outcomes. In this PhD project we disclose some aspects of the role of macrophages in GBM biology, as these cells are involved in tumor growth and can be targeted by therapeutic approaches. Indeed, the development of new therapeutic strategies based on nanoparticles that release selective drugs into the brain will be important to clinical assessment and give a hope to patients.

Il glioblastoma è il tumore più aggressivo tra i tumori che colpiscono il cervello negli adulti e dal punto di vista clinico rappresenta la tipologia di cancro con prognosi peggiore. Le strategie terapeutiche attuali si basano sulla chirurgia, anche se la sua natura altamente infiltrante spesso impedisce una rimozione chirurgica ottimale. Negli ultimi anni, diversi studi hanno riportato una significativa infiltrazione di cellule immunitarie nel microambiente tumorale. Tra questi, la microglia e i macrofagi associati al tumore, rappresentano fino al 30% della massa tumorale e la loro presenza ha dimostrato di essere correlata agli esiti clinici. In questo progetto di dottorato abbiamo approfondito il ruolo dei macrofagi nella biologia del glioblastoma, poiché queste cellule mieloidi sono coinvolte nella migrazione delle cellule tumorali e possono essere utilizzate come bersaglio da approcci immuno-oncologici. Inoltre, lo sviluppo di nuove strategie terapeutiche basate su nanoparticelle che rilasciano farmaci selettivi nel cervello rappresenta un importante strategia nella battaglia contro questi tumori in quanto queste nanoparticelle possono essere applicate in fase di terapia per somministrare farmaci sia in fase di diagnosi precoce.