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Intercellular communication and tumor microenvironment rewiring in glioblastoma pathophysiological response to radiotherapy

PhD Thesis

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1. Sommario

L'insorgenza e la progressione del glioblastoma (GBM) è supportata da un ampio range di fattori che influenzano lo stato metabolico, il setting genetico ed epigenetico del tumore e l'interazione con il microambiente circostante. Il complesso interplay tra cellule tumorali e microambiente innesca meccanismi di resistenza che incrementano la progressione del tumore a favoriscono processi di immunosoppressione. Un ruolo essenziale nel supportare l'aggressività del GBM è dato dalla de-regolazione di alcune pathways intracellulari, tra le quali la pathway di Sonic Hedgehog (SHH), direttamente convolte nel modulare la proliferazione cellulare e nel mantenimento della staminalità. Le evidenze di questo lavoro dimostrano che questa pathway sia correlata al ruolo esercitato dalle connessine, dimostrando un effetto sinergico nella regolazione dei processi di migrazione e delle capacità infiltranti delle cellule di GBM. In ambito clinico, la strategia terapeutica attuale si avvale dell'effetto combinato ottenuto dalla resezione chirurgica, chemioterapia e radioterapia. Nonostante questo approccio sia ampiamente condiviso, è stato documentato che la radioterapia rappresenti una delle principali fonti di effetti off-target che intaccano le cellule immunitarie. La microglia ed i macrofagi associati al GBM sono fortemente rappresentati a livello del microambiente, rivelando un ruolo cruciale nel favorire processi di immunosoppressione e di progressione tumorale. Questo lavoro di ricerca ha come obiettivo l'analisi degli effetti off-target, indotti dalla radioterapia, che interessano l'asse microglia-GBM. È stato osservato che i media condizionati derivanti da cellule microgliali irraggiate, con dosi pari a 2 e 15 Gy, inibiscano l'attivazione di processi apoptotici delle cellule di GBM naïve. L'esposizione a questi media ha preservato, inoltre, la fitness mitocondriale delle cellule tumorali, limitandone anche lo stress ossidativo. Infine, è stata testata la somministrazione di metformina, noto inibitore del complesso I della catena di trasporto finale degli elettroni. Questo farmaco, già impiegato in ambito clinico, è stato utilizzato in combinazione ai media condizionati derivanti da microglia irraggiata. Questo trattamento ha portato alla diminuzione della proliferazione tumorale. Le evidenze riportate avvalorano l'ipotesi che i media derivanti dalla microglia irraggiata favoriscano la progressione del GBM ed inducano un miglioramento dello stato mitocondriale, promuovendo processi di rimodulazione metabolica. Queste osservazioni hanno come obiettivo quello di incoraggiare ulteriori studi riguardanti gli effetti indotti dall'approccio radioterapeutico, il quale potrebbe svolgere un ruolo chiave nell'alterazione metabolica delle cellule tumorali, favorendo processi di riprogrammazione cellulare e meccanismi di immunoevasione.

1bis. Abstract

Tumor microenvironment (TME), composed by a milieu of stromal and immune cells, is one of the critical players in glioblastoma (GBM) progression and heterogeneity. GBM progression is supported by a wide range of mechanisms, which involve several intracellular processes that influence metabolic arrangement, genetic and epigenetic state and the tumor milieu. GBM represents a challenging clinical condition due to the plethora of dynamic processes occurring in TME, stimulating immunosuppression mechanisms and triggers therapy resistance. Current approach employed to counteract GBM progression takes into account the synergistic effect mediated by surgical resection, chemotherapy and involved field radiotherapy (RT). RT represents the gold-standard treatment for this WHO grade IV glioma. Unfortunately, RT is characterized by severe off-target effects, affecting central nervous system (CNS)-resident cells. Tumor associated microglia/macrophages (TAMs) are largely represented in TME of CNS tumors, showing a critical role in GBM progression, favouring immune-escape processes and stemness features. Herein, we first evaluated GBM proliferation and migration in response to specific stimuli, promoting intercellular communication. Then, we moved to evaluate the off-target effects induced by RT, focussing on the related consequences on the microglia-GBM axis. On one hand, CXs established critical interactions with Sonic Hedgehog signalling pathway, which were responsible for GBM infiltrative phenotype, promoting tumor aggressiveness and modulating the heterocellular crosstalk within the TME. On the other hand, GBM cells and its milieu undergo significant alterations due to the impact of radio treatment. We focussed on the interplay between 2-15 Gy irradiated (IR) microglia-derived conditioned media (CM) and naïve GBM cells. We observed that tumor cells survival was preserved, avoiding death mechanisms and favouring GBM clone formation. IR microglia-derived CM exposure showed also a key role not only in maintaining mitochondrial mass and fitness but also preventing mitochondrial ROS production. The administration of metformin, together with IR microglia CM exposure, induced a decrease in tumor cell proliferation. The evidence provided suggests that RT-derived off-target effects alter microglia state, promoting GBM aggressiveness and growth through the release of factors which are able to sustain metabolic rewiring toward oxidative phosphorylation. Our observations highlight the essential role of the interplay between tumor and healthy surrounding cells, which is the main cause of metabolic reshaping and epigenetic modifications, leading to the alteration of TME response and resulting in an immunosuppressive phenotype. This work aims to encourage further investigations on TME remodelling, focussing on immune response alteration and GBM-TME crosstalk reshaping.

2. Keywords and abbreviations

Keywords

Glioblastoma, Tumor Microenvironment, Connexin, Smoothened, GLI1, Microglia, Radiotherapy, Metformin, Immunosuppression, Immunometabolism, Epigenetics.

List of abbreviations

Akt: protein kinase B

Angio-TAMs: Pro-angiogenic TAMs

APOC1: Apolipoprotein C1

APOE: Apolipoprotein E

ARG1: Arginase 1

ASR: Age-Standardized Rate

BBB: Blood-Brain Barrier

BMDMs: Bone Marrow-Derived Macrophages

CAR T: Chimeric Antigen Receptor T

CCL2: C-C Motif Chemokine Ligand 2

CCL3: C-C motif Chemokine Ligand 3

CCL5: C-C Motif Chemokine Ligand 5

CCR2: C-C Motif Chemokine Receptor 2

CCR2: C-C motif Chemokine Receptor 2

CD11b: integrin α-M

CD28: Cluster of Differentiation 28

CD31: Cluster of Differentiation 31

CD45: Cluster of Differentiation 45

CD68: Cluster of Differentiation 68

CD8: Cluster of Differentiation 8

CD84: Cluster of Differentiation 84

CDK: Cyclin-Dependent Kinase

CDK1: Cyclin-Dependent Kinase 1

CDK4: Cyclin-Dependent Kinase 4

CM: Conditioned Media

CNS: Central Nervous System

COX-2: Cyclooxygenase-2

CSF-1: Colony Stimulating Factor 1

CTLA-4: Cytotoxic T-Lymphocyte-Associated Antigen 4

CX3CR1: C-X3-C motif Chemokine Receptor 1

CXCL1: C-X-C motif Chemokine Ligand 1

CXCL10: C-X-C motif Chemokine Ligand 10

CXCL16: C-X-C motif Chemokine Ligand 16

CXCL2: C-X-C motif Chemokine Ligand 2

CXCL8: C-X-C motif Chemokine Ligand 8

CXCR1: C-X-C motif chemokine receptor 1

CXCR6: C-X-C motif Chemokine Receptor 6

Cxs: Connexins

DAMPs: Damage-Associated Molecular Patterns

DCs: Dendritic Cells

ECM: Extracellular Matrix

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial–Mesenchymal Transition

ERK: Extracellular signal-Regulated Kinases

FDA: Food And Drug Administration

GABRA1: Gamma-AminoButyric acid type A Receptor subunit α1

GBM: Glioblastoma

G-CIMP: Glioma-Cpg Island Methylator Phenotype

GLUT3: Glucose Transporter 3

Gpnmb: Glycoprotein nmb

GTR: Gross Total Resection

HER2: Human Epidermal growth factor Receptor 2

HIF-1: Hypoxia Inducing Factor 1

HLA-DR: Human Leucocyte Antigen DR

HRE: Hypoxia-Response Element

IBA1: Ionized Calcium-Binding Adapter Molecule 1

ICAM-1: InterCellular Adhesion Molecule 1

IDH: Isocitrate Dehydrogenase

IFN- γ: Interferon-γ

IFN-TAMs: Interferon-Primed Tams

IGF1: Insulin-Like Growth Factor 1

IGFBP2: Insulin-like Growth Factor Binding Protein 2

IL-10: InterLeukin-10

IL-12: InterLeukin-12

IL-13R: InterLeukin-13 Receptor

IL13-Rα2: Interleukin-13 Receptor α2

IL-1β: InterLeukin-1β

IL-6: InterLeukin-6

Inflam-TAMs: Inflammatory cytokine-enriched TAMs

IR: Irradiated

JAK2: Janus Kinase 2

LA-TAMs: Lipid-Associated TAMs

LDH: Lactate Dehydrogenase

LET: Linear Energy Transfer

Lox-1: Low-density lipoprotein receptor-1

LPA: Lysophosphatidic Acid

MAPK: Mitogen-Activated Protein Kinase

MCTs: Monocarboxylate Transporters

MDSCs: Myeloid-Derived Suppressor Cells

MERTK: Tyrosine-Protein Mer

MGMT: O^6-Methylguanine-DNA Methyltransferase

MHCII: Major Histocompatibility Complex II

MIF: Migration Inhibitory Factor

M-MDSCs: Monocytic MDSCs

MMP9: Matrix Metalloproteinase 9

MRI: Magnetic Resonance Imaging

MSCs: Mesenchymal Stromal Cells

mTORC1: mammalian Target Of Rapamycin Complex 1

NEFL: neurofilament light chain

NF1: Neurofibromatosis type 1

Nf-kB: Nuclear factor kappaB

NKG2D: Natural Killer Group 2 member D

OVs: Oncolytic Viruses

OxPhos: Oxidative Phosphorylation

P2RY12: Purinergic Receptor 12

PAMPs: Pathogen-Associated Molecular Patterns

PD-1: Programmed Cell Death Protein 1

PDGFRA: Platelet Derived Growth Factor Receptor α

PD-L1: Programmed Cell Death Ligand 1

PI3K: Phosphoinositide 3-Kinase

PKM1/2: Pyruvate Kinase M1/M2

PMN-MDSCs: Polymorphonuclear MDSCs

Prolif-TAMs: Proliferating TAMs

Reg-TAMs: immune Regulatory TAMs

ROS: Reactive Oxygen Species

RT: Radiotherapy

RTK: Receptors Tyrosine Kinase

RTM-TAMs: Resident-Tissue Macrophages-like TAMs

S100A4: S100 calcium binding protein A4

S1P: Sphingosine-1-Phoaphatase

SASP: Senescence-Associated Secretory Phenotype

SDF-1: Stromal Derived Factor 1

SHH: Sonic Hedgehog

SK: Sphingosine Kinase

SLC12A5: SoLute Carrier family 12 member 5

SMO: Smoothened

SOX2: SRY-BOX transcription factor 2

SPP1: Secreted Phosphoprotein 1

STAT3: Signal Transducer and Activator of Transcription 3

SYT1: Synaptotagmin 1

TAMs: Tumor Associated Microglia/Macrophages

TANs: Tumor-Associated Neutrophils

TCGA: The Cancer Genome Atlas

TERT: TElomerase Reverse Transcriptase

TGF-β: Transforming Growth Factor-β

TME: Tumor Microenvironment

TMEM119: Transmembrane Protein 119

TMZ: Temozolomide

TNF: Tumor Necrosis Factor

TTF: Tumor Treating Field

VCAN: Versican

VEGF: Vascular Endothelial Growth Factor

VEGFR-2: Vascular Endothelial Growth Factor Receptor 2

XCL1: X-C motif Chemokine Ligand 1

ZEB1: Zinc finger E-box-Binding homeobox 1

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4. Introduction

4.1 GBM: epidemiology and classification

Glioblastoma (GBM) is known as the most widespread and aggressive primary glioma affecting adult population, with a median age at diagnosis of 65 years old. GBM represents approximately 48% of all primary central nervous system (CNS) tumors and 57% of all gliomas [1]. In 2020, the global incidence of this malignancy, reaches the highest rates in North and Eastern Europe, North and Latin America and Asia, with a median rate of 3.18 per 100'000 persons (WHO, 2020). Italy is represented by an incidence of about 5.7 per 100'000 and a 3.5 per 100'000 mortality age-standardized rates (ASR), reflecting an average condition as compared to whole Europe overview (WHO, 2020 - **Figure 1**). According to the evaluation of the estimated number of new cases in Europe, Italian contribution is equal to an 8.5% of the total amount of new CNS tumor cases in whole European country, preceded only by Russian Federation, Germany, France and United Kingdom (WHO, 2020).

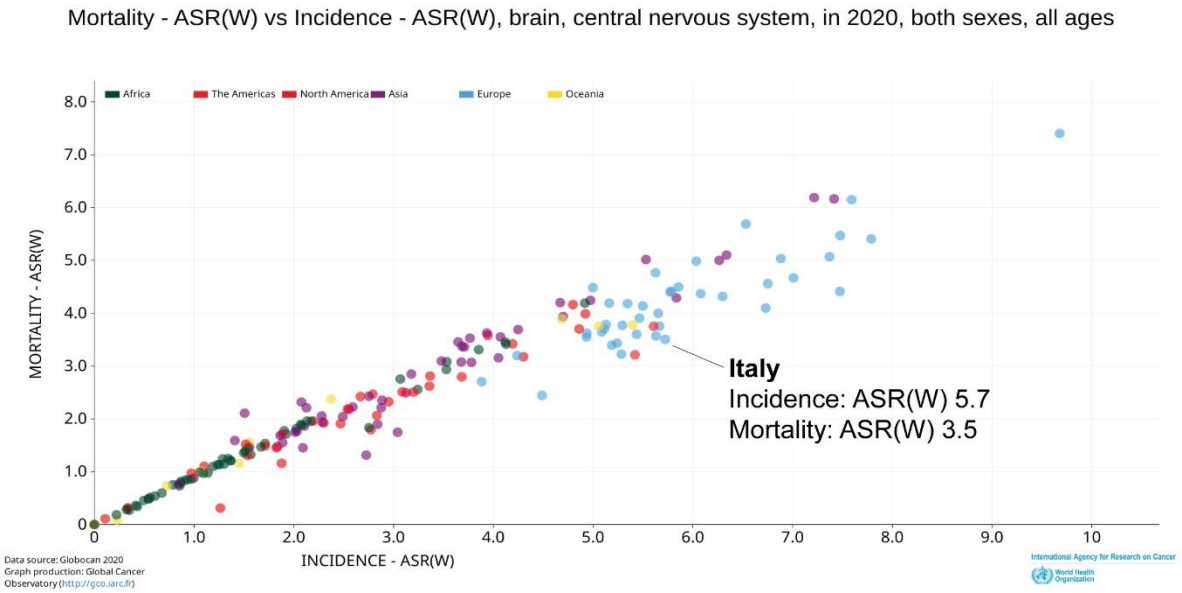

Figure 1: Epidemiologic evaluation of CNS cancers incidence and mortality indexes. *a) Scatter plot graph illustrating the relationship between global age-standardized mortality and incidence rates for brain and CNS tumors in 2020, with data points for Italy showing an incidence rate of 5.7 and a mortality rate of 3.5, both standardized to the world population ASRs(W).*

GBM prognosis is dismal and long-term survival can be considered a relatively rare event: at 5-years post-diagnosis, the overall relative survival is about 5.8%, depending by the age of diagnosis and by sex [1],[2]. Gender can affect GBM onset, indeed the worst prognosis is common in male compared to female with a M:F incidence ratio of 1.6:1 [3]. The cause of this substantial sex-based difference in terms of incidence and overall survival is due to the multiple genetic different features existing between male and female, including O6-Methylguanine-DNA Methyltransferase (MGMT) promoter

hypermethylation, more common in women than men, thus explaining their better survival outcome [3],[4]. Focussing on the data related to incidence and mortality rates in Europe, 2020 WHO statistic evaluation for both sexes shows ASM(W) incidence values of 7.0 and 4.9 and ASM(W) mortality rates equal to 5.2 and 3.4 for males and females, respectively. Regarding the Italian scenario, M:F incidence rates is 7:4.4, and M:F mortality rates is 4.3:2.8, showing an average condition which confirm the substantial sex-based difference observed in whole Europe (WHO, 2020).

A few sound hypotheses are validated in terms of gliomas risk factors. Congenital disabilities of CNS and neurological alterations, Turcot syndrome, Lynch syndrome and Li-Fraumeni syndrome are the main potential non-modifiable factors associated to gliomas and GBM onset. However, these account for <1% of cases [3],[1]. Furthermore, genome-wide studies evaluated the correlation between increased risk for glioma development and 25 single nucleotide polymorphisms, including 11 directly correlated to GBM. Nevertheless, the genetic and biological significance of this association remains to be clarified [5]. Familiarity and genetic components play a crucial role in the brain tumor occurrence. About 5-10% of gliomas occur in familial clusters, with a 2-fold increased risk to develop such a tumor [3]. In terms of potentially modifiable-risk factors, exposure to ionizing radiations is a well-established factor associated with gliomas onset [6].

The fifth edition of the WHO classification of tumors of the CNS, published in 2021, suggests a new designation for this brain tumor, for which GBM is classified as IV WHO grade tumor, isocitrate dehydrogenase (IDH)-wildtype [7],[8]. GBM is now integrated into the gliomas, glioneuronal and neuronal tumors category, part of adult-type diffuse gliomas [9]. The previously included IDH-mutant tumors, due to their lower malignancy and aggressiveness, are currently identified as astrocytoma, IDH-mutant, graded also as II, III or IV WHO grades tumors [10]. In diagnostic field, GBM has always been classified on histological features, which including elevated hypercellularity, nuclear atypia, microvascular proliferation and necrotic foci. Cell necrosis exhibits a surrounding palisade positioning of tumor cells [11]. Notwithstanding the remarkable importance of these histopathological markers in routine diagnosis, the most recent WHO classification highlights the potential prognostic power of certain molecular biomarkers, which should be considered, given the well-established GBM intra- and inter-cellular heterogeneity [8]. In the specific case of adult-type diffuse gliomas, a lot of molecular parameters, together with histological assessment, could be very useful in brain tumor grade evaluation [12]. 2021 edition of WHO classification introduced new

molecular criteria regarding GBM diagnosis including: telomerase reverse transcriptase (TERT) promoter mutation, epidermal growth factor receptor (EGFR) gene amplification, the combination of gain of chromosome 7 and loss of chromosome 10 (+7/-10) and the absence of IDH mutations [13],[2],[8],[12]. Therefore, whether the assessment of all high-grade histopathological features are not mandatory, at least one of those aforementioned molecular characteristics need to be detectable to allow GBM diagnosis [8],[12]. One of the most recent approach to increase the diagnostic accuracy of glioma is related to the identification of GBM subclasses based to the DNA methylation [2]. This advanced approach could drastically change brain tumor diagnostic classification, due to the robustness and reproducibility of DNA methylation profiling, even if the samples are qualitatively poor. Moreover, this approach takes into account of the critical impact on DNA-methylation profile of tumor microenvironment (TME) [14]. Whole-genome analysis and DNA methylomes profiling, together with the evaluation of MGMT gene methylation state, represent solid biomarkers in terms of prognosis, diagnosis and treatment of GBM [15]. The Cancer Genome Atlas (TCGA) takes into account studies that consider specific criteria, such as rarity of cancer type, poor prognosis and overall public health impact. GBM was the first kind of tumor to be evaluated by TCGA. The widespread GBM subclassification is traditionally referred to the analysis of three core pathways including p53, Rb and receptors tyrosine kinase (RTK)/Ras/phosphoinositide 3-kinase (PI3K) signalling [16].

Traditionally, GBM is graded in classical, proneural, neural and mesenchymal transcriptomic subtypes [17]. Each subclass is defined by a specific genetic signature, including platelet derived growth factor receptor α (PDGFRA) and EGFR amplification, MGMT status, whole-genome DNA methylation profile and glioma-CpG island methylator phenotype (G-CIMP) [18],[19].

- Classical subtype: MGMT promoter methylation represents a peculiar and unique predictive biomarker of this GBM subclass, which is also included in DNA methylation cluster M3. Moreover, classical type is characterized by extensive amplification of chromosomes 19 and 20. Classic subtype is also determined by EGFR amplification/mutation, that induces an increased EGFR expression and phosphorylation. Classical GBM shows overexpressed NOTCH1, NOTCH3 and Sonic Hedgehog (SHH) pathways and a downregulation of mitogen-activated protein kinase (MAPK) signaling as well as several proapoptotic pathways, such as Bak, caspase 7 and cleaved caspase 9 [17],[18],[20].

- Proneural subtype: a strong portion of proneural tumors are represented by G-CIMP methylation cluster, associated with a better prognosis. Moreover, M6 DNA methylation group is widely represented by proneural GBMs. This GBM subset is characterized also by an alteration of PDGFRA and it is represented by ATRX mutations. The amplification of MYC, PDGFRA, cyclin-dependent kinase 4 (CDK4) and SRY-Box transcription factor 2 (SOX2) genes expression is also largely described in this subtype. Proneural subset shows a high expression of PI3K pathways and protein kinase B(Akt)-regulated mammalian target of rapamycin complex 1 (mTORC1) activation site, and also an overexpression of several proteins as cyclooxygenase-2 (COX-2), insulin-like growth factor binding protein 2 (IGFBP2) and Annexin 1 [17],[18],[19].
- Neural subtype: neural GBM subclass is typified by neurofilament light chain (NEFL), solute carrier family 12 member 5 (SLC12A5), synaptotagmin 1 (SYT1) and gamma-aminobutyric acid type A receptor subunit α1 (GABRA1) neuron expression markers but it also shows association with oligodendrocytic and astrocytic differentiation [17].
- Mesenchymal subtype: this GBM subset is associated with a poor prognosis and it is represented by DNA methylation clusters M1 and M2, therefore it strongly shows Neurofibromatosis type 1 (NF1) inactivation. Mesenchymal subclass exhibits high levels on endothelial marker, including cluster of differentiation 31 (CD31) and vascular endothelial growth factor receptor 2 (VEGFR-2), and markers of inflammation such as fibronectin. Moderately increased activation of MAPK pathway has been also documented. Otherwise, mesenchymal GBM, in contrast with proneural features, displays a downregulation of PI3K pathway and a wide activation of S6 kinase, related to activated mTOR effector pathway [21],[17],[18].

4.2 GBM therapeutic strategies

The current therapy paradigm for newly diagnosed GBM includes safe maximum resection, followed by RT with concomitant temozolomide (TMZ)-based chemotherapy and final adjuvant chemo-treatment. Nevertheless, the prognosis remains poor, failing to significantly improve patient's overall survival [22].

4.2.1 GBM gold standard treatments

- Safe maximum resection: surgery represents the initial treatment for tumor mass debulking [23]. This strategy is the first line approach for GBM, increasing the window-of-opportunity to treat patients and mostly tackling tumor volume, alleviating symptoms related to intracranial pressure and compression caused by tumor mass [24],[25]. Patients undergo surgery for gross total resection (GTR), which is a highly effective and strongly suggested method included into the European guidelines for management of GBM. It consists in a safe surgical resection, preserving CNS functions and integrity of surrounding healthy tissue [24]. Nevertheless, GTR for GBM is technically challenging and not applicable in every case, due to the infiltrative phenotype of this malignancy [25]. This intrinsic feature of GBM makes the visualization of distinct tumor/brain margin hard. In this regard, magnetic resonance imaging (MRI)-guided surgical practice with the addiction of an optical imaging agent, named 5-aminolevulinic acid (5-ALA), could be very helpful in identifying healthy tissue/tumor mass borders [26].
- RT and concurrent chemotherapy: RT acts through linear energy transfer (LET) radiations in order to affect tumor cell viability. Irradiation is responsible for either DNA strand breaks direct damages or indirect effects induced by the production of free radicals [27],[28]. X-ray radiation therapy represents a necessary step and the most effective treatment in counteracting GBM progression [29]. X-ray irradiation is usually performed 3-5 weeks after surgery, and it is delivered through fractionated doses, thus maximizing patient's survival. For first-time treated GBM, 60 Gy dose of radiation is administrated in 2 Gy fractions 5 days a week, for 6 weeks [24],[25]. However, promising and innovative approaches in terms of radiation sources are recently considered. Positively charged particles such as protons could improve biological effects thanks to the released of higher quantity of energy on targeted area [27],[30]. Conventionally, X-ray treatment is combined with TMZ administration, 75 mg/m^2/day x 6 weeks [2]. This first-line treatment provides an increase of 2.5 months in terms of overall survival when combined to RT [31]. TMZ acts as DNA alkylating agent able to penetrate the blood-brain barrier (BBB).

Into the cytoplasmic level, TMZ undergoes hydrolysis reactions which allow the formation of the active cation form, that provides to purines and pyrimidines methylation, especially in guanine rich sites. These DNA alterations cause cell cycle arrest at G2/M phase and apoptosis [24]. The efficacy of this chemo treatment strongly depends on MGMT gene promoter methylation state, which, for this reason, represents an important prognostic marker [23]. Lack of methylation of MGMT promoter is responsible for the activation of methyltransferase function, acting as suicide DNA repair system, which acquires a methyl group from guanine to its cysteine residue. This condition reverses TMZ action and stimulates TMZ-resistance. Otherwise, methylated MGMT gene promoter inhibits MGMT activation, preserving TMZ effectiveness and TMZ-sensitivity [24],[23],[29].

- Adjuvant chemotherapy: TMZ maintenance, when possible, is performed with 6 adjuvant cycles, 150-200 mg/m^2/day for 5 days every 28 days. RT/TMZ followed by TMZ maintenance remains the undisputed treatment for the majority of GBM cases [2],[23]. During adjuvant TMZ administration, it is often applied the tumor treating field (TTF) therapy. This technique uses transducers directly applied to the scalp, which ensure low-intensity (1–3 V/cm) and intermediate-frequency (200 kHz) alternating electric fields, stimulating antimitotic processes in proliferating tumor cells [32],[1].

4.2.2 GBM biotechnological therapies

Even though the impact of current world-spread therapies against GBM, patients' survival remains poor. This dramatic epilogue is also the consequence of the intrinsic features of GBM, involving the remarkable intra and inter-cellular heterogeneity and the tumor cells propensity to infiltrate the health surrounding tissue. Moreover, the BBB role is crucial in hampering therapeutic sensitivity and in stimulating immunosuppressive environment and immune evasion [33]. For this reason, all of the recent advances in the field of GBM molecular pathogenesis play a crucial role in developing innovative and unconventional therapies. At this purpose, immunotherapy represents a promising advanced treatment for counteracting GBM progression. The field of immunotherapy offers a plenty of different strategies, including chimeric antigen receptor T (CAR T) cell therapy, immune checkpoint blockade, oncolytic virotherapy and vaccine therapy [34].

- CAR T cell therapy: this treatment has already shown an outstanding efficacy in the cure of haematological malignancies. CAR T approach is focused on the genetic engineering of T lymphocytes expressing CAR transmembrane receptors, able to recognize tumor antigens, in an

MHC-indipendent manner [34]. Those chimeric molecules have a tripartite structure, consisting in an extracellular domain, with a single chain tumor-binding moiety, a transmembrane region, represented by a variable spacer domain and a cytosolic signalling domain, related to T-cell activation. The intracellular domain is typically represented by CD3ζ but, in second and third generations CAR, it is modified by the addition of one or two co-stimulatory elements (such as cluster of differentiation 28, CD28, or 4-1BB), which enhance the activation processes [33]. T cell intended for engineering, are picked up from peripheral blood of the patient, *ex vivo* amplified and genetically remodelled to allow the expression of the targeted tumor-specific receptor. Therefore, CAR T cells are injected back to permit the action against the tumor mass [24]. The most common CAR T target receptors for GBM treatment include EGFR variant vIII, interleukin-13 receptor α2 (IL13-Rα2) and human epidermal growth factor receptor 2 (HER2). Despite the success showed by IL13Rα2 CAR T cells, which provided tumor regression and maintenance for almost 7.5 month after the initiation of the treatment, this approach still remains elusive for GBM. Such a limitation is linked to the fact that not all the targets are always expressed, confirming the existent differences between tumors subclasses and the strong intra-tumoral heterogeneity [35],[29].

- Immune checkpoint blockade: this approach takes advantage of the physiological processes involving T-cell activation and T-cell suppression balance, allowing the regulation of the immune system response. This mechanism is mediated by receptors or ligands complexes, termed immune checkpoints, which are responsible for immune homeostasis and regulation. Cancer cells are able to exploit this mechanism through the expression of different checkpoint molecules, evading T-cell immune-mediated destruction [36]. This promising treatment has been proven to be efficient for a wide range of solid tumors, but unfortunately the therapeutic success in counteracting GBM progression is modest. At this purpose a combination therapy, including immune checkpoint blockade and immune-stimulating agents administration, is currently under investigation in many clinical trials for GBM [33]. Checkpoint inhibitors revolutionized the management of many aggressive malignancies, such as renal carcinoma, metastatic melanoma and lung cancer. In particular programmed cell death protein 1 (PD-1), its ligand (PD-L1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, CD152), are among the main checkpoint molecules expressed in GBM cells [1]. PD-L1 is expressed in more than 80% of newly diagnosed GBM, CTLA-4 strongly characterizes high-grade gliomas and both of them are positively correlated with GBM severity and immune-evasion ability [24],[37]. PD-1 and CTLA-4

act as negative regulators, which are responsible for T-cells activity blocking. At this purpose, nivolumab and ipilimumab, targeting PD-1 and CTLA-4 respectively, represent the most widespread monoclonal antibody inhibitors tested in GBM clinical trials [38].

- Oncolytic virotherapy: oncolytic viruses (OVs)-based therapy consists in the injection of replication-competent viruses, which are able to specifically targeting cancer cells. Those viral particles are distinguished in natural and genetically modified virus, both selectively infecting tumoral cells preserving surrounding healthy cells [39]. Engineered OVs are genetically manipulated to induce the expression of tumoral-specific receptors, such as EGFRvIII, interleukin-13 receptor (IL-13R) and PDGFR, which enhance the affinity for cancer cells [24]. The employment of OVs could be very effective due to the potential multiple downstream effects, which include not only the direct killing of malignant cells, but also the turning from an immunosuppressive environment ("cold" tumor) to a responsive condition ("hot" tumor). This OV infection-derived switching is mediated by the transport of pro-inflammatory factors, the secretion of tumor antigens (i.e. pathogen-associated molecular patterns - PAMPs, and damage-associated molecular patterns - DAMPs), the inhibition of tumor suppressive genes and the TME disruption [40],[41]. GBM is suitable of OV treatment because of the lacking of non-CNS metastasis and the relatively localized tumor mass. Several kinds of viruses are already candidate for GBM treatment, such as adenovirus, herpes simplex virus, poliovirus and retrovirus [34],[29]. Ongoing clinical trials demonstrate that the combination of two immunologic approaches, DNX-2401 (adenovirus serotype) injection and anti-PD1 inhibitor administration, has revealed to be beneficial in terms of overall survival for recurrent GBM [42].

The importance of combination therapy is also demonstrated by the development of the molecular target strategy for the treatment of GBM, which involve the administration of bevacizumab. This monoclonal antibody is strongly related to tumor-associated angiogenesis and acts against the vascular endothelial growth factor (VEGF), widely expressed in GBM cells. This anti-angiogenic agent has been approved for the treatment of recurrent GBM by Food and Drug Administration (FDA) in 2009 in USA and Canada [43],[2]. Bevacizumab reduces peritumoral edema, allowing less steroid administration. Nevertheless, in the majority of the clinical trial, overall survival outcome remains uncertain [9]. For this reason, many clinical studies propose combination therapy, for newly diagnosed and recurrent GBMs, which involve the administration of bevacizumab with conventional and unconventional treatments, such as chemotherapeutic agents, TMZ or lomustine, re-irradiation and different types of monoclonal antibodies target therapy [44]. The efficacy remains to be

clarified, but oncological research and clinical studies are putting a significant effort on novel approaches.

Despite the advances in the field of therapeutic strategies employed for counteracting GBM development and progression, there is no established standard of care for progressive of recurrent GBM. The identification of effective therapies is challenging due to disease heterogeneity, and it should take into account tumor size and location, previous treatments, patient's age, Karnofsky performance score, patterns of relapse and prognostic factors. The integration of TTF with TMZ represents a noteworthy breakthrough in GBM therapy, the first major advancement in approximately a decade, and should be contemplated for newly diagnosed patients without contraindication. Given the bleak prognosis of this disease, the focus of GBM treatment should extend beyond mere survival improvement, aiming to preserve and enhance the patient's quality of life [45]. Several clinical trials are currently carried out in order to highlight potential therapeutic approaches for counteracting GBM recurrences. Following table shows the main treatments taken into account (**Table 1**).

Newly Diagnosed GBM	Recurrent GBM
Surgery	Additional surgery
Complementary treatment	Re-irradiation and special techniques
Dose-dense TMZ	Stereotactic radiosurgery and hypofractionated stereotactic radiotherapy
Duration of TMZ maintenance therapy	Conventionally fractionated radiation
Gliadel (carmustine) implantable wafers	Brachytherapy
Optimal dose-fractionation schedule for external beam radiation therapy	Combination treatments
Targeted therapy	Second-line chemotherapy
	Nitrosourea/ TMZ/ Bevacizumab monotherapy and combination regimens

Table 1: Therapeutic strategies employed for newly diagnosed and recurrent GBM (modified from [45])

4.3 GBM tumor microenvironment

The heterogeneity of GBM is a fundamental feature contributing to its characteristic aggressiveness. This high variability is attributed to the diverse genotypic, metabolic, and epigenetic features, creating a dynamic context that still lacks a comprehensive understanding in GBM pathophysiology. First and foremost, GBM stem cells (GSCs) represent one of the main players in GBM heterogeneity, exerting a pivotal role in tumor regrowth and recurrence and determining resistance to chemo- and radio-treatments [46]. GSCs maintain their capability of self-renewal adapting their metabolic, epigenetic and transcriptional settings to the multifaceted surrounding environment, resulting in a constant reprogramming [47]. The dynamic crosstalk established between GSCs and GBM milieu favours tumor growth, invasiveness and angiogenesis, favouring GBM aggressiveness [48]. For these reasons, GBM microenvironment is undoubtedly another preeminent factor which sustains intra-tumoral and inter-tumoral variability. TME is described as a complex and active milieu comprising stromal and immune cells, secreted molecules and the surrounding extracellular matrix (ECM). This intricate interplay with GBM cells establishes a mutual relationship. On one side, the multitude of molecular participants involved in GBM progression leads to a high degree of adaptability and substantial diversity within the adjacent microenvironment. Conversely, GBM growth heavily relies on the multifaceted processes taking place within this intricate TME setting. This critical and dual-sided interaction results in the formation of hypoxic regions within the tumor bulk, a stronger infiltrative phenotype, resistance to chemotherapy and RT and the initiation of angiogenesis. Consequently, the dynamics of the TME represents a major challenge that needs to be addressed in the pursuit of effective GBM therapy [49],[50]. It's worth noting that there are distinct non-immune and immune-related components within the TME, each with its unique characteristics and functions.

4.3.1 Non-immune TME-related component

In GBM, ECM represents the interface allowing pathophysiological processes required in enhancing GBM invasiveness and migration, representing the biological substrate for autocrine and paracrine intercommunication between tumor and non-tumor cells. In this scenario, ECM is deeply modified by the increased secretion of fibronectin, matrix metalloproteinase 9 (MMP9) and hyaluronic acid, important players in infiltrative and mobility processes mediated by glioma cells. Mesenchymal stromal cells (MSCs), through the releasing of cytokines as interleukin-6 (IL-6) and chemokine C-X-C motif ligand 2 (CXCL2), mediate the degradation of ECM, favouring the migration process [49]. Furthermore, it was established the interplay between ECM and immune cells, which result in the immunomodulator features related to tumor ECM [51].

BBB physically isolates CNS from the periphery and represents one of the main factors limiting chemotherapy efficacy. From a physiological point of view, BBB encompasses non-fenestrated endothelial cells surrounded by pericyte and astrocyte end-foot processes. Nevertheless, when BBB is compromised by GBM onset, this setting and integrity are lost. Because of the massive secretion of VEGF in the TME, brain's vasculature undergoes deep changes due to the extensive neo-angiogenetic processes, showing deep impairment in terms of vessels volume and permeability, which results in compromised organization and functionality [52].

Glial cells and neurons are also involved in GBM progression. Tumor-associated astrocytes act in multiple ways in promoting GBM growth. In one hand, astrocytes are able to regulate glioma proliferation through the production of neurotrophic factors, such as transforming growth factor-beta (TGF-β) and the insulin-like growth factor 1 (IGF-1), which directly sustain tumor cells trophism and metabolism. Astrocytes undergo reactive astrogliosis, which represents a potential contributor of GBM cell infiltration mediated by the activation of zinc finger E-box-binding homeobox 1 (ZEB1), resulting in epithelial–mesenchymal transition (EMT) [53],[54]. In the other hand, astrocytes seem to be related to the immune modulation within TME, by the releasing of PD-L1, which stimulates immune suppression mechanisms [55]. Moreover, neurons play an important role in promoting GBM progression. Neuronal cells contribute to the inhibition of T cells and microglia, supporting immunosuppressive and anti-inflammatory milieu [56].

4.3.2 Immune TME-related component

The widest and heterogeneous component of GBM TME is represented by the immune compartment, which is made up of different immune populations, such as myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TANs), dendritic cells and TAMs, consisting in bone marrow-derived macrophages (BMDMs) and resident microglia [57].

MDSCs play an important role in immunosuppression and therapy resistance. During GBM genesis and development, the damaged BBB allows the infiltration of inflammatory monocytes, which differentiate in MDSCs into the tumor tissue, strongly associated with $CD133^+$ GSCs subtype. These cells have been also found in the peripheral blood of GBM patients. This heterogeneous immune population consists in activated monocytes and neutrophils, that can be classified in monocytic MDSCs (M-MDSCs), similar to monocytes, and granulocytic or polymorphonuclear MDSCs (PMN-MDSCs), comparable to neutrophils. Low-density lipoprotein receptor-1 (Lox-1), cluster of differentiation 84 (CD84) and C-X-C motif chemokine receptor 1 (CXCR1) represent the newly designed markers for MDSCs identification [58],[59],[57]. Several studies pointed out that M-MDSCs and PMN-MDSCs in GBM patients are mainly distributed in peripheral blood, showing a predominant portion of PMN-MDSCs then M-MDSCs [60],[61]. It has been established that MDSCs act in order to suppress T cells activation through the stimulation of catabolic processes of L-arginine and the production of reactive oxygen species (ROS), preventing cluster of differentiation 8 $(CD8)^+$ T cell proliferation. Moreover, MDSCs induce the secretion on interleukin-10 (IL-10) and TGF-β, favouring immunosuppressive TME [62].

Growing body of evidence highlights the role of TANs. High frequency of TANs represents a negative prognostic marker and it is inversely correlated to the overall survival of GBM patients. They are mostly located within the GBM bulk, recruited by interleukin-8 (IL-8), C-X-C motif chemokine ligand 8 (CXCL8) and migration inhibitory factor (MIF) [49]. TANs act stimulating GSCs spreading and cancer invasion upregulating S100 calcium binding protein A4 (S100A4)-dependent mechanisms, which is the main cause of EMT and anti-VEGF therapy resistance (**Figure 2**) [63].

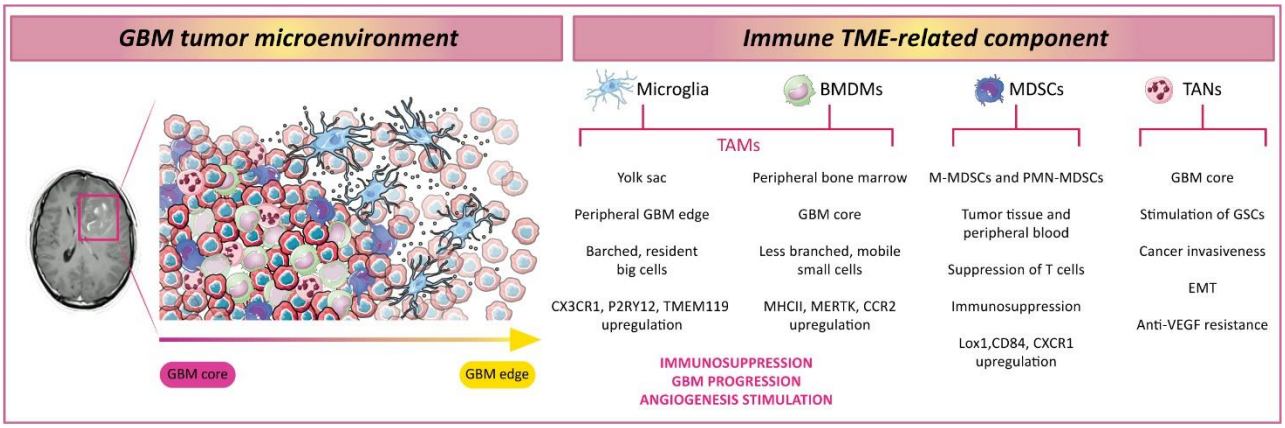

Figure 2: GBM milieu and immune TME-related components. *The key immune components within GBM TME primarily consist of TAMs, which encompass both microglia and BMDMs, as well as MDSCs and TANs. GBM cells are depicted in red. These immune cells are distributed within the GBM tumor mass, including its core and the peripheral regions, interested by infiltration and invasion processes. Remarkably, immune cells in the GBM TME play a facilitating role in promoting GBM progression. They provide support for the proliferation of GSCs and contribute to the establishment of an immunosuppressive environment within the TME.*

Dendritic cells (DCs) represent a potential anti-tumor immune cell type due to their production of pro-inflammatory cytokines, such as interleukin-12 (IL-12), which can favour the recruitment of $CD8^+$ T cells. DCs are potentially recruited by C-C motif chemokine ligand 5 (CCL5) and X-C motif chemokine ligand 1 (XCL1), but they are poorly represented in GBM TME, as their functionality is compromised by immunosuppressive effects mediated by tumor cells [64],[62].

In this scenario, TAMs play a crucial role in gliomagenesis and GBM progression. TAMs reach up to 30-40% of the total cells within the TME, representing the most abundant no-tumoral cell population into the tumor bulk. For this reason, TAMs represent a crucial target in the field of immunotherapy [65].

4.4 Tumor-associated macrophages/microglia

Glioma grade is directly correlated with the number of TAMs present within TME, revealing their fundamental pro-tumoral role, lowering overall survival in glioma patients [66]. High-grade tumors share the ability to secrete a wide range of chemo-attractive molecules, such as C-C motif chemokine ligand 2 (CCL2), colony stimulating factor 1 (CSF-1) and stromal derived factor 1 (SDF-1, also known as CXCL12), which recruit TAMs into the hypoxic regions of TME, favouring migration and invasion processes [67]. The spectrum of TAMs used to be really dynamic and active depending on the types of cues received from GBM cells and the other TME compartments. Due to the high plasticity of TAMs, it is potentially possible to distinguish two opposite TAMs-related phenotypes,

even if this classification results overly simplistic: M1, pro-inflammatory and anti-tumorigenic phenotype, and M2, anti-inflammatory and pro-tumorigenic phenotype. M1 polarization is triggered by cytokines such as IFN-γ, with a consequential turning in a pro-inflammatory phenotype, which allows the releasing of inflammatory factors, as tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-6 and IL-12. M2 shifting is led by IL-10, interleukin-4 (IL-4) and IL-13 secretion, resulting in anti-inflammatory cells capable to express anti-inflammatory cytokines as TGF-β, arginase (ARG1) and IL-10 [57],[68]. It has been also observed that GBM cells are able to manage the inflammatory state of TAMs through the production of C-X-C motif chemokine ligand 26 (CXCL16), which modulates CXCL16/C-X-C motif chemokine receptor 6 (CXCR6) signaling toward an M2-like phenotype [69]. It is worth noticing that *in vivo* studies discourage the M1/M2 dichotomy, which highlight the lack of a clear identification of either phenotype. The miss of a clear binary classification is potentially due to the co-expression of either M1 or M2 genes in individual cells and because of the no-totally differentiated state of GBM-related TAMs [68],[70].

4.4.1 TAMs: a two blades knife

TAMs in GBM environment include BMDMs, deriving from peripheral bone-marrow, and microglia, which arise from the haematopoietic yolk sac and migrate in the CNS during the embryonic development. Microglia are mainly distributed in glioma peripheral edge, closed to the regions of infiltration, while BMDMs are spatially distributed in the tumor core, in the close proximity of necrotic and perivascular areas [57]. Infiltrating BMDMs stimulate angiogenesis and support glioma cell invasiveness and suppressive mechanisms through the expression of secreted phosphoprotein 1 (SPP1) and Glycoprotein nmb (Gpnmb). It has been also established the substantial presence of microglial cells in newly diagnosed GBM, whereas macrophages are mainly represented as a consequence of GBM recurrences, mostly in hypoxic niches [71],[69]. BMDMs and microglia show a significant difference in terms of morphological and molecular features. BMDMs are represented by small cells, with short branches and remarkable migratory capacities, whereas microglia are resident and bigger cells, which show a greater number of branches extending into the tumor mass [49]. These immune cells share regulatory characteristics and common biomarkers, such as integrin α-M (CD11b), ionized calcium-binding adapter molecule 1 (IBA1), cluster of differentiation 68 (CD68) and F4/80 (in murine model). Cluster of differentiation (CD45) expression has traditionally been used to differentiate microglia ($CD11b^{+}CD45^{lo}$) from BMDMs ($CD11b^{+}CD45^{hi}$). However, the expression levels of CD45 result an inadequate marker to define a clear difference between these

immune populations, since microglia can upregulate CD45 expression as a consequence of pathological conditions, which certainly include gliomagenesis and tumor progression [67],[57]. Thanks to the advances in genome-wide microarray and single-cell RNA sequencing analysis, microglia and macrophages can be clearly identify by specific biomarkers. Macrophages show high expression of major histocompatibility complex II (MHCII), tyrosine-protein Mer (MERTK) and C-C motif chemokine receptor 2 (CCR2), while microglia, poorly expressing MHCII and CCR2, upregulate C-X3-C motif chemokine receptor 1 (CX3CR1), purinergic receptor P2RY12 and transmembrane protein 119 (TMEM119) expression [68],[62].

4.4.2 TAMs role in supporting GBM progression

TAMs stimulate immunosuppression mechanisms releasing anti-inflammatory molecules, including IL-10 and TGF-β, and promote angiogenesis, through the production of factors as VEGFα. IL-10 promotes neoplastic growth and a suppressive milieu via janus kinase 2/ signal transducer and activator of transcription 3 (JAK2/STAT3) pathway. STAT3 activation enhances the suppression of the neighbouring immune cells by the decrease of MHCII expression, leading to the reduction of TNF and interferon-γ (IFN-γ) in GBM, fostering anti-tumor mechanisms and sustaining a suppressive environment. Moreover, TAMs upregulate PD-L1 expression, positively correlated to gliomas grade, which is another crucial player within GBM immunosuppressive milieu [72],[73]. A further contribution in maintaining an immunosuppressive microenvironment, is also played by TGF-β production, mainly mediated by microglia. TGF-β affects T cells activity, resulting in the blockade of human leucocyte antigen DR (HLA-DR) surface receptor, crucial in tumor-associated antigen presentation to T cells. TGF-β, released by microglia, has several roles in immune-escape mechanisms, leading to the inhibition of natural killer group 2 member D (NKG2D), which mediates tumor host-response, and the promotion of tumorigenesis, allowing GSCs invasion and EMT process [67].

Tumor progression is largely promoted by the high expression of Autotaxin mediated by microglial cells. This enzyme synthetizes lysophosphatidic acid (LPA), a signaling molecule responsible for microglia-GBM interplay which stimulates tumor progression [74]. Furthermore, microglia play a key role in GBM cell migration and infiltration, stimulating the degradation of ECM components, as collagens and proteoglycans, through the upregulation of MMP2 and MMP4 [62]. TAMs are strongly related to GSCs promotion, inducing stemness features by the secretion of IL-1β and IL-6, which in

turn enhances the recruitment of M2-like macrophages [75]. The main TAMs-derived effects in favouring GBM progression are summarized in **Figure 3**.

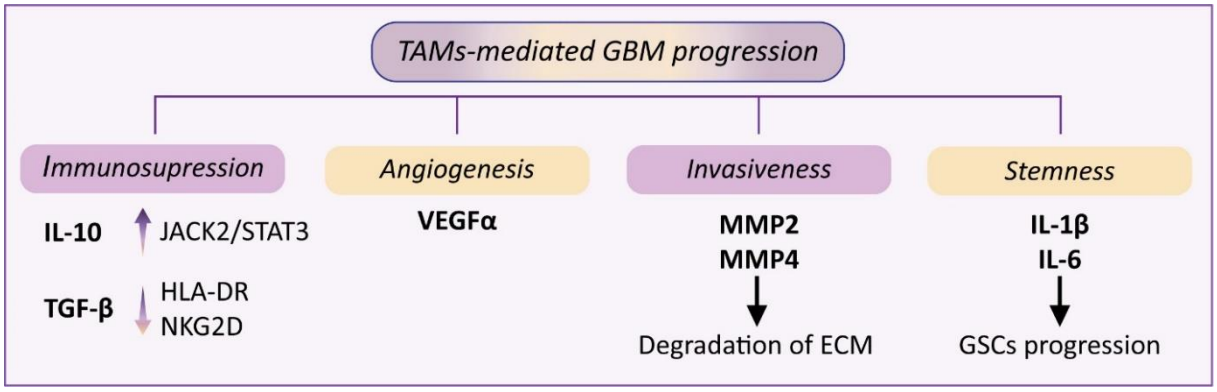

Figure 3: Brief summary of the main TAMs-derived processes responsible for GBM progression. *TAMs play a crucial role in managing and supporting GBM growth and aggressiveness, by activating mechanisms related to immunosuppression, angiogenesis, invasiveness, and stemness. Immunosuppression is orchestrated through the secretion of IL-10 and TGF-β. IL-10 promotes the activation of STAT3, thereby inhibiting the immune response. TGF-β, primarily released by microglia, contributes to immune evasion mechanisms and fosters tumor promotion. Angiogenetic process is triggered by the production of VEGFα by TAMs, promoting the formation of new blood vessels within the tumor. Additionally, TAMs facilitate the infiltrative phenotype of GBM by mediating the degradation of ECM protein components through MMP2 and MMP4 activity. Lastly, TAMs stimulate stemness characteristics, enhancing the proliferation of GSCs.*

4.4.3 Metabolic rewiring in GBM cells and TAMs

Within the tumor bulk, GBM cells are characterized by crucial differences in terms of function, localization and metabolism, showing a remarkable heterogeneity. Moreover, the wide range of inter relationship established between tumor bulk and surrounding environment, allows to identify two different GBM areas: the perivascular niche and the hypoxic niche. In this scenario, it has been proposed that GBM shows a “metabolic plasticity”, which is represented by the coupling between the Warburg effect and reverse Warburg effect [76].

The presence of oxygen and the availability of nutrients and substrates in perivascular niche, GBM cells of this area are able to deliver glucose and substrates in the hypoxic niches. Hypoxia may represent one of the key condition responsible for the metabolic switch from oxidative phosphorylation and glycolysis, resulting in the overexpression of glycolytic pathways, stem-like signature and sustaining of GSCs proliferation as a stress response mediated by cancer cells [77],[76]. This condition involves several pathways and molecules, leading to the up-regulation of the transcription factor named hypoxia inducing factor 1 (HIF-1), PI3K/Akt/mTOR pathway and tumor suppressor p53, generally mutated in tumor cells [78]. Hypoxic condition stabilizes the

regulatory subunit of HIF-1, called HIF-1α, permitting its translocation into the nucleus and allowing the coupling with HIF-1β. This heterodimer binds a specific enhancer sequence, the hypoxia-response element (HRE), leading to the downstream expression of hypoxia-responsive genes [79]. The overexpressed HIF-1 stimulates the expression of glycolysis-involved genes, such as pyruvate kinase M1/M2 (PKM1/2) and lactate dehydrogenase (LDH). Furthermore, HIF-1 exerts a down-regulation of oxidative phosphorylation through the activation of NADH dehydrogenase 1α subcomplex, resulting in the inhibition of complex I of the electron transport chain. Hypoxic tumor cells also upregulate the expression of the glucose transporter 3 (GLUT3), mostly involved in glucose intake and tumor promotion, and of the monocarboxylate transporters (MCTs), such as MCT1 and MCT4, involved in lactate transport [80].

Glycolytic process leads to the production of pyruvate, which can be converted into lactate by PKM2 and LDH. This process is known as Warburg effect, which promotes anaerobic processes, even in the presence of oxygen. This condition implies mitochondrial dysfunctions and oxidative phosphorylation impairment, resulting in a metabolic shift toward glycolysis process [56]. This metabolic rewiring seems to be developed as an adaptive strategy implemented by high proliferative cancer cells to face up the significant energy demand. These cells need to produce energy in a fast and efficient fashion. To this purpose, glycolysis represents a valid metabolic choice. Moreover, glycolysis ensures high production of lactate leading to the enhancement of tumor growth and invasion [81]. Therefore, this metabolic rearrangement increases macromolecules biosynthesis, which provides to DNA and lipid anabolism, sustaining tumor growth. It has been proposed that lactate represents a key molecule in fulfilling ATP and biosynthetic needs into the perivascular niche, which is largely composed by oxidative GBM cells. Hypoxic GBM cells play a crucial role in sustaining oxidative GBM cells by releasing lactate molecules into the perivascular area, permitting its conversion into pyruvate. Through this coupled process, these cells satisfy their energetic demand by the so-called “reverse Warburg effect”, where glycolysis taking place in hypoxic cells supports oxidative metabolism in normoxic GBM cells. Therefore, oxidative phosphorylation represents a potential biomarker of differentiated tumor state, whereas anaerobic processes characterize stemness phenotype. This mutual relationship proves a metabolic symbiosis between these two tumor cell compartments [62],[82]. Taken together, these observations demonstrate the central role of lactate as a key metabolite and signalling molecule, rather than a “waste” product.

Within the CNS, either in physiological or in pathological condition, lactate is recently recognized as a crucial substrate of metabolic flexibility. Concerning brain tumors, this small molecule shows important implications as a bioenergetic fuel not only for oxidative cancer cells, as above described, but also for TME components [83]. The TME acidification, caused by lactate releasing, appears to be related to the functional and metabolic rewiring of recipient cells, including microglial cells (**Figure 4**) [84],[85].

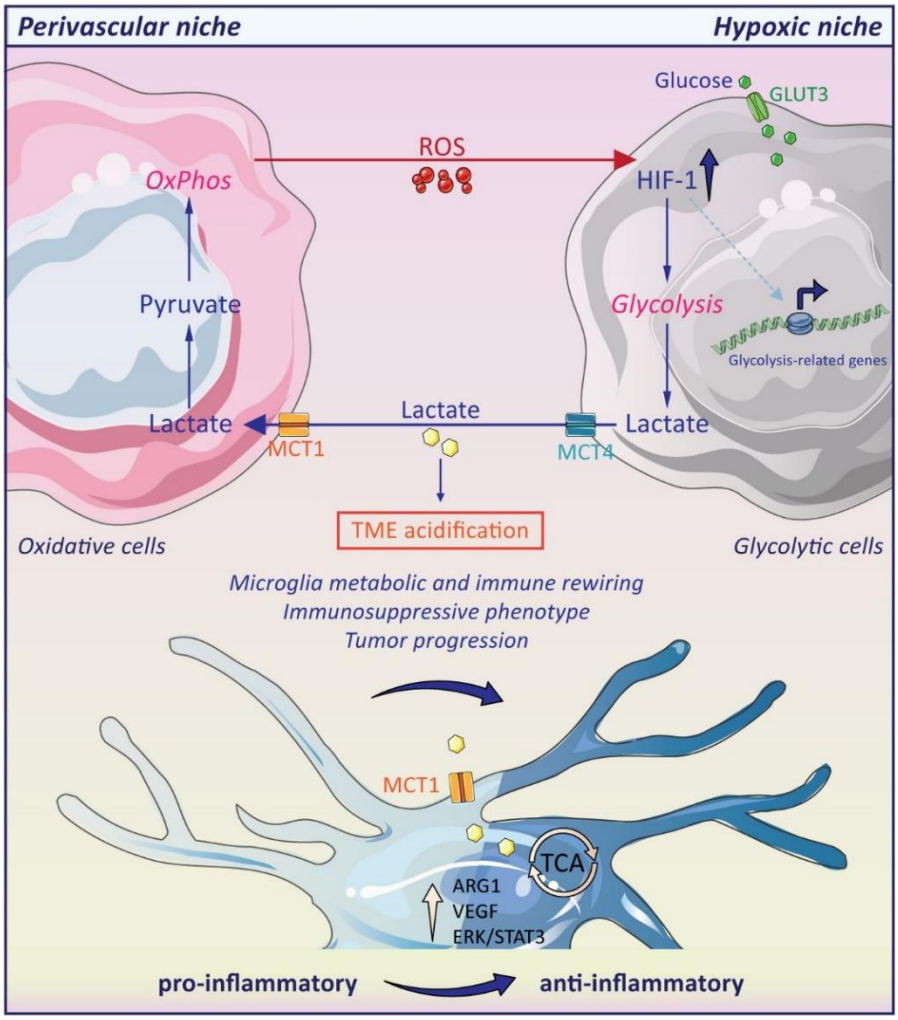

Figure 4: Schematic representation of the crosstalk between metabolically different GBM cells and the effects of lactate release on microglia. *Herein, Warburg effect and reverse Warburg effect are shown (upper part). Different cancer cells in GBM mass are metabolically coupled. In hypoxic cancer cells, stimulated by ROS derived from perivascular niche, glycolytic processes take place. This mechanism allows the production of lactate which is diverted to the perivascular area and there converted in pyruvate. Pyruvate molecules are completely oxidized by oxidative phosphorylation. In turn, this process sustains ROS production. Lactate release leads to TME acidification, which is responsible for metabolic and functional rewiring of cell TME components, including microglia (lower part). Lactate intake is guaranteed by the activation of microglial MCT1, favouring a metabolic rewiring toward TCA cycle and oxidative processes. Moreover, lactate molecules seem to be related to the increase in ARG1, VEGF expression and the activation of ERK/STAT3 pathway. These lactate-induced conditions trigger an immunosuppressive/pro-tumoral microglial phenotype.*

It has been observed that microglial cells, similarly to oxidative cancer cells, import lactate molecules by the activation of transmembrane transporter, MCT1, and specific lactate receptors, including GPR81. Intracellular lactate is promptly oxidized by LDH to pyruvate and then used as substrate for tricarboxylic acid (TCA) cycle. Therefore, lactate intake induces the expression of several genes related to Oxidative Phosphorylation (OxPhos), and anti-inflammatory/immunosuppressive phenotype, which leads to immune-tolerance and support tumor growth [86],[83]. In this scenario, Colegio et al. found that these lactate-derived processes are certainly mediated by HIF-1 expression [87].

Microglial reshape is linked to the activation of a number of pathways, including ARG1 and VEGF, related to immunosuppression, neovascularization and tumor growth, and extracellular signal-regulated kinases (ERK)/STAT3, which is responsible for angiogenesis and cancer cell proliferation, migration and invasion [88],[87]. In GBM-related microglia it has been described a significant downregulation of TGF-β pathway and a decrease in microglia sensing capacity [89]. Nevertheless, most of the derived intracellular processes remains to be elucidated. Microglial metabolic rewiring and immune switch are strongly inter-depended and undoubtedly associated to lactate intake [85].

Due to the competition among TME cell components in terms of nutrients availability, tumor and immune cells need to redirect their metabolic programs implying alternative substrates. The significant consumption of glucose, related to the metabolic activity of glycolytic cancer cells, determines low glucose levels into the TME, which contribute to TAMs exhaustion, resulting in immunosuppression [84]. This anti-inflammatory condition rewires TAMs metabolism triggering fatty acid oxidation (FAO) and OxPhos. FAO and OxPhos processes are supported and enhanced by glutamine consumption, which is employed for anaplerotic reactions to provide intermediate molecules of TCA cycle, such as α-ketoglutarate (α-KG). α-KG is also involved in M2-like phenotype switch in immune cells, blocking the expression of inflammatory genes through the inhibition of nuclear factor kappaB (Nf-kB) activation. The most aggressive GBM subtype, which is represented by mesenchymal tumors, uses glutamine molecules as the major ATP supplier [80],[76]. It has been observed that glutamine deprivation in TME leads to the activation of HIF-1 and the production of IL-23, involved in the suppression of immune response. Moreover, *in vitro* experiments on renal and bladder cancer cell lines, demonstrated that the lack of extracellular glutamine promotes the overexpression of PD-L1, showing immunosuppressive activity after binding PD-1 receptor on immune cells membrane [90]; [91]. As such, it is not surprising that TAMs-related anti-inflammatory

features are mainly observed in hypoxic GBM core, due to the HIF-1 stabilization mediated by lactate intake, while pro-inflammatory phenotype is mostly found in normoxic GBM regions [76],[92].

GBM cells show also an intricate metabolic competition, which pushes GBM cells to quickly adapt and switch their metabolic pathways. This environmental pressure leads to fulfil cell needs employing different sources of energy, including amino acids and fatty acids. It has been well documented that GSCs are able to meet their energetic demands reverting their metabolism toward the utilization of lipids [93]. Cholesterol and sphingolipids represent the most abundant portion of lipid content into the brain tissue. Sphingosine-1-phoaphatase (S1P) is a bioactive molecule regulated by sphingosine kinase 1 and 2 (SK) enzymes, which shows high levels in glioma compared to the healthy brain tissue. S1P demonstrates an important role as signaling molecule in GBM, in which enhance proliferation, migration and cell survival [94].

4.4.4 Single cell omics-based TAMs classification

Single-cell sequencing and cell-omics analysis has allowed not only the identification of TAMs biomarkers but also the classification of TAMs groups, which show wide heterogeneity due to the tumor origins, genetic and epigenetic features, sex and age of the patient and therapeutic approaches [62].

The most recent classification takes into account signature genes, activated pathway and the related functions for which Ruo-Yu Ma and colleagues established seven groups of different TAMs, widely represented in gliomas and GBM: interferon-primed TAMs (IFN-TAMs), immune regulatory TAMs (Reg-TAMs), inflammatory cytokine-enriched TAMs (Inflam-TAMs), lipid-associated TAMs (LA-TAMs), pro-angiogenic TAMs (Angio-TAMs), resident-tissue macrophages-like TAMs (RTM-TAMs), and proliferating TAMs (Prolif-TAMs) [95].

- IFN-TAMs are characterized by INF-regulated genes overexpression, as C-X-C motif chemokine ligand 10 (CXCL10) and PD-L1. Even if, these TAMs show M1-like phenotype, they act suppressing immune response via recruitment of immunosuppressive regulatory T cells.
- Reg-TAMs are closely related to M2-like macrophages, whose phenotype is characterized by ARG1 and CX3CR1 expression. This TAMs subclass plays an important role in immunosuppression mechanisms.

- Inflam-TAMs seem to be associated to pro-inflammatory signature due to the expression of cytokines such as IL-1β, CXCL1, CXCL2 and C-C motif chemokine ligand 3 (CCL3). This profile might be related to the regulation of immune cells into the TME.
- LA-TAMs are strongly characterized by the expression of lipid catabolism-related genes, including apolipoprotein C1 (APOC1) and apolipoprotein E (APOE). Lipid consumption in macrophages significantly supports an immunosuppressive milieu.
- Angio-TAMs represent a subtype correlated to angiogenetic processes, due to the high expression of VEGF-α; SPP1 and versican (VCAN). This TAMs type is responsible for tumor cell intra- and extravasation and therapy resistance, resulting in a positive association with the poor prognosis of GBM patients.
- RTM-TAMs are represented by RTM phenotype, expressing monocyte-associated signature. This subtype presents a significant heterogeneity and is responsible for the promotion of tumor invasion through EMT simulation and regulatory T cell recruitment.
- Prolif-TAMs mainly express proliferation markers, such as Ki-67 and cyclin-dependent kinase 1 (CDK1). This TAMs subtype promotes tumor progression and growth and may represent the precursors of the other six TAMs subclasses.

4.5 RT-induced off-target effects on GBM TME

RT represents one of the first-line therapeutic approaches in many solid tumors. It is certainly the most powerful treatment in terms of cytotoxic effects, causing either direct effects, as single- and double-strand DNA breaks and protein alterations, which induce mitotic and chromosomal aberrations, senescence and death processes, or indirect effects, such as ROS production which in turn leads to DNA damage, protein oxidation and lipid peroxidation. Despite its well-established effectiveness, increased by the improved accuracy of irradiation field, this approach strongly affects not only cancer cells but also surrounding healthy tissue [96],[97].

RT usually results in severe consequences on healthy cells, extracellular milieu and structures composing TME. RT-related side effects include oxidative stress, hypoxia and crucial changes in inflammatory state. These alterations are responsible for angiogenesis-vasculogenesis, cellular senescence, compromised pharmacokinetics and pharmacodynamics, immune modulation and ECM alterations (**Figure 5**). Taken together, all of these effects result in tumor recurrences and increased aggressiveness [98].

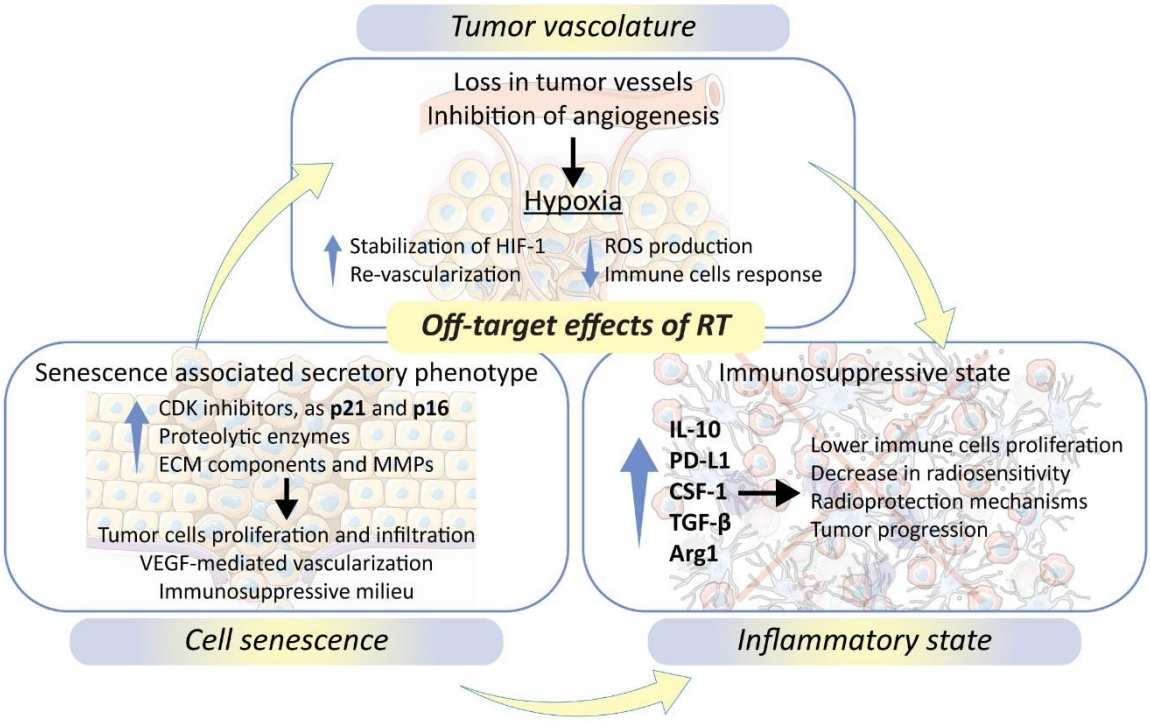

Figure 5: Schematic summary of the main off-target effects induced by RT. *Tumor vasculature constitutes one of the main indirect targets of RT, resulting in a significant reduction in angiogenesis. This condition leads to insufficient oxygen supply, which in turn induces hypoxia. Activation and stabilization of HIF-1, in response to hypoxia, contribute to a significant decrease in RT effectiveness. This is evidenced by a decrease in ROS production, increased vascularization, and heightened immunosuppression. As a consequence of HIF-1 activation, immune escape mechanisms are triggered, leading to the production of IL-10 and PD-L1, which inhibit the proliferation of immune cells. Several factors, such as CSF-1, TGF-β, and Arg1, sustain immunosuppressive polarization, resulting in reduced radiosensitivity, enhanced radioprotection and ultimately promoting tumor growth. Cell senescence also plays a key role in fostering an immunosuppressive environment. Both senescent tumor and stromal cells release factors like CDK inhibitors, proteolytic enzymes, MMPs, and ECM components. This supports GBM aggressiveness, leading to increased survival, infiltration and VEGF-mediated vascularization of tumor mass.*

4.5.1 Off-target effects on tumor vasculature and inflammatory state

RT causes several consequences in pericytes and endothelial cells, compromising tumor vasculature. Radiation-induced alterations result in endothelial cells dysfunctions, including increased permeability, senescence, fibrosis and apoptosis. In GBM, the disruption of vessels and the inhibition of angiogenetic processes may be considered an exploitable effect of RT. Nevertheless, the loss in vasculature leads to adverse outcomes such as the reduced chemotherapy distribution and the decrease of oxygen supply [99]. Indeed, hypoxia is strongly associated with a drop in RT efficacy, due to the reduced oxygen-dependent DNA damage, resulting in a loss of ROS production. Intra-tumoral hypoxic areas promote the activation and stabilization of HIF-1 signalling in GBM cells, leading to an increase of stemness features, radio-resistance and re-vascularization. HIF-1 leads

angiogenesis and vasculogenesis, which are further sustained by VEGF, SDF-1 and angiotensin levels, overexpressed in post-irradiated GBM. SDF-1, in turn, is able to promote the recruitment of macrophages following RT [100],[101]. Nevertheless, hypoxic conditions can also inhibit immune cells proliferation via IL-10 production and PD-L1 up-regulation, which are related to the promotion of immunosuppressive mechanisms [102]. Therefore, the activation of immunosuppressive state, which involves TAMs, TANs and MDSCs, induces the production of several factors such as CSF-1, TGF-β and CCL2. TGF-β is a key molecule in the inhibition of radiosensitivity in tumor cells and plays an important role in supporting and maintaining an immunosuppressive milieu, as well as CSF-1, an overexpressed chemokine, which acts stimulating an anti-inflammatory response. It has been also observed that RT stimulates MDSCs which plays an essential role in increasing radioprotection activity, stimulating tumor progression processes through the up-regulation of ARG1 [103].

4.5.2 Off-target effects induce cell senescence and ECM alteration

Analysing the plethora of the RT-derived side effects, it has been observed an increase of senescence-associated secretory phenotype (SASP) related to both tumor and stromal cells. Senescence is strongly related to the up-regulation of cyclin-dependent kinase (CDK) inhibitors, as p21 and p16. In GBM, these inhibitors are amplified in a dose- and time-dependent manner. SASP is characterized by the production and secretion of inflammatory molecules, ECM components and proteolytic enzymes. These processes allow and sustain survival, proliferation and infiltrative phenotype, promoting the aggressiveness of GBM relapses [99]. SASP shows a paracrine spreading as well, which has a significant impact also on neighbouring cells. Fletcher-Sananikone and colleagues observed that astrocytes, microglia and endothelial cells, in irradiated brain, undergo senescence sustaining GBM progression and contributing to more invasive and aggressive tumor cells compared to mock-irradiated brain. Moreover, these lethal effects increase neighbouring tumor cells proliferation through the establishment of an immunosuppressive milieu, production of ECM components and MMPs, support of VEGF-mediated vascularization, promotion of tumor growth secreting interleukins and growth factors. Senolytic drugs could be a valid adjuvant treatment in managing GBM growth because of their action on pro-survival mechanisms, adopted by both surrounding and tumoral senescent cells [104]. This evidence contributes to increase RT-derived bystander effects on radiation-naïve healthy cells creating a vicious circle in which ROS production and mitochondrial alterations are enhanced, resulting in DNA damage. This leads to the activation of DNA repairing systems, irreversible cell cycle arrest and cellular senescence [105].

Furthermore, radiation can also induce critical alterations in ECM composition, which represents essential factor in favouring tumor cells infiltration and migration. It has been established that RT leads to the up-regulation of proteins and enzymes related to ECM biosynthesis and degradation such as hyaluronin and MPP, respectively and ECM-glioma cell interplay, including integrins and intercellular adhesion molecule 1 (ICAM-1). ECM alterations and the mutated ECM-cell interactions derived from radiation therapy might be considered as novel therapeutic targets [99].

5. Aims

GBM aggressiveness is not only associated with the intrinsic capabilities of tumor cells related to self-renewal regeneration and growth promotion, but it is strongly coupled to the interactions that elapse with its microenvironment. TME represents a crucial player in ensuring tumor progression, stemness and therapy resistance, and it deserves to be deeply investigated.

GBM triggers several processes leading to immunosuppressive TME. Many of these mechanisms can be considered as straight consequences of RT-based approaches.

Our studies aim at analysing the mechanisms underlying the genesis and maintenance of tumor aggressiveness and immunosuppression. We focussed on the intercellular communication between GBM cells and microglia following RT exposure, analysing the potential intracellular and intercellular processes which regulate GBM metabolic reshaping and TAMs-related physiology conditioning

The aims and the key points on which our research has been focussed are defined and summarized hereafter:

a. Analyse the intracellular processes involved in GBM progression and aggressiveness, focussing on the interplay established with the extracellular milieu and on the interaction with the surrounding cell components.
b. Evaluate the significance and the outcome of the crosstalk between GBM cells and its microenvironment, highlighting the role of TAMs in managing tumor growth and metabolic features after the employment of radiation treatment.
c. Underline the off-target effects derived from radiation on the microglia-GBM cells interplay, in particular investigating the derived consequences on GBM cell intracellular mechanisms in terms of metabolic state and mitochondrial fitness.
d. Point out the processes triggered by radiotherapeutic approach into tumor cells and how these mechanisms are associated with the increase of immunosuppressive features and epigenetic modifications.

6. Description of results

GBM pathophysiology is characterized by unmet challenges, which are determined by the high cellular heterogeneity and intricate set of intra- and inter-relationships between tumor and no-tumor neighbouring cells. The multifaceted environment characterizing GBM tumor bulk is a crucial regulator in managing the plethora of processes involved in tumor proliferation, infiltration and immune-escape [50]. In this scenario, the analysis of the biological cell mechanisms which are responsible for tumor progression and the major players that favour an ever-growing complexity is an essential point to develop new therapeutic strategies. Our research work aims at examining some of the main processes within the GBM-microenvironment interplay and biological complexity.

One of the critical process which determines GBM heterogeneity is represented by the widely analysed metabolic reprogramming [106]. The plethora of reshaped metabolic processes, which occurs in tumor cells, represents not only the main cause of tumor resistance and immunosuppressive milieu, but also a side effect of therapeutic strategies adopted to inhibit tumor progression. Even though current GBM therapies are indispensable for limiting tumor growth, they have a strong impact not only on malignant mass but also on the surrounding environment, inducing unavoidable and deep alterations, which mainly regard nutrients availability and immune response remodelling [107],[99].

It has been observed that microenvironment is able to alter tumor cell state through the modulation of many essential intracellular pathways, which exerts a key role in regulating glioma proliferation and invasiveness, thus promoting stemness features. Many pathways play a key role GBM aggressiveness, including Notch, Wnt and SHH signalling pathway, which regulate GBM progression and heterocellular crosstalk between tumor and surrounding healthy tissue [108]. In this scenario, the activity of SHH signalling pathway is deregulated, inducing cell growth and favouring infiltrative phenotype, angiogenesis and self-renewal processes. One of the critical components of SHH pathway is represented by smoothened (SMO), which allows the non-canonical activation of SHH [109]. SMO function can be direct regulated by several pharmacological modulators that show inhibitory and stimulatory activity. One of the main SMO agonists is purmorphamine, which leads to the GLI-mediated expression of genes involved in the suppression of apoptosis and in the promotion of proliferation and stemness [110]. On the contrary, a noteworthy SMO antagonist is represented by cyclopamine, which bind SMO transmembrane domain in order to avoid SMO

activation. Preclinical studies demonstrated the cyclopamine-mediated blocking effects on tumor growth [111],[112].

Due to the importance of the inter-relationship between surrounding environment and intracellular signalling regulation, we moved to evaluate one of the main classes of trans-membrane proteins which allow intercellular communication, which is represented by connexins (Cxs). Cxs function, which includes cell-to-cell interaction and paracrine molecules exchanging, is accomplished after the formation of gap junctions and hemichannels [111],[113].

Our evidence suggests that aberrant and overexpressed SHH signalling is strongly related with Cxs activity. We observed that this axis seems to be associated with an increased GBM progression and invasiveness. CX43 represents a crucial mediator in tumor trophism, which strongly influences GBM milieu and intercellular communication. We evaluated SHH-CX43 interplay, through the assessment of cyclopamine/purmorphamine-related modulation of SMO, in order to understand the potential involvement in GBM cells proliferation and their migratory capabilities. We found that the administration of purmorphamine promoted GBM cells proliferation, instead cyclopamine acted reducing the percentage of surviving fraction compared to the no-treated control. Moreover, we found that SMO agonist stimulated not only the expression of SHH-related proteins, including GLI1 transcription factor, but also of CX43, thus suggesting an interconnection between these two pathways. Otherwise, our results showed that cyclopamine administration had no effects on both GLI1 and CX43 expression.

The correlation between CX43 expression and SHH downstream activation was confirmed by the evaluation of GBM cells migration, which resulted enhanced after SMO-mediated SHH activation. Purmorphamine activity was reverted by the administration of a specific CX43 inhibitor, namely ioxynil octanoate, indicating a synergistic role of SHH pathway and CX43 in favouring GBM cells motility and invasiveness.

It has been established that SMO-mediated SHH activation has significant consequences in tumor progression, stimulating EMT and angiogenetic processes, leading to immunosuppression and diverting TAMs polarization through M2-like/pro-tumoral phenotype [114]. Furthermore, the role of gap junctions and hemichannels in conditioning and managing the cellular relationships of TME, and their role in cell-to-cell crosstalk and paracrine signalling has been well-documented. Taken together, all of these observations support the hypothesis of a SHH-CX43 axis and its potential

coordinated and coupled role in the regulation GBM aggressiveness and in managing GBM-TME synergy [115],[116].

Focussing on the plethora of existing interactions within GBM TME components, it should be underlined the crucial interplay between tumor and immune cells, which shows a multitude of significant effects in modulating TME pathophysiology. This mutual influence leads to a wide range of effects, favouring tumor progression and supporting the establishment of immune-escape mechanisms, resulting in a stronger tumor development [67],[68]. Dramatic changes in cellular interplay and TME reprogramming frequently occur as side effects of therapies, which induce an increase in cell stress response and the reshaping of metabolic and epigenetic features [107]. It has been well documented that RT, more than others therapeutic strategies, leads to a multitude of side effects, that trigger GBM progression, angiogenesis and immunosuppressive phenotype [117]. At the basis of these pro-tumoral effects, it could be identifying a radical rewiring of intra- and inter-cellular processes which compromise oxygen and substrates availability, promoting a metabolic cell state remodelling [96].

We focussed our research on the off-target effects induced by radiation treatment on the heterocellular interplay between GBM cells and microglia. We observed that irradiated microglia-derived conditioned media (CM), used as a treatment for naïve GBM cells, were able to sustain tumor proliferation, preventing death processes and promoting GBM clone formation. On the contrary, the exposure to irradiated GBM-derived CM induced a decrease of naïve GBM cells survival, causing apoptosis and cell death. Additionally, our evidence showed that irradiated microglia CM preserved GBM mitochondrial mass, morphology and fitness, maintaining mitochondrial ROS production at the same levels of the no-treated cells. This evidence suggested a protective effect mediated by irradiated microglia on GBM cells health.

Moreover, the effects of metformin were assessed on GBM clone formation, concurrently administrated with irradiated microglia-derived CM treatment. Metformin is an FDA-approved drug which acts inhibiting complex I activity, thus arresting oxidative phosphorylation process. When co-treated, radiation-naïve GBM cells showed a significant decrease in terms of GBM cells proliferation and percentage of surviving fraction. Therefore, we speculated that irradiated-microglia CM were able to divert GBM cells metabolic state toward oxidative phosphorylation, thus favouring cell energy production and proliferation. This observed process could be identified as the well-known “reverse Warburg effect”, for which TME cell components, including tumor and no-tumor cells, are

involved in a metabolic symbiosis which stimulates and sustains metabolic plasticity [118]. RT-induced release of paracrine factors and small molecules act as potential mediators of metabolic oxidative reshaping, reverted by metformin-induced oxidative phosphorylation blockage.

Our observations confirm the crucial impact of RT on TME and GBM cells biology and metabolism and highlight the reinforced pro-tumoral interplay between TAMs and GBM cells, especially after repeated and/or high dose radiation treatment.

The heavy changes characterizing tumor and no-tumor cells metabolism are strongly associated to immunosuppression. Immune response is significantly compromised by the high dynamism of metabolic conditions, resulting in a failure of immunotherapeutic-based approaches. The high demand of glucose by GBM cells, induced by the Warburg effect which increases glycolytic process, determines constant changes in substrate availability, leading to a continuous and inescapable metabolic reprogramming. Such a phenomenon leads to oxidative stress response which mainly causes a decrease of oxygen availability [73],[56]. Hypoxic environment promotes HIF activation which produces downstream effects on transcription processes, favouring the expression of genes related to metabolic rewiring and immunosuppression.

The coupled action, resulting from epigenetic modifications and metabolic rewiring, favours significant increase of cell stress response, immune-escape mechanisms and tumor progression, promoting tumor-resistance and cell senescence [76],[119]. It has been well-established that several enzymes and oncometabolites are able to induce a plethora of epigenetic changes. For instance, isocitrate dehydrogenase (IDH) activity, catalysing α-KG and NADPH production, is strongly associated with the hypermethylation state of GpC islands, which determines the methylation profile and the malignance grade of glioma. Mutations in IDH structure lead to the production of the oncometabolite 2-hydroxyglutarate (2-HG), inducing the blockage of demethylation processes and the activation of DNA damage response systems [120].

Moreover, it is noteworthy the role of lactate as oncometabolite, which acts as a mediator of either metabolic reshaping, inducing TME acidification and a subsequent switch toward oxidative metabolism, or epigenetic mechanisms, acting at the level of lactylation sites on histone core. Lactylation processes promote tumorigenesis through the modulation of cell metabolism, proliferation and invasiveness. Furthermore, acetyl-CoA and succinyl-CoA play a key role in producing epigenetic changes associated to acetylation and succinylation processes, which promote tumor-resistance, metabolic reassessment and a stronger tumor development [85],[121].

Epigenetic deregulation, resulting from oncometabolites activity, is also related to immunosuppressive state of GBM milieu. Hypermethylation is one of the main consequences of 2-HG production that sustains the transcription of HRE, resulting in the promotion of immune escape processes which induce anti-inflammatory cytokines production and immunosuppressive MDSCs recruitment [122]. The methylation state also regulates the expression of glycolytic enzymes, such as PKM1/2, which promotes MYC expression [123]. This oncogene manages PD-L1 expression, enhancing the activity of this immune checkpoint inhibitor in GBM cells and braking T cells proliferation [124].

GBM glycolytic processes lead to low extracellular levels of glucose, increasing lactate production and sustaining the exhaustion process of immune cells. Immunosuppression is further supported and maintained by the chronic exposure to chemokines and cytokines released by resident microglial cells and the plethora of the immune cells recruited at the level of GBM microenvironment [125],[67]. The inhibition of immune response is also encouraged by the disruption of myeloid cells, which is mediated by the invasive chemo- and radio-therapeutic approaches employed [126].

GBM cells are frequently characterized by mitochondrial impairment, which is responsible for the increase of superoxide radical production, thus resulting in increased oxidative stress. This condition induces the activation of antioxidant systems as defensive mechanism or leads to the increase of genomic instability, favouring tumor development. Protection mechanisms trigger resilience processes, in a condition defined mitohormesis, which promote a recovery of cell survival after ROS-derived mitochondrial damage, through the adaptation of cell metabolism [127],[128].

Taken together, this body of evidence confirms the strong interconnection between metabolic reprogramming, epigenetic changes and immunosuppressive features that determine GBM TME pathophysiology. All of these complex and intriguing fields result in an intricate synergism, which should be deeply analysed in order to overcome the obstacles and the adverse outcome of immunotherapeutic approaches.

7. Concluding Remarks

Due to the magnitude of GBM progression, TME pathophysiology and the intricate plethora of existing correlations, the multiple biological mechanisms leading to this crucial crosstalk remain still unclear. The interplay between immunosuppression and metabolic rewiring results crucial in pathophysiological processes that govern GBM and TME biology [99]. On the one hand, RT represents one of the most powerful and effective strategies for the treatment of brain tumors, on the other hand the well-known GBM complexity becomes even more cryptic following this therapeutic approach [107],[129]. In this scenario, TAMs diversity in GBM milieu strongly increases the plasticity of GBM TME, determining an even more complex tumor background. Target TAMs populations could be a promising approach, which takes into account the diversity of the wide range of immune cells, considering also the critical role exerted by chemokine and cytokines pools. These secreted molecules play an essential role not only in relation to inflammatory response but also as mediators of radiation-induced effects [130],[131]. This evidence should be emphasized to better address tumor heterogeneity and development. Therefore, the challenging nature of the GBM microenvironment and the related interplay with its milieu need to be further investigated to develop innovative therapeutic approaches.

8. References

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9. Articles in extenso

Paper 1*:* ***Connexin 43 and Sonic Hedgehog Pathway Interplay in Glioblastoma Cell Proliferation and Migration.*** *Torrisi F, Alberghina C, Lo Furno D, Zappalà A, Valable S, Li Volti G, Tibullo D, Vicario N, Parenti R. Biology (Basel). 2021 Aug 12;10(8):767. doi: 10.3390/biology10080767. PMID: 34439999; PMCID: PMC8389699.*

Paper 2*:* ***Microglia and glioblastoma heterocellular interplay sustains tumor growth and proliferation as an off-target effect of radiotherapy****. Manuscript is currently under revision on Cell proliferation (Wiley).*

Paper 3*:* ***Epigenetics and Metabolism Reprogramming Interplay into Glioblastoma: Novel Insights on Immunosuppressive Mechanisms.*** *Torrisi F, D'Aprile S, Denaro S, Pavone AM, Alberghina C, Zappalà A, Giuffrida R, Salvatorelli L, Broggi G, Magro GG, Calabrese V, Vicario N, Parenti R. Antioxidants (Basel). 2023 Jan 18;12(2):220. doi: 10.3390/antiox12020220. PMID: 36829778; PMCID: PMC9952003.*

Paper 1

biology

MDPI

Article

Connexin 43 and Sonic Hedgehog Pathway Interplay in Glioblastoma Cell Proliferation and Migration

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Simple Summary: Glioblastoma is the product of accumulated genetic and epigenetic alteration where tumor cells support each other through cellular communication mechanisms and deregulated signalling processes. The autocrine and paracrine pathways between the intracellular and extracellular milieu is mediated by connexin 43, the main gap junction-forming protein driving glioblastoma progression. In this scenario, sonic hedgehog pathway, a key deregulated pathway involved in cell network signalling may affect connexin 43 expression, promoting glioblastoma pathobiology. In this study, we sought to explore how the modulation of the sonic hedgehog affects connexin 43 inducing glioblastoma hallmarks. To do this we evaluated biological effects of sonic hedgehog pathway modulation by purmorphamine and cyclopamine, a smoothened agonist and antagonist, respectively. We revealed that cell migration and proliferation are associated with connexin 43 expression upon sonic hedgehog modulation. Our study suggests that sonic hedgehog and connexin 43 axis may represent a potential therapeutic strategy for glioblastoma.

Abstract: Glioblastoma (GBM) represents the most common primary brain tumor within the adult population. Current therapeutic options are still limited by high rate of recurrences and signalling

axes that promote GBM aggressiveness. The contribution of gap junctions (GJs) to tumor growth and progression has been proven by experimental evidence. Concomitantly, tumor microenvironment has received increasing interest as a critical process in dysregulation and homeostatic escape, finding a close link between molecular mechanisms involved in connexin 43 (CX43)-based intercellular communication and tumorigenesis. Moreover, evidence has come to suggest a crucial role of sonic hedgehog (SHH) signalling pathway in GBM proliferation, cell fate and differentiation. Herein, we used two human GBM cell lines, modulating SHH signalling and CX43-based intercellular communication in in vitro models using proliferation and migration assays. Our evidence suggests that modulation of the SHH effector smoothened (SMO), by using a known agonist (i.e., purmorphamine) and a known antagonist (i.e., cyclopamine), affects the CX43 expression levels and therefore the related functions. Moreover, SMO activation also increased cell proliferation and migration. Importantly, inhibition of CX43 channels was able to prevent SMO induced effects. SHH pathway and CX43 interplay acts inducing tumorigenic program and supporting cell migration, likely representing druggable targets to develop new therapeutic strategies for GBM.

Keywords: GBM; connexin; gap junction; smoothened; GLI1

Introduction

Despite the multimodal and synergistic approaches, combining surgical, pharmacological and radiotherapeutic strategies, glioblastoma (GBM) is still the most lethal brain tumor, characterized by high rate of recurrences and a dismal prognosis [1,2]. Emerging research in the field, has led to the investigation of molecular pathogenesis in order to find the biological mechanisms underlying GBM growth, aggressiveness and resistance [3-7].

Sonic Hedgehog (SHH) represents one of the most relevant signalling pathways mediating both cell fate and differentiation [8–10]. The activation of 7-transmembrane protein Smoothened (SMO) determines the GLI-Kruppel family member 1 (GLI1) nuclear translocation promoting proliferation, stem cell renewal and survival [10-12]. SHH dysregulation has been reported in brain tumors, including GBM [13-15]. Indeed, proliferation and self-renewal of GSCs are regulated by SHH signalling activation [16-17], whereas their inhibition have been associated to an increased chemotherapy response, reducing of GSCs resistance and maintenance [18-20]. Moreover, infiltrative growth of GBM has been associated to the aberrant activation of SHH pathway by the enhancement of migration ability [21]. SHH pathway is also a key component of the autocrine and

paracrine signalling promoting tumor progression, due to uncontrolled proliferation, sustained angiogenesis and invasiveness [22].

Intercellular communication in GBM represents an active research field. Indeed, cell network and autocrine/paracrine signalling were found to modulate the molecular mechanisms of GBM proliferation [23]. In particular, cell-to-cell and cell-to-extracellular fluid communication stimulate GBM cells migration, enhancing the infiltrative pattern, preluding to therapeutic failure and inevitable recurrences [24,25].

Connexins (Cxs) are integral membrane proteins that assemble to form gap junctions (GJs), mediating a direct cytoplasmic connection between adjacent cells, and hemichannels (HCs), providing autocrine and paracrine pathways between the intracellular and extracellular milieu [26]. Allowing exchanges of ions, metabolites, second messengers and small molecules (less than 1000 kDa), GJs and HCs represent key cellular substrates of many significant biological processes throughout life in both physiological and pathological conditions [27-29]. Particularly, GJs- and HCs mediated intercellular crosstalk represents an undoubted way through which different cell types regulate tumor development and progression. Controlling Cxs expression and activity implies significant changes in microenvironment composition and intercellular signalling, a major contributor in tumor cell stimulation and stress resistance.

Among the most important Cxs involved in tumor trophism, connexin 43 (CX43) implication is supported by a plethora of data, describing CX43-based channels as major microenvironmental conditioning mediators [26,28,30]. It has been proposed that CX43 regulates the expression levels of proteins involved in cell growth independently by their channel forming properties, given the multifaceted role of CX43 carboxyl tail, which exerts a number of effects ranging from controlling the translocation of transcription factor regulators into the nucleus to the enhancing of the migration of glioma cells out of the tumor core by interacting with cytoskeleton elements [31]. The close relationship between CX43 and SHH consistently stands out in several scenarios. It appears primarily during embryonic development, when direct cell-to-cell communication is the key mechanism for structures patterning; in this context, for instance, synergistic SHH signalling and CX43 expression, establishing GJs networks synchronizing the Ca^{2+} profile among cells, coordinate collective cell movements, strictly dependent on SHH signalling activation [32]. This phenomenon also occurs in tumor-associated conditions characterized by cell invasion, further confirming a mechanistic link between development and tumorigenesis [33]. Based on such evidence, we hypothesized that a close association between CX43 and SHH pathway in promoting cell network,

cell renewal and tumor microenvironment may be critical to sustain the GBM malignancy progression.

For this purpose, we first evaluated in vitro the metabolic and cytotoxic effect of purmorphamine and cyclopamine, a SMO agonist and antagonist, respectively [34,35], and the biological effect of SHH pathway modulation with particular regard to the expression of CX43 and cell migration.

Materials and Methods

Cell lines Culture and SMO Modulation

Experiments were performed using U-251 MG and T98-G human glioblastoma cell lines. Cells were purchased from European Collection of Authenticated Cell Cultures (ECACC, Public Health England, Porton Down Salisbury, Wiltshire, UK) and cultured in DMEM high glucose supplemented with 10% Serum Fetal Bovin (FBS), 100 IU/mL penicillin (Penicillin-Streptomycin solution) and 2 mmol/L glutamine. Cells were maintained in an exponentially growing culture condition in an incubator at 37 °C in a humidified atmosphere (95% air and 5% CO_2) and were routinely sub-cultured in standard tissue culture flasks. All experiments employed cells at a passage $n < 25$.

Purmorphamine (Cat#72204, Stem Cell Technologies), cyclopamine (Cat#S1146, Selleckchem, Rome, Italy) and ioxynil octanoate (Cat#33381-100MG, Merk, Milan, Italy) were prepared as a stock solution at 10 mM and stored at −20° C. For cells treatment, drugs were diluted at the final concentration in culture medium not exceeding 0.5% of Dimethyl sulfoxide (DMSO, Merk, Milan, Italy) in order to not affect cell viability. Untreated cells were supplemented with DMSO, as vehicle.

Rate of Cell Growth Assay

The rate of cell growth (R.C.G.) was calculated as previously described [11]. Briefly, cells were seeded on six multiwell plates at a final density of 2×10^4 cells/cm^2. Cells were counted and seeded at the same density every 2 or 3 days for a total of five consecutive passages. The R.C.G. was calculated by counting the number of viable cells by trypan blue vital stain exclusion and dividing it by the number of plated cells; ratios were divided by the number of days per passage.

Immunofluorescence

Immunofluorescence analysis on GBM cell lines was performed as previously described [36]. Briefly, cells were seeded in cover glass placed into multiwell 24 plates at final density of 2×10^4 cells/cm^2. Cells were fixed in 4% paraformaldehyde (PFA) at room temperature for 10 min. Then, cells were incubated with blocking solution (10% normal goat serum, NGS, in PBS) for 1 h at room temperature.

Samples were then incubated overnight at 4 °C with the following primary antibodies diluted in incubating solution (1% NGS in PBS): mouse anti-GFAP (1:500, Cat#MAB16117, RRID: N/A, Immunological Sciences); rabbit anti-CX43 (1:200, Cat#3512S, RRID: AB_2294590, Cell signalling); rabbit anti-SMO (1:1000, Cat# ab72130, RRID: AB_1270802, Abcam, Cambridge, UK), rabbit anti-Ki67 (1:200, Cat#ab15580, RRID: AB_443209, Abcam, Cambridge, UK). Then, after three washes in PBS, samples were incubated for 1 h at room temperature with the appropriate combination fluorescence goat secondary antibodies: Goat anti-mouse Alexa Fluor 488 (1:1000, Cat# A-11001, RRID: AB_2534069, Thermo Fisher Scientific); Goat anti-mouse, Alexa Fluor 546 (1:1000, Cat# A11003, RRID: AB_2534071, Thermo Fisher Scientific); Goat anti-rabbit, Alexa Fluor 488 (1:1000, Cat# A27034, RRID: AB_2536097, Thermo Fisher Scientific); Goat anti-rabbit, Alexa Fluor 546 (1:1000, Cat# A11010, RRID: AB_143156, Invitrogen). Nuclei were counterstained with 4',6-diamidino-2phenylindole (Dapi, 1:1000, Cat# D1306, Invitrogen) for 5 min at room temperature. Slides were mounted with fluorescent mounting medium Permafluor (ThermoScientific) and digital images were acquired using a Leica DM IRB (Leica Microsystems, Buccinasco, Milano, Italy) fluorescence microscope or the Leica TCS SP8 confocal microscope. In order to quantify the fluorescence intensity of CX43, n = 4 representative regions of interest (ROIs) were quantified by application of a Isodata threshold using ImageJ analysis software. Data were normalized over ROI total area and expressed as mean fluorescence intensity fold change (FC) over control ± SEM. CX43 frequency distribution was calculated on ROI based on GFAP immunofluorescence signal and the mean fluorescence intensity of CX43 was divided by the ROI area. Data are shown as violin plots of this ratio for $n \geq 20$ cells per group.

Cytotoxicity and Metabolic Turnover Assays

To assess cytotoxicity and metabolic turnover, we used Lactate dehydrogenase (LDH) assay (i.e., cytotoxicity) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (i.e., metabolic turnover) as previously described [28,37], with minor modifications. Cells were distributed in 96-well plates (Costar, Milan, Italy) at a final density of 10,000 cells/well/100 µL and incubated for 24 h. The day after, cells were exposed to drugs as above described, and incubated for 4, 24 and 48 h. On the day of each time point, medium was removed and processed as manufacturer's instructions for the LDH-viability assay (CytoSelectTM LDH cytotoxicity assay kit, Cell Biolabs, Milan, Italy). For metabolic turnover, MTT at a final concentration of 1 mg/mL was added to each well and incubated for 3 h under standard culture conditions. Media were then gently removed, 200 µL of MTT solvent (DMSO) was added, and cells were stirred on an orbital shaker for

10 min at room temperature. The absorbance was measured using a Varioskan Flash spectrophotometer (Thermo Scientific, Milan, Italy) at 570 nm. Data were expressed as the percentage of MTT reduction versus control cells. Each experiment was performed three times with six replicates per condition during each experimental run.

Clonogenic Assay

Clonogenic assay was performed as previously described [38]. Briefly, U-251 MG and T98-G cells were plated at 50/cm^2 cells per well in triplicate, and were incubated for 8 h, allowing them to attach on the well plate. Then, cells were treated with either vehicle, purmorphamine or cyclopamine at a final concentration of 0.1 µM, 1 µM and 10 µM. Each plate was incubated for 7–10 days, and after colonies were formed, they were fixed with 4% PFA for 15 min and stained with 1% crystal violet for 30 min at room temperature. Colonies which accounted for more than 50 cells were considered as clones. Each assay was repeated in triplicate in three independent experiments. Surviving fractions were calculated as the ratio of colonies counted over the cell plated multiplied for the plating efficiency of the vehicle, according to the protocol of Franken et al. [39].

Immunoblotting

For Western blot analysis, cells were seeded in six multiwell plates at final density of 3 × 10^6/well and incubated at 37 °C before drug exposure. Drugs were added at the final concentration of 1 µM on cells and maintained for 24 h. Then, cells were washed in PBS, detached by scraper and centrifuged for 5 min at 1200 rpm to collect dry pellet, that were stored at −80 °C until use. Proteins were extracted using RIPA Lysis Buffer (50 µL/sample; Abcam, Cambridge, UK) supplemented with protease inhibitor (1:100, Cat# P8340, Merk, Milan, Italy). Briefly, samples were incubated for 20 min at room temperature and centrifuged at 13,000× *g* for 3 min. An equal amount of proteins (30 µg) were electrophoresed on 4−20% SDS-PAGE gels and transferred to nitrocellulose membranes as previously described [40,41]. Membranes were incubated for 1 h at room temperature with blocking buffer (5% non-fat milk in 0.1% tween-20 in PBS) and then overnight at 4 °C with primary antibodies diluted in blocking buffer. The following primary antibodies were used for immunoblotting: Rabbit anti-GLI1 (1:1000, Cat# ab49314, RRID: AB_880198, Abcam, Cambridge, UK), Rabbit anti-CX43 (1:1000, Cat# C6219, RRID: AB_476857, Merk, Milan, Italy), and mouse anti-GAPDH (1:1000, Cat# ab181602, RRID: AB_2630358, Abcam, Cambridge, UK). Then, membranes were washed three times in 0.1% tween-20 in PBS and then incubated for 1 hr at room temperature with the appropriate

secondary antibody: IRDye 800CW Goat anti-mouse (1:5000, Cat# 925-32210: RRID: AB_2687825, LI-COR Biosciences) or goat anti-rabbit (IRDye 680RD; LI-COR Biosciences, Cat# 926-68071, RRID: AB_2721181, 1:10,000). Proteins bands were imaged using an Odyssey Infrared Imaging Scanner (LICOR Biosciences, Milan, Italy) and protein levels were quantified by densitometric analysis. The density of each band was quantified using ImageJ analysis software and the band density was normalized to the GAPDH optical density measured in the same membrane. For immunoblotting quantification, the density of each band was quantified using ImageJ analysis software and band density was normalized to the GAPDH optical density measured in the same membrane. All values are shown as the mean fold change (FC) over control ± SEM.

Migration Assay

U-251 MG and T98-G cells were seeded in 24-well plates at a final concentration of 3×10^5/well and grown until cells reached about 90% confluency. The day after a central and linear scratch was created using a 200 µL tips and samples were washed with PBS to remove residuals cells; media were then replaced with migration medium (DMEM high glucose, 100 IU/mL Penicillin-Streptomycin and 2 mmol/L glutamine) containing vehicle, 1 µM purmorphamine and/or 1 µM cyclopamine added alone or in combination with ioxynil octanoate at a final concentration of 10 µM. For the quantification of migration index, the total scratch area was segmented from one edge to the opposite edge and the total size of the wound was then measured at 0 and 24 h post scratch and migration index was calculated as percentage ratio of scratch area at 0 h -scratch area at 24 h over scratch area at 0 hrs. All values are shown as the mean FC over 0 hrs ± SEM.

Statistical Analysis

All tests were performed in GraphPad Prism (version 5.00, GraphPad Software, San Diego, CA, USA). Data were tested for normality using a D'Agostino and Pearson omnibus normality test and subsequently assessed for homogeneity of variance. For multiple comparison, one-way ANOVA was used where appropriate, followed by Holm–Šídák post-hoc test.

Results

Human GBM Cell Lines Actively Express SMO

We first characterized two GBM-derived cell lines, studying their stability over passages in vitro (**Figure S1**) and their R.C.G. (**Figure 1a**). Our data showed that U-251 MG and T98-G displayed a similar profile in terms of total number of cells over passages (P1–P6) and that no statistical

differences were observed in R.C.G. (respectively: 2.15 ± 0.10 vs. 2.17 ± 0.14 for U-251 MG and T98-G). We further expand our characterization to study the potential of our cell lines to respond to known modulators of SHH signalling pathway acting on SMO. To do that, we performed an immunofluorescence analysis for GFAP and SMO, confirming the astroglial origin of analysed cells and the presence of SMO on all tested cell lines (**Figure 1b**). This evidence indicated that U-251 MG and T98-G expressed the druggable target SMO.

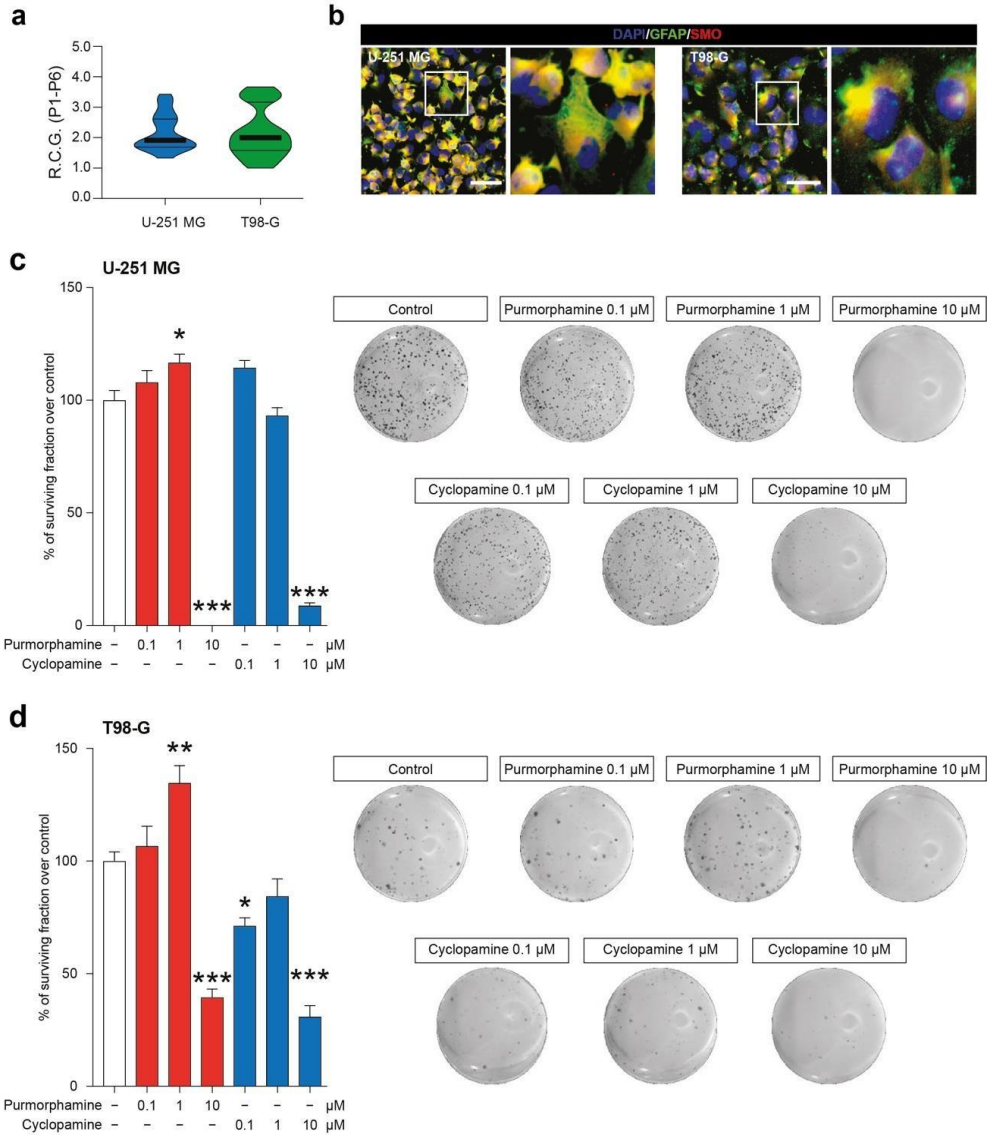

Figure 1. SMO modulation in U-251 MG and T98-G cells impacts on surviving fraction. *(a) Rate of cell growth of human U-251 MG and T98-G cell lines, data are shown as violin plot of R.C.G. of n = 3 replicates over n = 6 passages. (b) Representative microphotographs of human U-251 MG andT98-G cells expressing GFAP and SMO; nuclei were counterstained with DAPI; scale bar = 20 µm. (c,d) Surviving fraction and representative wells of U-251 MG (c) and T98-G cell lines (d) exposed to increasing concentration of purmorphamine and cyclopamine (0.1–10 µM). Data are expressed as mean ± SEM of n = 3 independent experiments; * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001 versus control cell cultures treated with vehicle; one-way ANOVA with Holm–Šídák post-hoc test. R.C.G.: rate of cell growth. P: passage.*

Modulation of SHH Signalling Pathway Impact Cytotoxicity, Metabolic Turnover and Cell Proliferation

Given the presence of SMO, we decided to test cytotoxicity at 4, 24 and 48 h on human GBM cell lines exposed to SMO modulators using a known agonist (i.e., purmorphamine) and a known antagonist (i.e., cyclopamine) at concentration ranging from 0.01 to 10 µM. We performed a lactate dehydrogenase assay showing no significant effects in U-251 MG and T98-G as compared to vehicle treated controls (**Figure S2**). These results showed that, in our experimental conditions, neither purmorphamine nor cyclopamine exerted significant cytotoxic effects on GBM cell lines as a single dose of 0.01 to 10 µM.

In order to evaluate a potential impact of SMO modulation on metabolic and mitochondrial function, we performed an MTT turnover assay. Notably, in U-251 MG cells a statistically significant increase in MTT turnover was observed as soon as 4 h post-exposition in cultures treated with 10 µM of purmorphamine (129.6 ± 5.2% purmorphamine 10 µM; *p*-value < 0.05; **Figure S3**). This effect was also present at 24 h with both 1 µM and 10 µM of purmorphamine treated cultures (135.6 ± 6.7% purmorphamine 1 µM and 132.5 ± 12.3% purmorphamine 10 µM; *p*-value < 0.05; **Figure S3**). In T98-G cell line we were not able to observe any significant effect on MTT turnover at 4 hrs post-exposition with purmorphamine. Interestingly, at 24 h T98-G cells exposed to purmorphamine significantly increase their MTT turnover (138 ± 7.7% purmorphamine 10 µM; *p*-value < 0.01; **Figure S3**). This effect was retained for 48 hrs post purmorphamine exposition (136 ± 9.1% purmorphamine 10 µM; *p*-value < 0.01**; Figure S3**). In addition, in T98-G, but not in U-251 MG, a transient but significant reduction of MTT turnover at 4 hrs after 10 µM cyclopamine exposition was observed (34.9 ± 1.7% cyclopamine 10 µM; *p*-value < 0.001; **Figure S3**).

This evidence prompted us to perform clonogenic assay in order to evaluate the long-term effects of SHH pathway modulation in colonies formation and, particularly, the ability of cyclopamine to reduce cell proliferation. The colonies formation assay revealed that both cell lines were significantly impacted by 1 µM purmorphamine as compared to the untreated cells (117 ± 3.8%; *p*-value < 0.05 vs. 100 ± 3.8% vehicle for U-251 MG; 135 ± 7.7%; *p*-value < 0.01 vs. vehicle 100 ± 4.1% for T98-G; **Figure 1c,d**). This concentration was able to increase the % of surviving fraction, thus supporting the idea that SHH pathway foster GBM proliferation. Of note, despite LDH and MTT data, clonogenic assay on U-251 MG and T98-G cell lines revealed that long-term exposition to 10 µM of purmorphamine strikingly reduced the % of surviving fraction (**Figure 1c,d**). Of note, a reduction of

surviving fraction was observed in both cell lines exposed to increasing concentration of cyclopamine, in particular with 10 µM of drug (**Figure 1c,d**).

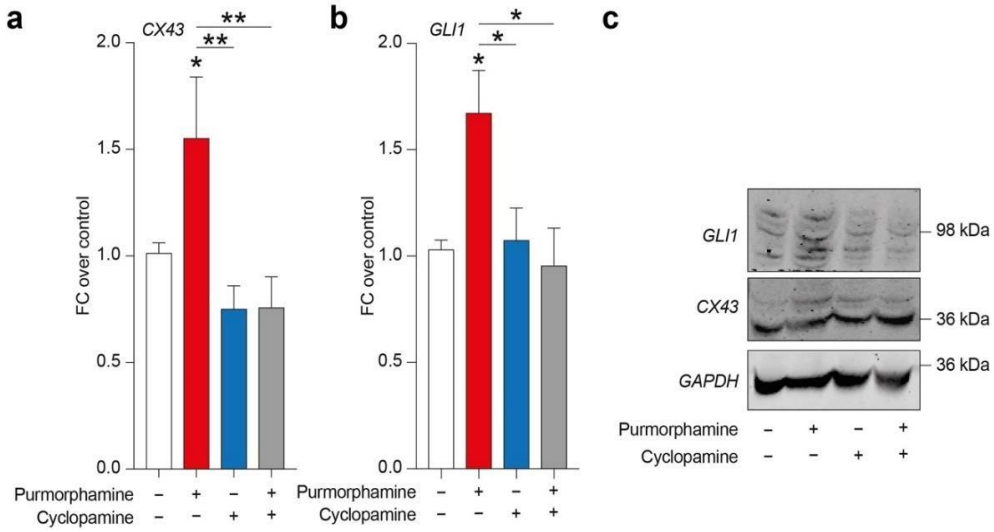

Figure 2. SHH pathway activation is related to CX43 increased expression levels in U-251 MG. *(a–c) CX43 and GLI1 expression levels (a,b) and representative blots (c) on human U-251 MG cells exposed to 1 µM of purmorphamine and/or 1 µM cyclopamine. Data are mean FC over control ± SEM of n = 3 independent experiments; * p-value < 0.05, ** p-value < 0.01 versus control or between groups; one-way ANOVA with Holm–Šídák post-hoc test. FC: fold change*

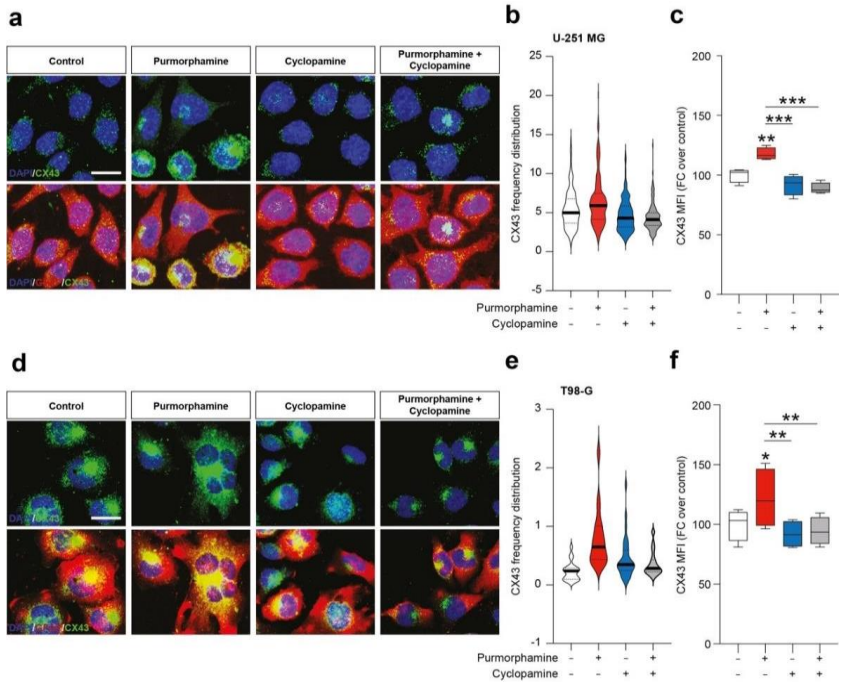

Figure 3. SHH pathway activation increase immunofluorescence intensity of CX43 in human GBM cell lines. *(a–c) Representative microphotographs of CX43 and GFAP (a), CX43 frequency distribution (b) and quantification of CX43 MFI (c) of control human U-251 MG cells and U-251 MG cells exposed to 1 µM of purmorphamine and/or cyclopamine. (d–f) Representative pictures of CX43 and GFAP (d), CX43 frequency distribution (e) and quantification of CX43 MFI (f) of control human T98-G cells and T98-G cells exposed to 1 µM of purmorphamine and/or cyclopamine. Data in (b,e) are shown as violin plot of n ≥ 20 cells and data in (c,f) are shown via standard box and whiskers plot of n = 4 independent experiments; * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 versus control or between groups; one-way ANOVA with Holm–Šídák post-hoc test. In (a,d) nuclei were counterstained with DAPI; scale bar in (a,d) = 10 µm. MFI.*

SHH Pathway Activation is related to CX43 and Ki-67 in Human GBM Cell Lines

In order to evaluate a potential relation between SHH signalling pathway and CX43, we tested purmorphamine, cyclopamine and combination of these drugs in inducing CX43 expression levels and GLI1, a main intracellular effector of canonical SHH signalling pathway. Analysis of protein expression levels revealed a significant increase in both CX43 and GLI1 in cells exposed to 1 µM of purmorphamine, and this effect was not observed in cyclopamine- or purmorphamine + cyclopamine-treated cells (**Figure 2a–c**). whole Western blot bands are shown in **Figure S4**

To further expand this evidence, we performed an immunofluorescence analysis on U-251 MG and T98-G cell lines for CX43 and GFAP. We found purmorphamine induced an overall increase of CX43 Mean Fluorescence Intensity (MFI) in U-251 MG (**Figure 3a-c**) and that this phenomenon was particularly pronounced in some of the cells that expressed particularly high levels of CX43, while GFAP immunopositivity was comparable among cells (**Figure 3a**). We observed similar phenomena in T98-G cells, even if results showed high levels of CX43 also in control condition (**Figure 3d**). Additionally, in this cell line, purmorphamine significantly increased CX43 MFI and this effect was reverted by co-treatment with cyclopamine (**Figure 3e-f**).

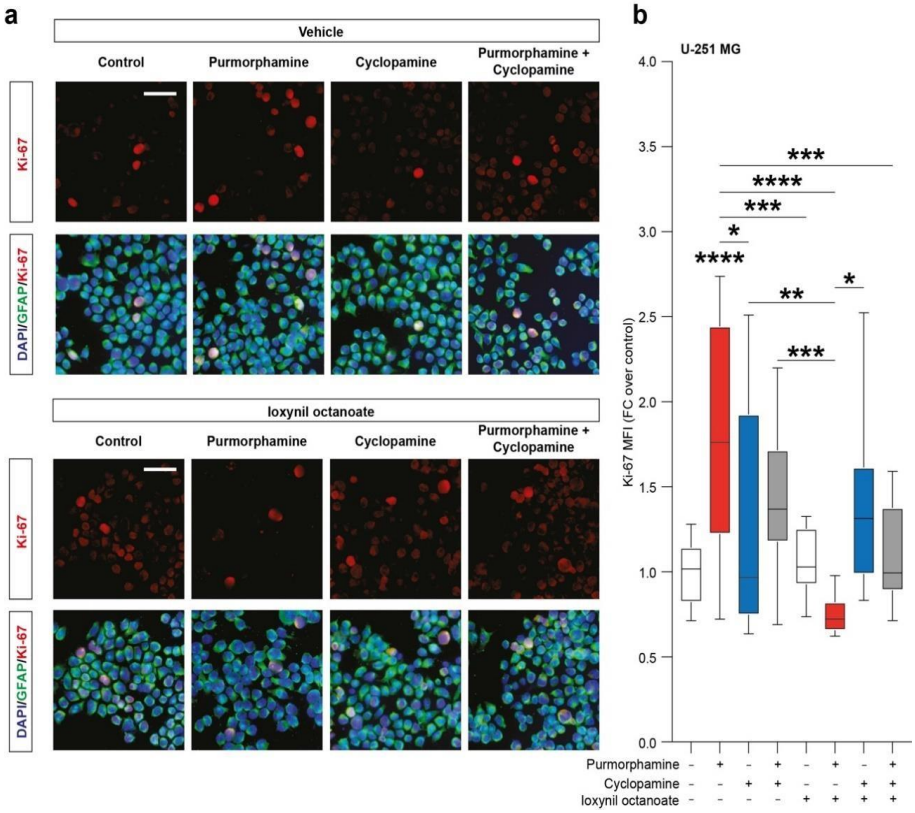

Figure 4. SHH pathway activation increase Ki-67 in U-251 MG cell line. *(a,b) Representative microphotographs of Ki-67 and GFAP immunofluorescence analysis (a) and quantification of Ki-67 MFI (b) of control U-251 MG cells and U-251 MG cells exposed to 1 µM of purmorphamine and/or cyclopamine, treated with either vehicle and ioxynil octanoate; nuclei were counterstained with DAPI; scale bar = 20 µm. Data are mean ± SEM of n = 3 independent experiments; * p-value < 0.05 and ** p-value < 0.01 *** p-value < 0.001 versus control or between groups; one-way ANOVA with Holm–Šídák post-hoc test. MFI.*

To evaluate the effects of SHH signalling pathway modulation on the proportion of proliferating cells we then performed an immunofluorescence analysis of the proportion of Ki-67 positive cells. Our analysis pointed out a significant increase in the nuclear Ki-67 MFI in purmorphamine treated cells (1.77 ± 0.2 purmorphamine versus 1.0 ± 0.04 control, p-value < 0.0001, **Figure 4a,b**) and this effect was not observed in cultures cotreated with cyclopamine (1.45 ± 0.1 cyclopamine, **Figure 4a,b**). Interestingly, in U-251 MG cultures exposed to purmorphamine and treated with ioxynil octanoate, a selective inhibitor of CX43-based GJs, the nuclear Ki-67 MFI was not increased versus control nor versus ioxynil octanoate-treated cells (0.75 ± 0.02 purmorphamine + ioxynil octanoate versus 1.1 ± 0.04 control ioxynil octanoate, **Figure 4a,b**). Of note, cyclopamine and purmorphamine + cyclopamine cotreatment cells treated with ioxynil octanoate showed not significant changes in Ki67 MFI (1.38 ± 0.1 cyclopamine + ioxynil octanoate and 1.11 ± 0.1 purmorphamine + cyclopamine + ioxynil octanoate**, Figure 4a,b**).

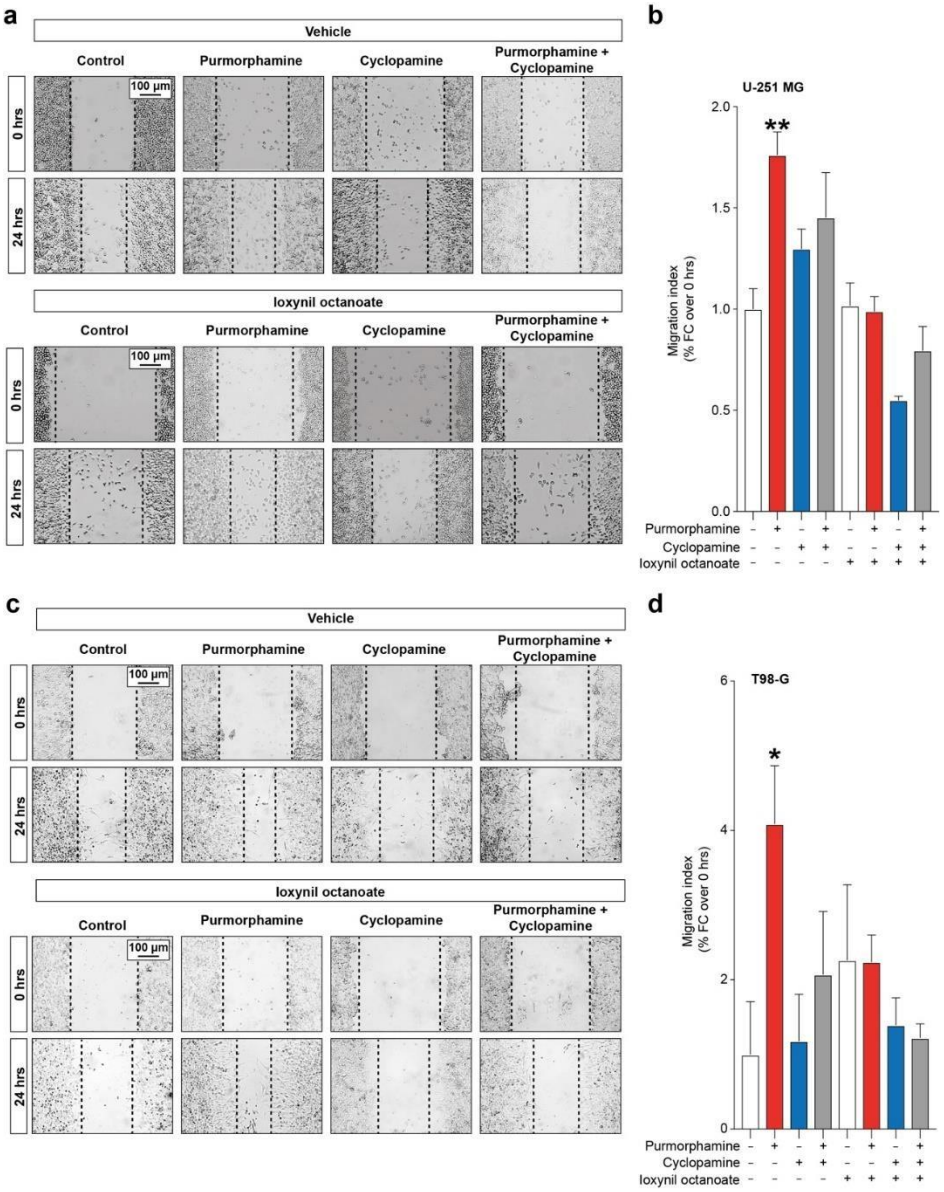

Figure 5. SHH-CX43 axis induces migration enhancement in human GBM cell line. *(a,b) Representative images (a) and migration index (b) of control human U-251 MG cells and U-251 MG exposed to 1 µM of purmorphamine and/or cyclopamine, treated with either vehicle or ioxynil octanoate. (c,d) Representative images (c) and migration index (d) of control human T98-G cells and T98-G exposed to 1 µM of purmorphamine and/or cyclopamine, treated with either vehicle or ioxynil octanoate. Data are shown as mean fold change over 0 hrs ± SEM of n = 3 independent experiments; * p-value < 0.05 and ** p-value < 0.01 versus control; one-way ANOVA with Holm–Šídák post-hoc test.*

SHH-CX43 Axis Induces Migration Enhancement in Human GBM Cell Lines

Given the importance of both SHH signalling pathway and CX43-based channels in modulating proliferation and cell migration, we moved to investigate the interplay between these factors in influencing GBM cell lines migration. Our data showed a significant increase in migration index in U251 MG cells exposed to purmorphamine 1 µM (1.76 ± 0.11 purmorphamine versus 1.00 ± 0.10 control, *p*-value = 0.0083**, Figure 5a,b**), which was not observed in cyclopamine cotreated cells (1.45

± 0.22 purmorphamine + cyclopamine, **Figure 5a,b**). Such an effect was not observed in cell cultures exposed to ioxynil octanoate, that abolish purmorphamine-induced migration increase (1.02 ± 0.11 control + ioxynil octanoate, 0.99 ± 0.08 purmorphamine + ioxynil octanoate 0.55 ± 0.02 cyclopamine + ioxynil octanoate, 0.80 ± 0.12 purmorphamine + cyclopamine + ioxynil octanoate, **Figure 5a,b**). These results were also confirmed in T98-G cells lines, in which we observed a significant increase in migration index upon purmorphamine exposition (4.09 ± 0.78 purmorphamine versus 1.00 ± 0.71 control, *p*-value = 0.03, **Figure 5c,d**) that was abolished by cyclopamine (1.18 ± 0.63 cyclopamine, **Figure 5c,d**). Moreover, in this cell line no significant changes were observed in cultures exposed to ioxynil octanoate (2.26 ± 1.01 control + ioxynil octanoate, 2.24 ± 0.36 purmorphamine + ioxynil octanoate, 1.39 ± 0.37 cyclopamine + ioxynil octanoate and 1.23 ± 0.19 purmorphamine + cyclopamine + ioxynil octanoate, *p*-values > 0.05 for all comparisons, **Figure 5c,d**).

Discussion

GBM, a WHO grade IV glioma, represents the most common primary brain tumor within the adult population. There is an urgent need to develop novel therapeutic approaches to reduce overall morbidity, mortality and short-term and long-term adverse effects of current therapeutic approaches [42]. The path towards the development of druggable targets and effective therapeutic approaches is particularly challenging due to the infiltrative nature of this malignant glioma and its marked heterogeneity with the warring presence of self-renewing cancer stem cells. Therefore, a valid approach is represented by an in-depth knowledge of the mechanisms and players controlling its complex tumor microenvironment.

SHH signalling pathway is a crucial player not only in cell proliferation, self-renewal and differentiation modulation during central nervous system development and patterning [8], but also contributing to the development of various malignancies, including GBM [18,22]. It has been suggested that SHH signalling pathway promotes GBM-cell migration and invasion by increasing matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9) production via the PI3K/AKT pathway [43,44] and by regulating the stem cell fraction in GBM cell lines [45]. The invasive behaviour of glioma cells induced by CX43-dependent signalling has been previously described by studies reporting that CX43 increases the secretion and activation of proteins associated with cell motility and extracellular matrix remodelling, also due to the tumorigenicity activation of neural progenitor spheroids and glioblastoma stem cells [46–48]. Indeed, inhibitors of the SHH pathway effector SMO have been successfully tested in both in vitro and in vivo GBM models and

demonstrated to efficiently counteract self-renewal and tumor progression [20,45]. Another noteworthy factor is that SHH-GLI signalling has been associated to proliferation, survival, self-renewal and tumorigenicity of cancer stem cells with several markers of stemness differently characterizing the grade, growth and survival of pathology [49–51], so that pharmacological modulation of SHH pathway has been proposed in different therapeutic plans to prevent tumor proliferation and recurrence [52].

A large amount of data supports the hypothesis that GJs- and HCs-mediated intercellular communication, by regulating the exchanges between adjacent cells and conditioning extracellular environment, also modulates survival, development and progression of tumor microenvironment as much as channel-independent mechanisms via the Cxs relationships with multiprotein complexes and pathways. The significant role exerted by GJs in GBM invasiveness and progression is not surprising since the condition in which a tumor microenvironment develops, is determined by the altered activity of GJs and HCs among the various cells, including endothelial cells, astrocyte, pericytes and neurons, coexisting in the so-called neurovascular unit (NVU) [53]. The dysregulation of homeostatic NVU microenvironment dramatically affect blood brain barrier (BBB) function, resulting in increased peripheral derived detrimental stimulation and peritumoral vasculature, finally inducing migration of GBM cells. In particular, CX43 proved to be an attractive target for GBM since it is dynamically expressed by highly invasive glioblastoma cells, showing a multifaceted appearance dependent on both GBM growth level and malignant phase so that some tumor cells would be expected to migrate (CX43 expressing cells) and others to proliferate (CX43 no expressing cells) [31]. Indeed, alongside the traditional role, it needs to be considered the effect of Cxs independent of GJs and HCs but correlated to the close collaboration with partners of different signalling pathways, or gene expression regulation, involved in cellular transformation processes [54,55].

Over the years, increasing interests have been placed in clarifying the crosstalk between GJs- and HCs-mediated communication and SHH pathway [33]. A finely regulated relationship between SHH and CX43 has been suggested during developmental processes. Decreased or absent gap junctional coupling in Cx43 mouse mutants leads to altered expression of morphogens including SHH conducing to different phenotypic abnormalities [56,57].

Similarly, morphogenic factors including WNT and SHH play important roles in microglia/astrocytes glioma crosstalk, recapitulating developmental programs of the tissues and organs during early

embryogenesis [16,58]. It has been documented that the plasticity of tumor progression critically depends on reciprocal interaction of tumor cells with the different players of microenvironment including connexin, morphogens and cytoskeleton elements which participate dynamically to malignant transformation [59].

Growth factors, cytokines and matrix proteins, released from tumor cells and host stromal cells, through different subsets of cellular interactions, give the tumor microenvironment the identity of a dynamic niche for tissue remodelling, where glioma cells can hijack the molecular programs involved in normal tissue development, including immune signalling pathways, to promote their own survival and expansion [60]. Moreover, it has been shown that SHH in tumor microenvironment is important for controlling epithelial–mesenchymal transition (EMT) in the pathogenesis, and progression of tumors, including prostate, bladder and brain cancers [58,61–64].

In this scenario, interfering with SHH pathway has been proposed as a promising strategy in GBM, even if co-players and mechanisms are largely unclear. Here we sought to investigate a relationship between CX43 and SHH signalling pathway in the complex GBM microenvironment.

Our results showed that two different human GBM cell lines actively express SHH signalling pathway effector SMO and we were able to modulate and interfere with canonical SHH pathway using purmorphamine and cyclopamine. In our study, cyclopamine on GBM cells was able to revert CX43 and GL1 expression induced by purmorphamine, but we did not observe a reduction of CX43 and GL1 expression compared to the control in cyclopamine exposed group. These effects were reflected in the functional experiments where we reported a significant increase of nuclear Ki-67 MFI and migration index in purmorphamine treated cells reverted by cyclopamine treatment. Previously studies demonstrated that cyclopamine interferes with GBM cell viability and stemness showing a synergistic effect with temozolomide [45,65]. However, in these studies, higher concentrations and long-time exposures of cyclopamine were used, whereas in our study we evaluated acute effects of both SMO stimulation and CX43 inhibition in order to find whether these treatments prelude to increasing migration and proliferation of GBM cell lines and may be reverted by cotreatments. SHH pathway inhibition by cyclopamine reduced clonogenicity in both GBM cell lines without affecting the metabolic turnover; it is worth noticing that MTT assay is not reflecting necessarily cell proliferation and growth, but viable cell metabolism [66]. Interestingly, we demonstrated that 1 µM purmorphamine was able to increase the clonogenicity of both GBM cell lines, while their surviving fraction was strikingly reduced with 10 µM. This similar result has been

reported by our group on neuronal stems cells and it may be associated with a rebound effect and/or receptor desensitization [11]. Besides, confirming the modulatory function of SHH pathway on GBM cell machinery, we also found a potential relationship between purmorphamine exposition and CX43 levels. Such a link has been investigated in developmental studies and CX43 knock down models, in which a reduction of SHH levels and morphogens signalling activation have been observed upon CX43 inhibition and/or manipulation [33], thus suggesting a potential bidirectional interplay between CX43 and SHH signalling. Indeed, we found that even if a known SHH agonist (i.e., purmorphamine) induced a significant increase of CX43 levels, such an evidence was coupled with a loss of function in cotreatment with ioxynil octanoate, an inhibitor of CX43-based channels. Future studies are warranted to investigate CX43 phosphorylation and its channel-independent role, which may also affect cell machinery and have critical effects on proliferation and migration [67]. It is worth noticing that, in our experimental setting, inhibition of smoothened through cyclopamine does not affect the levels of CX43 nor the overall migration capabilities of GBM cell lines. This evidence supports the hypothesis that microenvironment in GBM may play a role in fostering GBM cells migration and proliferation, by stimulating SHH signalling and deregulating CX43 expression levels, thus modifying channels selectivity and microenvironmental conditioning [68].

Conclusions

Our results suggest a potential axis between CX43 and SHH signalling pathway at least on two main aspects: (i) “permissive”, similarly to developmental programs in which SHH-GLI signalling favours intercellular communication and patterning that leads to microenvironmental modification and disease/tumorigenic onset; (ii) “supporting” the stemness signature of GBM, so that aberrant SHHGLI pathway, through modified CX43-mediated subsets cellular interaction, promotes cancer stem cell population critical for GBM self-renewal.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Growth curves. Figure S2: Cytotoxicity assay. Figure S3: MTT turnover assay. Figure S4. Whole western blot showing all bands.

Author Contributions: conceptualization: N.V., R.P.; project administration: F.T., N.V., R.P.; methodology: F.T., C.A., D.L.F., A.Z., S.V., G.L.V., D.T., N.V., R.P.; investigation: F.T., C.A., D.L.F., A.Z., N.V.; formal analysis: F.T., S.V., G.L.V., D.T., N.V., R.P.; supervision: S.V., G.L.V., D.T., N.V., R.P.;

writing—original draft: F.T., N.V., R.P.; writing—reviewing and editing: F.T., C.A., D.L.F., A.Z., S.V., G.L.V., D.T., N.V., R.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Materials

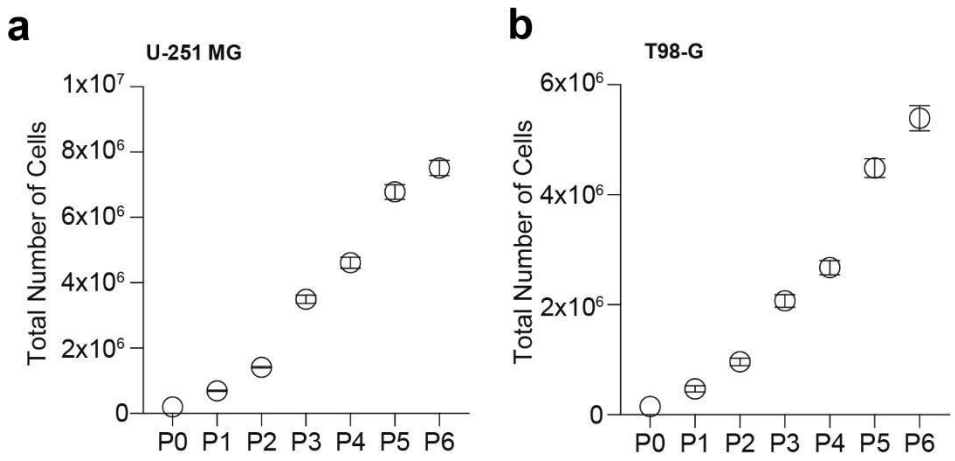

Figure S1. Growth curves. *(a-b) Growth curve over six passages of human U251-MG (a) and T98-G (b) cell lines. Data are mean ± SEM of n = 3 independent cultures. P: passage.*

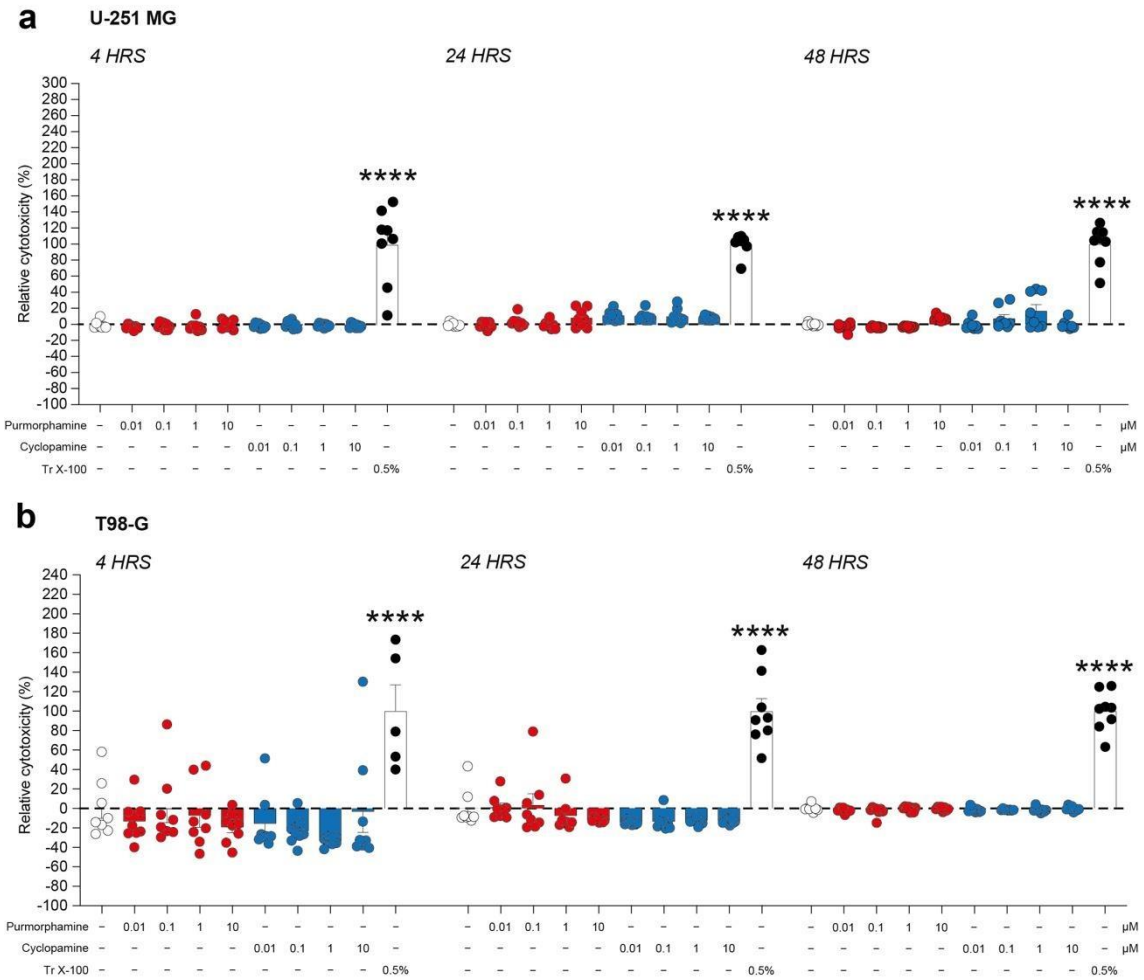

Figure S2. Cytotoxicity assay. *(a-b) Cytotoxicity assay of positive and negative controls of human U251MG (a), and T98-G (b) cell lines and cultures exposed to increasing concentration of purmorphamine and cyclopamine (0.01–10 μM). Data are mean ± SEM of n = 3 independent experiments; ****p-value < 0.0001 versus control; one-way ANOVA with Holm–Šídák post-hoc test. Tr X-100: Triton X-100.*

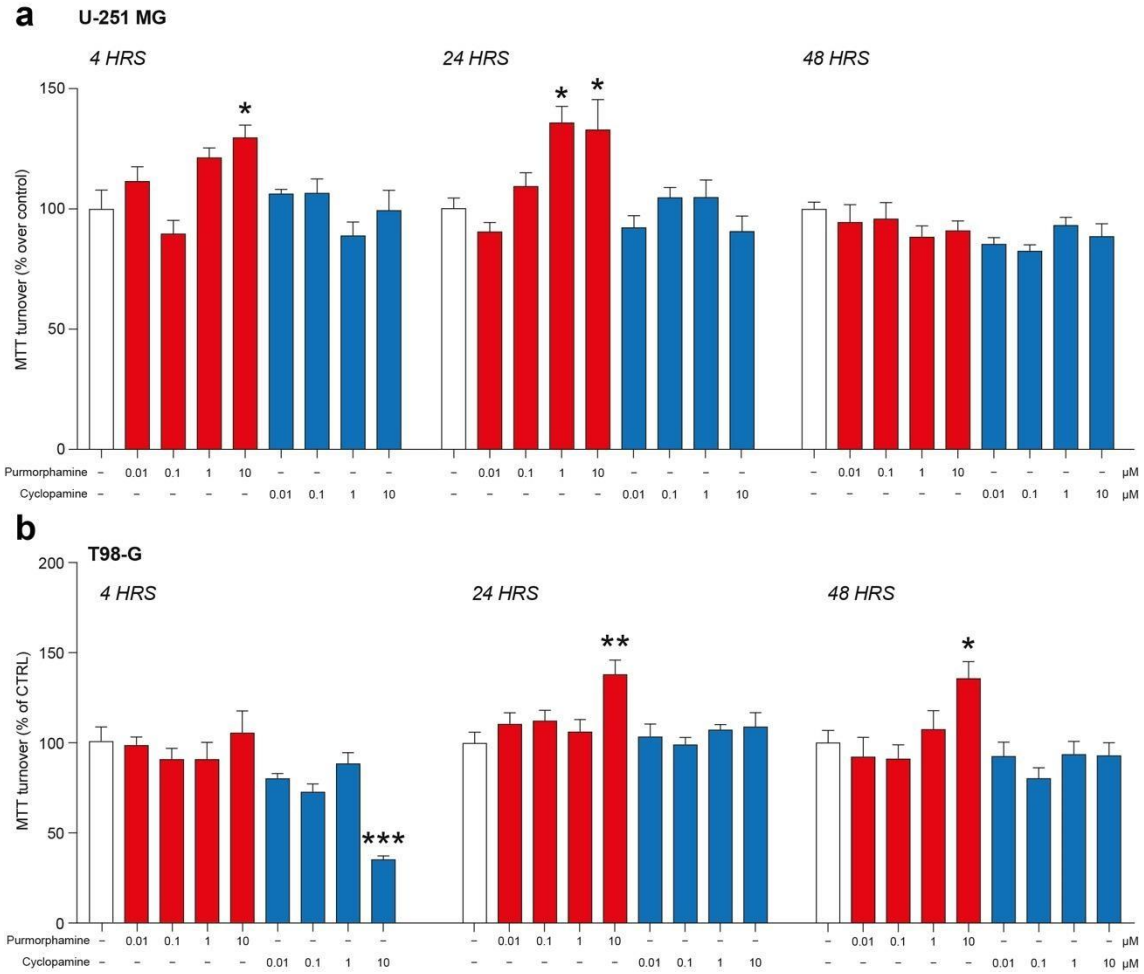

Figure S3. MTT turnover assay. *(a-b) 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) turnover of control human U251-MG (a) and T98-G (b) cell lines and cultures exposed to increasing concentration of purmorphamine and cyclopamine (0.01–10 µM). Data are mean ± SEM of n = 3 independent experiments; *p-value < 0.05, **p-value < 0.01 and ***p-value < 0.001 versus control; oneway ANOVA with Holm–Šídák post-hoc test.*

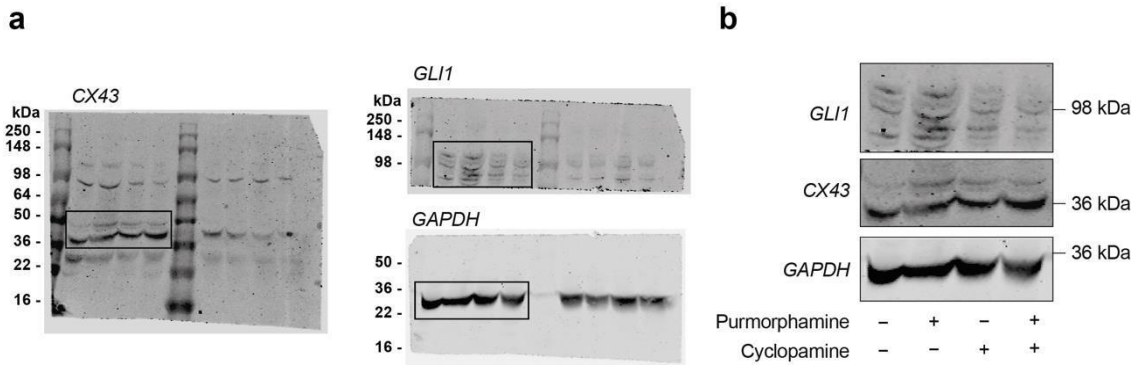

Figure S4. Whole Western blot showing all bands. *(a) Uncropped membranes for CX43, GLI1 and GAPDH. (b) Cropped membranes showed in Figure 2c.*

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Paper 2

Under revision on Cell proliferation (Wiley)

Microglia and glioblastoma heterocellular interplay sustains tumor growth and proliferation as an off-target effect of radiotherapy

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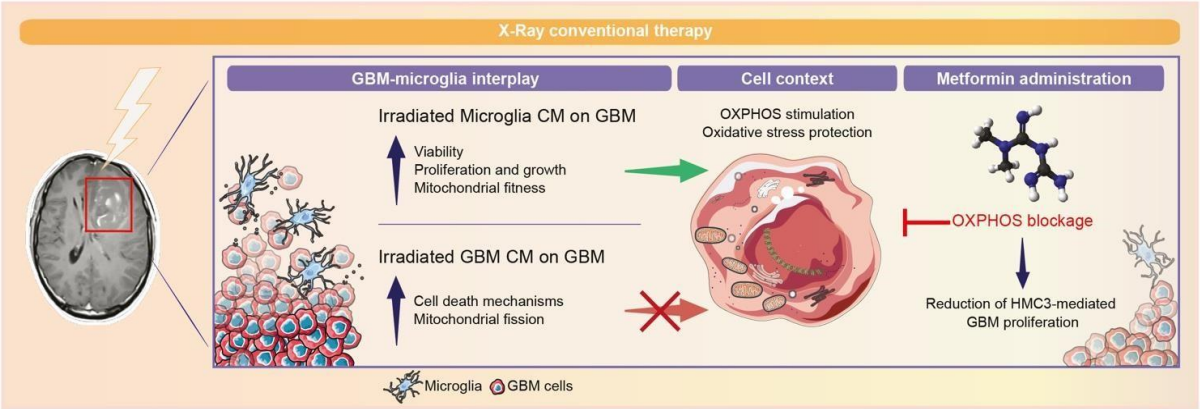
Abstract: Glioblastoma (GBM), a WHO grade IV glioma, is a malignant primary brain tumor for which com-bination of surgery, chemotherapy and radiotherapy is the first-line approach despite adverse effects. Tumor microenvironment (TME) is characterized by an interplay of cells and soluble factors holding a critical role in neoplastic development. Significant pathophysiological changes have been found in GBM TME, such as glia activation and oxidative stress. Microglia play a crucial role in favouring GBM growth, representing target cells of immune escape mechanisms. Our study aims at analysing radiation-induced effects in modulating intercellular communication and identifying the basis of protective mechanisms in radiation-naïve GBM cells. ***Methods:*** Tumor cells were exposed to conditioned media (CM) derived from 0 Gy, 2 Gy or 15 Gy irradiated GBM cells or 0 Gy, 2 Gy or 15 Gy irradiated human microglia. ***Results:*** We demonstrated that irradiated microglia promote an increase of GBM cell lines proliferation through paracrine signaling. On the contrary, irradiated GBM-derived CM affect viability, triggering cell death mechanisms. In addition, we investigated

whether these processes involve mitochondrial mass, fitness and oxidative phosphorylation and how GBM cells respond at these induced alterations. ***Conclusion***: Our study suggests that off-target radiotherapy modulates microglia to support GBM proliferation and induce metabolic modifications.

Keywords: Glioblastoma; tumor microenvironment; microglia; radiotherapy; metformin.



Introduction

Glioblastoma (GBM) is the most aggressive primary malignant brain tumor affecting the adult population, with a prognosis that remains dramatically poor and about the 5% of patients survive five years after diagnosis [1]. GBM heterogeneity limits the efficacy of the current therapeutic approaches including surgical resection, followed by radiotherapy (RT) and temozolomide chemotherapy [2]. Therefore, developing new therapeutic strategies and revealing the mechanisms responsible for the failure of current therapies, become undisputable to improve the outcomes of this devastating disease.

The effects induced by RT on GBM tumor microenvironment (TME) represent a critical field of investigation and the main factor in inducing therapeutic failure [3, 4]. TME of GBM is an intricate network where, in a hypoxic milieu, different cell types coexist, including tumor cells, immune cells, fibroblasts, resident glial and endothelial cells and various secretory factors [5, 6]. RT triggers specific responses within GBM TME, such as cell death, senescence, activation, survival and migration [7, 8].

Tumor-associated microglia and macrophages (TAMs) are the most abundant non-neoplastic cells in the TME of GBM. They consist of both brain-resident microglia and bone marrow-derived myeloid cells from the periphery, constituting about 40% of the tumor mass in GBM [9]. In particular, the feedback from microglia, activated by related inflammatory signaling is imprinted

by the TME, playing a central role in favouring immunosuppression and immune escape mechanisms promoting tumor resistance [8, 10].

It has been reported that metformin acts as anti-cancer agent within the complex microenvironment of cancer, particularly in breast cancer [11]. Metformin belongs to biguanide pharmacological class and represents a first line therapy for Type 2 diabetes [12]. This drug acts reducing gluconeogenesis process and stimulating glucose uptake and consumption [13]. Regarding tumor suppressor mechanisms, metformin is involved in altering cell metabolism, blocking mitochondrial respiratory chain complex I and inhibiting the tricarboxylic acid (TCA) cycle and oxidative phosphorylation [14]. Moreover, metformin sensitizes cells to temozolomide, inhibits cell proliferation and invasion and decreases hypoxia-inducible factor (HIF-1α) and vascular endothelial growth factor (VEGF), key elements for GBM angiogenesis and malignance [15, 16].

Here we investigated the irradiation-induced alterations on microglia and the indirect effects mediated by off-target irradiation on spared GBM cells, aiming at highlighting exploitable mechanisms to improve tumor control and increasing radiosensitivity.

Materials and methods

Cell cultures, conditioned media and treatments

Experiments were performed using U-87 MG and U-251 MG human GBM cell lines and HMC3 human microglia cell line. GBM cells were purchased from European Collection of Authenticated Cell Cultures (ECACC, Public Health England, Porton Down Salisbury, UK) and cultured with growth medium [DMEM high glucose supplemented with 10% Foetal Bovine Serum (FBS), Penicillin Streptomycin 100 IU/mL and L-glutamine 2 mmol/L]. HMC3, were purchased from European Collection of Authenticated Cell Cultures (ECACC, Public Health England, Porton Down Salisbury, UK) and cultured with HMC3 growth medium [MEM supplemented with 10% FBS, Penicillin Streptomycin 100 IU/mL and L-glutamine 2 mmol/L]. Cells were maintained in growing culture condition in an incubator at 37 °C in a humidified atmosphere (95% air and 5% CO_2). Conditioned media (CM) were collected from U-87 MG, U-251 MG and HMC3 cultures at 24 or 48 hours post irradiation with 0 Gy (mock-IR), 2 Gy and 15 Gy doses of X-ray irradiation, filtered with a 0.22 µm syringe filter unit and stored at – 80 °C until use. For boiled CM, CM were incubated at 100 °C for 10 min. Then, CM and/or boiled CM were used at a final concentration of 25% with growth medium to

culture U-87 MG or U-251 MG cell lines in experimental settings according to the described procedures. Metformin (1,1-dimethylbiguanide hydrochloride, Cat#317240, Sigma-Aldrich) was prepared as a stock solution at 40 mM and stored at -20 °C until use. For cell treatment, metformin was diluted and at a final concentration of 100 µM in PBS. The effects of metformin were tested in GBM cell lines cultured with growth medium, irradiated GBM cells CM or irradiated HMC3 CM. For clonogenic assay, vehicle (i.e. PBS) or metformin were administrated every 48 hours. All experiments employed cells at a passage n < 25.

RNA extraction and RT-qPCR for gene expression analysis

Total RNA was extracted by Trizol® reagent following manufacturer's instructions (Invitrogen). Firststrand cDNA was then synthesized with reverse transcription kit (Applied Biosystem). Quantitative real-time PCR was performed in Step One Fast Real-Time PCR System, using the SYBR Green PCR MasterMix (Life Technologies). The specific PCR products were detected by SYBR Green fluorescence. The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with that of ACTB by using a comparative $2^{-\Delta\Delta Ct}$ method [17]. The sequence of primers used are shown in **Supplemental Table 1**.

Immunocytochemistry analysis

Immunocytochemistry was carried out as previously reported [18, 19]. Briefly, cells were irradiated with 0 Gy (i.e. mock-IR control group) and 15 Gy doses of irradiation, or exposed to irradiated HMC3 CM for 24 hours. U-87 MG and U-251 MG cell lines were stained with 200 nM MitoTracker Red CMXRos probe (Thermo Fisher Scientific) for 30 min at 37 °C in order to detect mitochondria, according to the manufacturer's instructions. The dye was removed and cells were washed 3 times in PBS. Nuclei were stained with NucBlue 2% in PBS (Thermo Fisher Scientific, Milan, Italy) for 15 min at 37°C. High-content analysis on cell cultures was performed using Operetta (Perkinelmer). Images were acquired at 24 hours after treatment and quantifications of MitoTracker mean fluorescence intensity (MFI), mitochondrial fragmentation and mitochondrial integrity were obtained using Operetta Harmony software (Perkinelmer).

LDH assay

The relative cytotoxicity was assessed using lactate dehydrogenase (LDH) activity assay (Abcam), following the manufacture's instructions. Briefly, cells were seeded in 96-well plates (Costar) at a

$$\%\, relative\ cytotoxicity = \left[\frac{(ODsample - ODnegative\ control)}{(ODpositive\ control - ODnegative\ control)}\right] \times 100$$

final density of $1x10^4$ cells/well/100 µL. In order to assess cytotoxicity induced by CM, we considered performed LDH assay at 24 hours post-CM treatment. Cells were treated with either homocellular or heterocellular CM collected at 24- or 48-hours post-irradiation. Cells treated with 1% of lysis solution (10% triton X-100 in PBS) were used as positive controls (100% relative cytotoxicity). Vehicle treated cells were used as negative control (0% relative cytotoxicity). At given timepoints, quantification of the LDH activity was performed on supernatants following manufacture's instructions. The absorbance was measured using a Multiskan SkyHigh Microplate spectrophotometer (Thermo Scientific, Milan, Italy) at 450 nm. The percentage of relative cytotoxicity was calculated using the following formula:

MTT turnover

For 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) turnover, MTT at a final concentration of 1 mg/mL was added to each well and incubated at 37 °C in a humidified atmosphere (5% CO_2) for 2 hours and 30 min under standard culture conditions, as previously

$$\%\ MTT\ turnover\ = (\frac{OD\ sample}{average\ control}) \times 100$$

described [20]. MTT turnover was evaluated at 24- and 72-hours post-CM treatment. Then, media were gently removed, 200 µL of MTT solvent (dimethyl sulfoxide - DMSO, Sigma) were added and placed on an orbital shaker for 10 min at room temperature. The absorbance was measured using a Multiskan SkyHigh Microplate spectrophotometer (Thermo Scientific, Milan, Italy) at 570 nm. Metabolic turnover was calculated as:

Cells cultured with 0 Gy CM, derived from both GBM and HMC3 cell cultures, were used as positive control (100% MTT turnover). Each experiment was performed three times, with n > 4 replicates per condition during each experimental run.

Clonogenic assay

Clonogenic assay was performed on U-87 MG or U-251 MG cell lines. 400 cells (U-87 MG) or 600 cells (U-251 MG) were plated in a 6 multiwell plate with a culture surface of 9.5 cm^2 per well. Cells were incubated with 2 mL of either 100% growth medium or 25% CM and 75% growth medium. For both naïve U-87 MG and naïve U-251 MG, the following experimental conditions were tested: 0 Gy irradiated GBM and HMC3 CM, 15 Gy GBM and HMC3 irradiated CM, 0 Gy irradiated HMC3 CM

$$PEcontrol\ = \frac{number\ of\ clones}{number\ cell\ plated}$$

boiled, 15 Gy irradiated HMC3 CM boiled. Clonogenic growth was allowed for 13 days for U-87 MG and 7 days for U-251 MG. Cells were then fixed with methanol for 15 min at room temperature. Colonies were stained with 1% crystal violet for 25 min at room temperature [21]. Colonies which accounted for more than 50 cells were considered as clones. Plating efficiency (PE) of controls was calculated as:

The percentage of surviving fraction was calculated as:

$$\%\ of\ surviving\ fraction\ = (\frac{PE\ sample}{PE\ control})\ \times\ 100$$

Flow cytometry

For flow cytometry-assisted viability analysis, $2.5x10^5$ cells were plated in T25 cell plate with a culture surface of 25 cm^2. GBM and microglia cells were cultured for 24 hours and then irradiated with 0 Gy, 2 Gy or 15 Gy. After 24 hours from radiation treatment, cells were stained in order to asses Annexin V/Propidium Iodide (PI) assay. GBM cell lines were also treated for 24 hours with 0 Gy, 2 Gy and 15 Gy irradiated cells-derived CM. CM were collected after 24 hours from X-ray treatment. After treatments, cells were washed and resuspended in 100 µL of PBS at 4 °C. 1 µL of Annexin V-FITC solution and 5 µL of PI (Beckmam Coulter) were added to cell suspension and mixed gently. Cells were incubated for 15 min at room temperature. Finally, 400 µL of binding buffer were added and cell preparation was analyzed by flow cytometry (MACSQuant Analyzer 10, Miltenyi Biotech) and analyzed using Flowlogic software (Miltenyi Biotech) [22]. To determine the mitochondrial ROS levels, cells were stained with 2.5 µM of MitoSOX probe for 30 min at 37 °C, and fluorescence intensity was measured according to the fluorescence detection conditions of PEMitoSOX-A B2-A using the MACSQuant Analyzer (Miltenyi Biotech).

Irradiation

Full experimental procedures are described in the Supplemental Data. Irradiation was performed in a linear accelerator, Elekta Synergy, at Radiotherapy Department of Cannizzaro Hospital, Catania, Italy with a dose rate of 3 Gy/min, using a 6 MV x-ray. GBM cell irradiation was carried out using dose values of 0 Gy (mock-IR group), 2 Gy and 15 Gy.

Statistical considerations

Data were tested for normality using a D'Agostino and Pearson omnibus normality test and subsequently assessed for homogeneity of variance. Data that passed both tests were further

analysed by two-tailed unpaired Student's t-test, that was used for comparison of n = 2 groups. For comparison of n ≥ 3 groups, one-way analysis of variance (ANOVA), followed by Holm-Sidak posthoc test for multiple comparisons were used. All tests were performed using GraphPad Prism (version 5.00 for Mac, GraphPad Software). For all statistical tests, p-value < 0.05 was considered statistically significant and symbols indicating statistical differences are reported in figure legends.

Results

Irradiated HMC3 CM preserve GBM cells viability

We first evaluated the effects of irradiation on cell viability on U-87 MG, U-251 MG and HMC3 at 24 hours post-treatment, using a cytofluorimetric assisted Annexin V/PI assay (**Figure S1**). Mock-IR cells (i.e. 0 Gy) were used as controls. As expected, our results suggested that 15 Gy dose induced a significant reduction of the percentage of viable cells in all tested cell line, as compared to 2 Gy treated cells and as compared to mock-IR cells (**Figure S1**).

In order to evaluate the potential effects of secretome of irradiated cells on radiation-naïve cells, we exposed cell cultures to either 0 Gy (mock-IR), 2 Gy and 15 Gy irradiation and we collected their CM at 24 hours, exposing naïve cells to their media and evaluating cell viability after 24 hours of conditioning (**Figure 1a**). Analysis on U-87 MG showed an increased proportion of dead cells when treated with 15 Gy U-87 MG CM as compared to naïve U-87 MG treated with mock-IR U-87 MG CM (**Figure 1b-c**) and versus 2 Gy U-87 MG CM treated cells (**Figure 1b-c**). Naïve cells incubated with 2 Gy U-87 MG CM showed a near-normal levels of viable, early/late apoptotic and dead cells as compared to control (**Figure 1b-c**). We then moved to analyse the effects of CM from irradiated HMC3 on radiation-naïve U-87 MG cells. Interestingly, in this case we did not observe any significant differences in terms of cell viability among groups (**Figure 1f-g**), indicating that CM of irradiated microglia do not influence U-87 MG cells viability. We also assessed this effect on U-251 MG cells (**Figure 1d-e** and **1h-i**). We found that 15 Gy U-251 MG CM induced a decrease of cell viability as compared to the mock-IR U-251 MG CM (**Figure 1d-e**) and also versus 2 Gy U-251 MG CM (**Figure**

1d-e), coupled with a significant increased proportion of dead cells in 15 Gy U-251 MG CM treated cells as compared to 2 Gy and mock-IR U-251 MG CM of about 1.6 fold (**Figure 1d-e**).

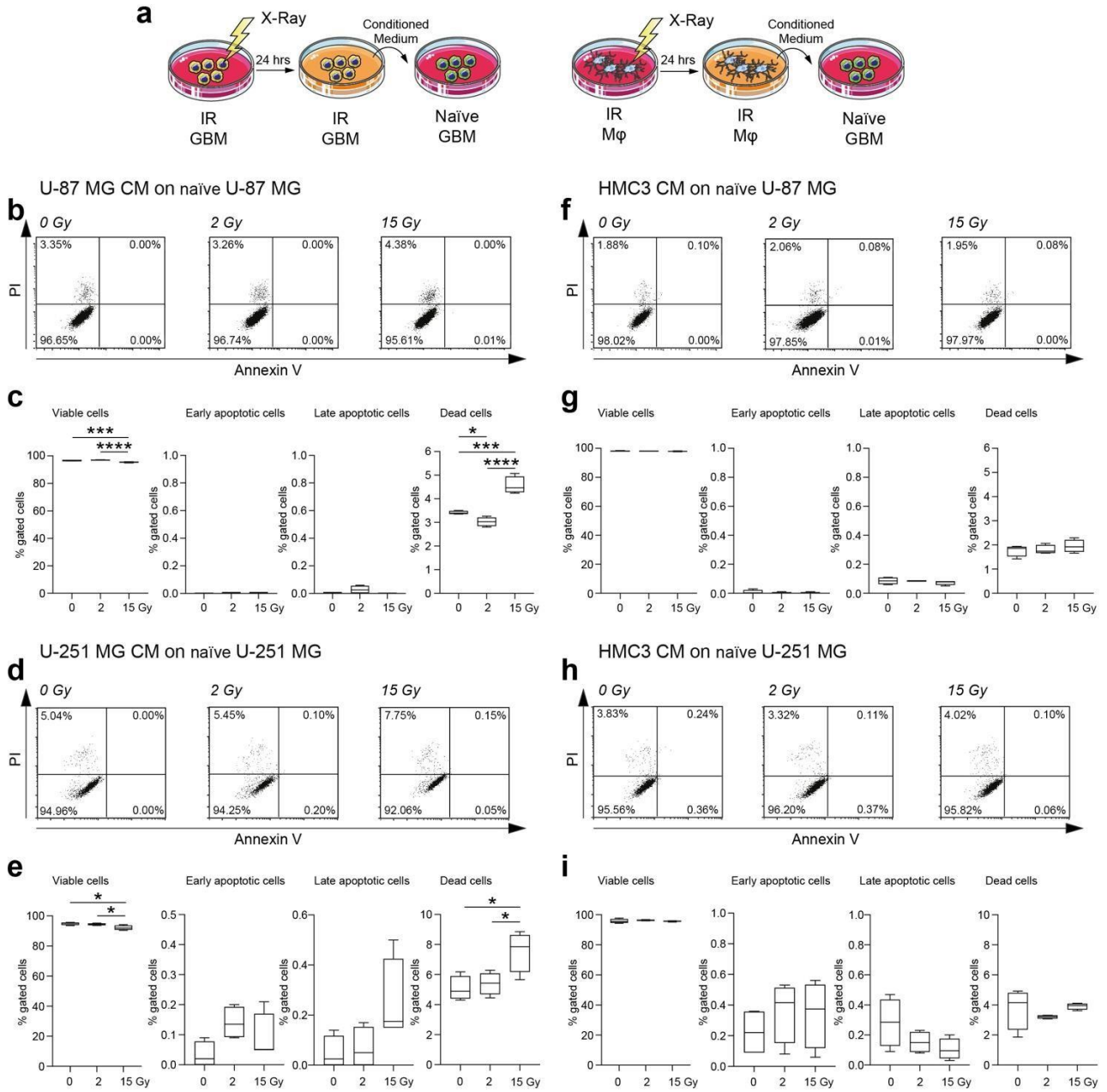


Figure 1. Irradiated HMC3 CM preserve radiation-naïve U-87 MG and U-251 MG cell lines viability. *a) Experimental workflow and CM treatment. b-i) Cytofluorimetric analysis of viability evaluated with Annexin V/PI assay on U-87 MG treated with U-87 MG CM (b-c), U-251 MG treated with U-251 MG CM (d-e), U-87 MG treated with HMC3 CM (f-g) and U-251 MG treated with HMC3 CM (h-i). Data are shown as standard box and whiskers and viability is expressed as the percentage of gated cells, n = 4 independent replicates for each experimental condition. * p-value < 0.05; *** p-value < 0.001; **** p-value < 0.0001.*

We also assessed the effects on cytotoxicity and metabolic turnover after incubation with irradiated GBM and microglia CM on U-87 MG and U-251 MG cell lines, using LDH and MTT assays at different timepoints (**Figure S2**). LDH assay revealed limited cytotoxicity in cells cultured with irradiated CM

and similar results were observed for MTT turnover at 24 hours (**Figure S2**). Notably, CM collected 48 hours post-irradiation, induced an increased relative cytotoxicity in U-87 MG CM treated naïve U-87 MG and U-251 MG CM treated naïve U-251 MG, but not in cells treated with HMC3-derived CM (**Figure S3**).

15 Gy irradiated HMC3 CM stimulate GBM clone formation

In order to find a potential effect on clonogenicity of GBM cells induced by homocellular or heterocellular communication via CM, we performed a clonogenic assay on U-87 MG and U-251 MG cells. Interestingly, 15 Gy U-87 MG CM was not able to induce significant effects on radiation-naïve U-87 MG cells (**Figure 2a**). Conversely, an increase of the surviving fraction was observed in radiation-naïve U-87 MG cells treated with 15 Gy HMC3 CM as compared to mock-IR HMC3 CM (**Figure 2b**). Similar results were observed on U-251 MG cell line, in which 15 Gy U-251 MG CM induced clone formation comparable to control cultures (**Figure 2c**). A significant increase on U-251 MG surviving fraction was detected after treatment with 15 Gy HMC3 CM versus control treated with mock-IR HMC3 CM (**Figure 2d**). In addition, analysis of fresh versus boiled CM derived from HMC3 showed no significant differences (**Figure S4**), indicating that the effects observed are mediated by thermo-stable molecules.

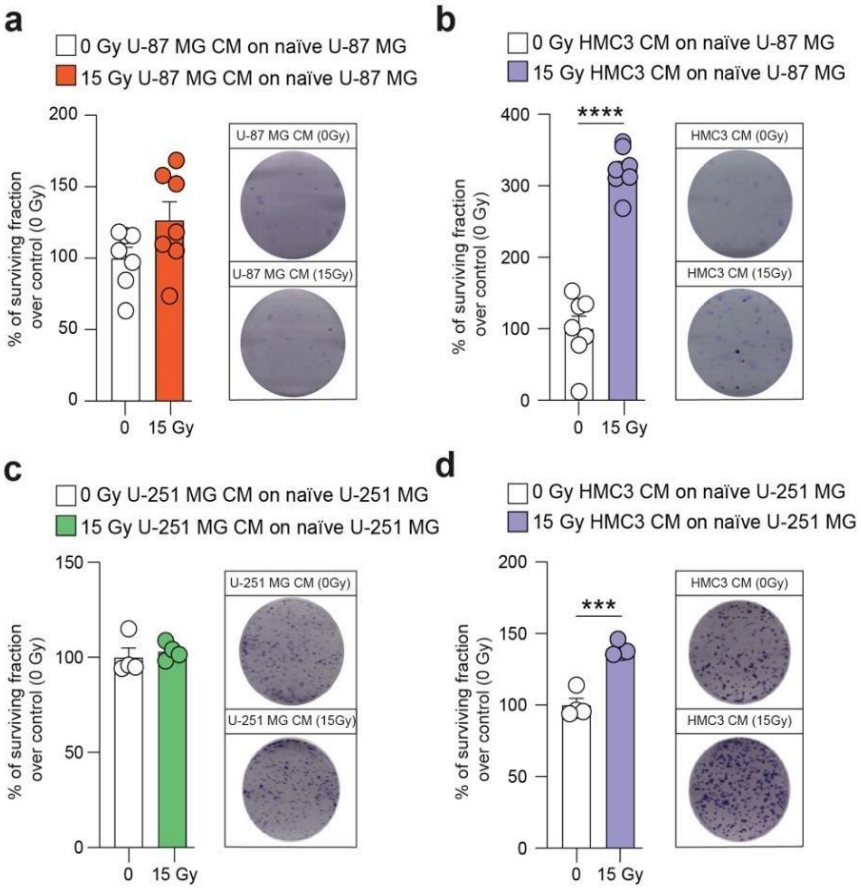


Figure 2. Irradiated HMC3 CM stimulate naïve U-87 MG and U-251 MG clone formation. a-b) *Surviving fraction and representative pictures of U-87 MG treated with 0 Gy and 15 Gy U-87 MG CM (a) or 0 Gy and 15 Gy HMC3 CM (b). c-d) Surviving fraction and representative pictures of U-251 MG treated with 0 Gy and 15 Gy U-251 MG CM (c) or 0 Gy and 15 Gy HMC3 CM (d). Data are expressed as scattered dot-plot and mean ± SEM of n ≥ 4 independent experiments. ***p-value < 0.001 and ****p-value < 0.0001.*

Irradiated HMC3 CM sustain mitochondrial fitness in GBM

In order to analyse mitochondrial fitness, we evaluated the effects of direct irradiation on GBM cell lines treated with mock-IR or 15 Gy HMC3 CM. We used a high-content analysis of mitochondrial mass, mitochondrial fragmentation and mitochondrial integrity on mock-IR versus 15 Gy directly irradiated GBM cells. Analysis on whole cell mitochondrial mass showed no significant differences between tested conditions on both U-87 MG and U-251 MG, although a slight decrease of mitochondrial fragmentation was observed in 15 Gy U-251 MG (**Figure S5**). Interestingly, HMC3 that underwent direct irradiation, showed a significant decrease of both mitochondrial mass (**Figure S5**) and mitochondrial fragmentation as compared to mock-IR HMC3 (**Figure S5**).

In order to analyse the effect of irradiated HMC3 CM treatment on mitochondrial fitness of GBM cell lines, we tested the CM-induced effects on U-87 MG and U-251 MG mitochondrial state and structure after 24 hours incubation with either mock-IR and 15 Gy HMC3 CM (**Figure 3**). Our results

suggested that HMC3 CM treatment had no significant effects of mitochondrial mass, fragmentation and on percentage of intact mitochondria in tested GBM cell lines, U-87 MG (**Figure 3a-b**) and U251 MG (**Figure 3c-d**), indicating that HMC3 CM do not affect mitochondrial function, fitness and preserves mitochondrial mass at near-normal levels.

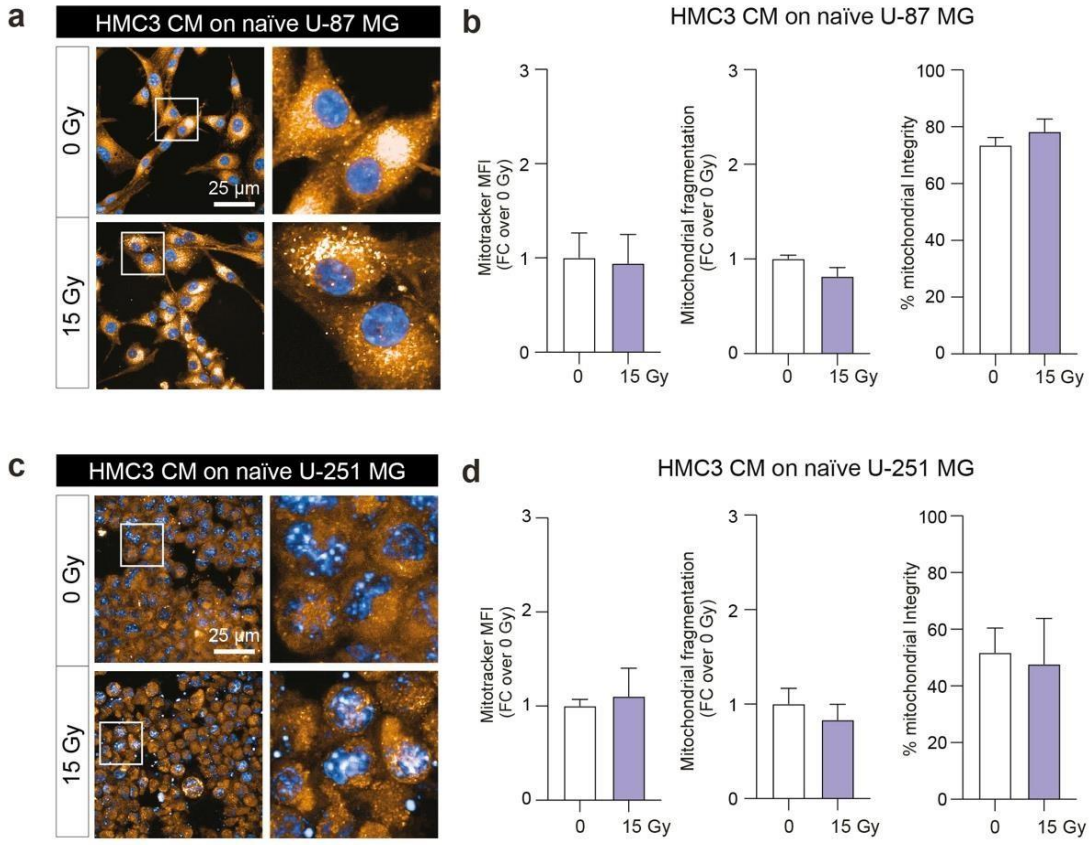


Figure 3. Irradiated HMC3 CM do not influence mitochondrial mass, fragmentation and integrity in naïve U-87 MG and U-251 MG cells. *a-b) Representative pictures of immunofluorescence staining with Mitotracker of U-87 MG treated with 0 Gy or 15 Gy HMC3 CM (a) and high-content analysis of Mitotracker MFI, mitochondrial fragmentation and percentage of mitochondrial integrity (b). c-d) Representative pictures of immunofluorescence staining with Mitotracker of U-251 MG treated with 0 Gy or 15 Gy HMC3 CM (c) and high-content analysis of Mitotracker MFI, mitochondrial fragmentation and percentage of mitochondrial integrity (d). Data are shown as bar plot and expressed as mean ± SEM of n = 4 independent replicates.*

HMC3 derived CM modulate mitochondrial fusion-fission mechanisms

In order to clarify the involvement of mitochondria and the effects observed on GBM cells cultured in irradiated microglia CM, we moved to evaluate the mRNA expression levels of the main genes involved in mitochondrial fusion, fission and stability. We analysed the levels of dynamin 1 like (DNM1L), ubiquinol-cytochrome c reductase complex assembly factor 2 (MNF1), mitofusin (MNF2), OPA1 mitochondrial dynamin like GTPase (OPA1) and fission, mitochondrial 1 (FIS1) on U87 MG and U-251 MG exposed to homocellular (U-87 MG or U-251 MG, respectively) or heterocellular (i.e. HMC3) CM (**Figure 4**).

GBM cells treated with 15 Gy GBM CM showed a significant increase of DNM1L, MNF1 and FIS1 expression levels as compared to mock-IR GBM (**Figure 4a-b**). A similar approach was used to evaluate genes involved in mitochondrial fusion/fission and stability on radiation-naïve GBM cells cultured with irradiated HMC3 CM. On the one hand, evaluation of mRNA expression levels showed that U-87 MG treated with 15 Gy HMC3 CM exhibit a slight reduction of DMN1L and MNF2 levels as compared to mock-IR HMC3 CM (**Figure 4c**) and no significant differences were observed in other tested genes. On the other hand, U-251 MG showed significantly higher levels of OPA1 and FIS1 when cultured with 15 Gy HMC3 CM (**Figure 4d**), no changes were observed in other tested genes.

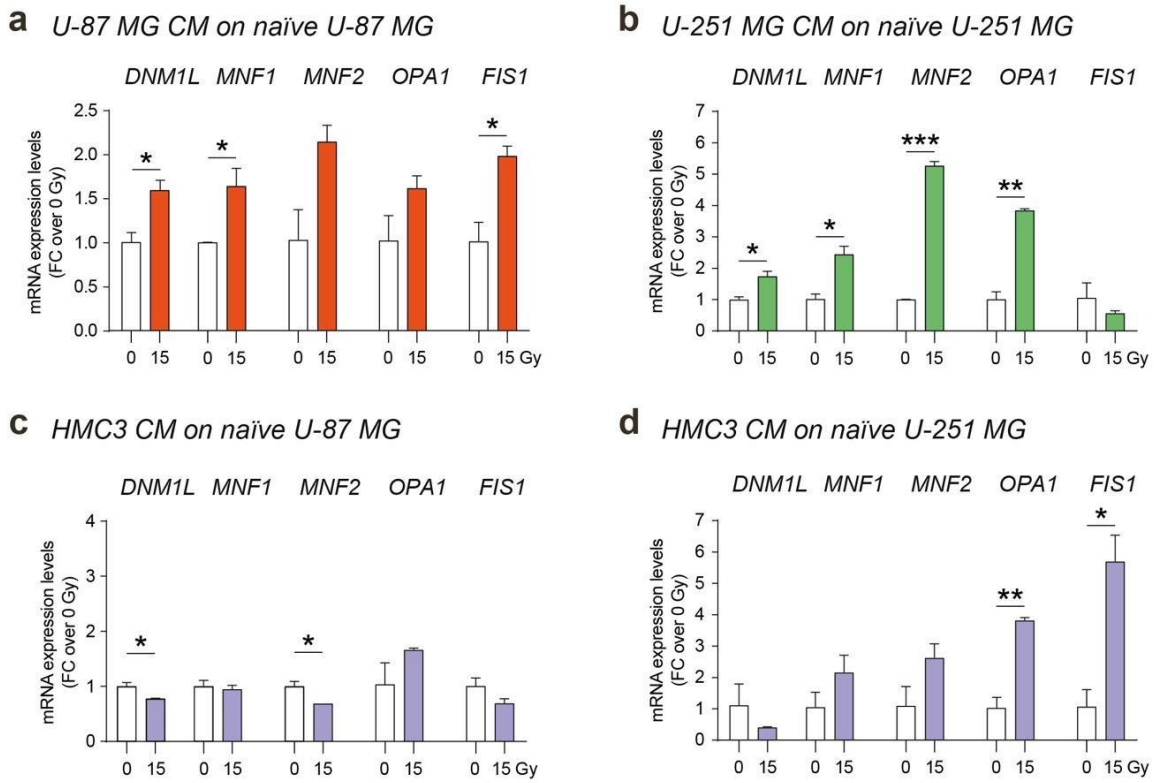


Figure 4. Irradiated cells-derived CM modulate mitochondrial fusion-fission mechanisms. *a) qRT-PCR analysis of mRNA expression levels of DNM1L, MNF1, MNF2, OPA1 and FIS1 in U-87 MG treated with 0 Gy or 15 Gy U-87 MG CM; b) qRT-PCR analysis of mRNA expression levels of DNM1L, MNF1, MNF2, OPA1 and FIS1 in U-251 MG treated with 0 Gy or 15 Gy U-251 MG CM; c) qRT-PCR analysis of mRNA expression levels of DNM1L, MNF1, MNF2, OPA1 and FIS1 in U-87 MG treated with 0 Gy or 15 Gy HMC3 CM; d) qRTPCR analysis of mRNA expression levels of DNM1L, MNF1, MNF2, OPA1 and FIS1 in U-251 MG treated with 0 Gy or 15 Gy HMC3 CM. Data are shown as bar plot and expressed as mean ± SEM of n = 4 independent replicates. *p < 0.05; **p < 0.01.*

HMC3 CM protect radiation-naïve GBM cell lines from mitochondrial oxidative stress

To assess the role of mitochondrial reactive oxygen species (ROS), we analysed the MitoSOX levels on directly irradiated cells finding a significant increase of the proportion of MitoSOX positive cells in all tested cell lines irradiated with 15 Gy as compared to mock-IR (**Figure 5a-c**). Particularly, we

observed an increased MitoSOX positive cells proportion in all tested irradiated cells versus mockIR cells (**Figure 5a-c**). Notably, mitochondrial ROS evaluation on 15 Gy HMC3 CM-treated GBM cell lines showed no significant changes as compared to mock-IR HMC3 CM group (**Figure 5d-e**), indicating that CM from irradiated microglia do not affect ROS production on radiation-naïve GBM cells.

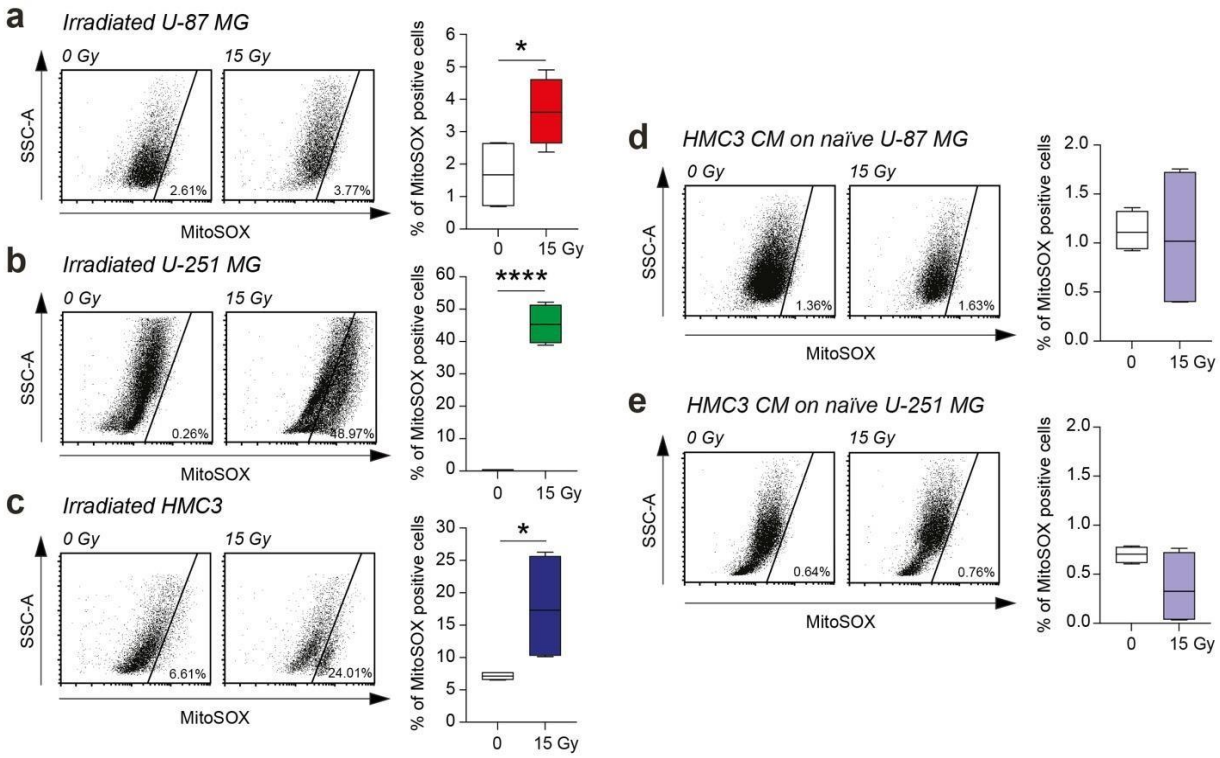


Figure 5. Radiation increases mitochondrial reactive oxygen species and irradiated HMC3 CM preserve mitochondrial oxidative state of U-87 MG and U-251 MG cells. *a-c) Cytofluorimetric analysis of MitoSOX positive cells in 0 Gy or 15 Gy U-87 MG (a), 0 Gy or 15 Gy U-251 MG (b) and 0 Gy and 15 Gy HMC3 (c). d-e) Cytofluorimetric analysis of MitoSOX positive cells in U-87 MG cultured with 0 Gy or 15 Gy HMC3 CM (d) and in U-251 MG cultured with 0 Gy or 15 Gy HMC3 CM (e). Data are shown via standard box and whiskers and are expressed as percentage of MitoSOX positive cells of n = 4 replicates for each experimental condition. * p-value < 0.05; **** p-value < 0.0001.*

Metformin administration reduces GBM clone formation mediated by irradiated HMC3 CM

In an effort to find potential modulators of heterocellular communication mediated by CM of irradiated HMC3, we tested whether oxidative phosphorylation mechanisms were involved in this phenomenon. We performed a clonogenic assay on U-87 MG and U-251 MG cell lines cultured with irradiated GBM CM (**Figure 6a-b**) and cultured in standard growth medium (**Figure 6c-d**), treated or not with metformin. Our results showed that metformin administration did not affect clone formation when U-87 MG and U-251 MG were cultured in growth medium. A similar result was observed for GBM cell lines exposed to irradiated GBM CM (**Figure 6a-b**).

We then tested the effects of metformin administration on GBM treated with 15 Gy HMC3 CM using clone formation assay. U-87 MG and U-251 MG showed a similar response to pharmacological blockage of mitochondrial oxidative phosphorylation system mediated by metformin. Particularly, U-87 MG showed a reduction of surviving fraction after metformin administration in 15 Gy HMC3 CM (**Figure 6e**). Similar results were observed in U-251 MG cell line, showing a significant reduction of the surviving fraction after treatment with metformin in 15 Gy HMC3 CM-treated U-251 MG cells (**Figure 6f**). Taken together, these data indicate that the decreasing of clonogenicity of GBM cell lines treated with irradiated HMC3 CM were a specific downstream result of metformin and that irradiated HMC3 CM stimulate oxidative phosphorylation mechanisms that can be targeted by metformin.

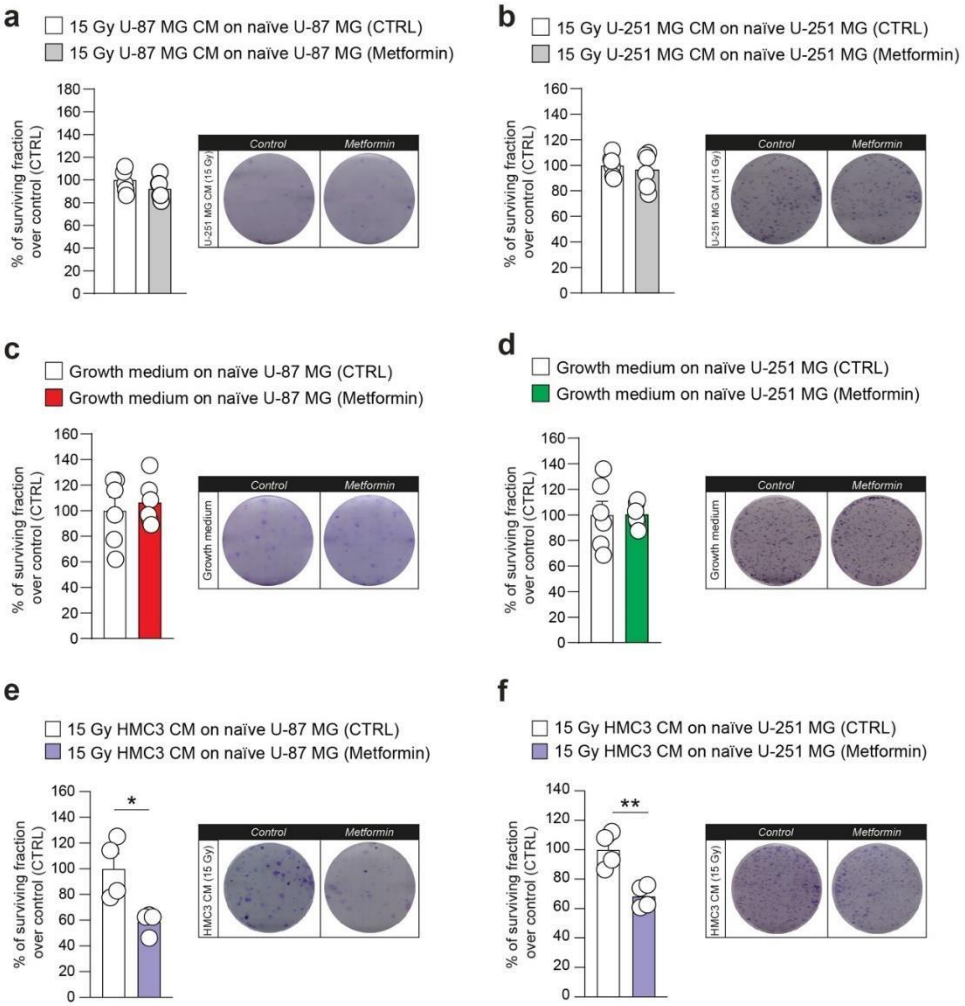


Figure 6. Metformin administration reverses irradiated HMC3 CM-induced effects. *a-b) Surviving fraction and representative pictures of U-87 MG (a) and U-251 MG (b) cell lines ± metformin, cultured with 15 Gy GBM CM. c-d) Surviving fraction and representative pictures of U-87 MG (c) and U-251 MG (d) cell lines ± metformin, cultured with standard growth medium. e-f) Surviving fraction and representative pictures of U-87 MG (e) and U-251 MG (f) cell lines ± metformin, cultured with 15 Gy HMC3 CM. Data are expressed as scattered dot-plot and mean ± SEM of n = 4 independent experiments. * p-value < 0.05 and ** p-value < 0.01.*

Conclusion

TME acquires different phenotypes and is differentially modulated by TAMs, contributing to GBM molecular subtyping [23]. Typically, TAMs have a pivotal role in leading tumor progression by limiting T cell-mediated anti-tumor immune response and stimulating cell proliferation and angiogenesis [24]. Metabolic changes are coupled with modifications of oxygen and nutrients availability, resulting from the complex relation among the different cellular components that mediate tumor metabolic rewiring [25]. This condition appears radical in GBM, so much so that is possible to distinguish GBM subtypes from a metabolic point of view, identifying mitochondrial GBM subset mainly based on oxidative mechanisms, characterized by an increase of oxidative

phosphorylation process at the expense of glycolysis [26]. Therefore, controlling the metabolism both in cancer and resident central nervous system (CNS) cells is a promising approach to limit resistance to therapy and/or to sensitize tumor [27].

Herein, we focused on the alteration induced by irradiation on TME, especially examining microglia-GBM interplay. Our results are in accordance to previously reported evidence on RT-induced GBM recurrences and proliferation [28] and expand on the biological effects of irradiated microglia via extracellular milieu mediated signaling. We found that microglial cells release factors that protect and preserve GBM viability, limiting apoptotic and cell death processes that affect radiation-naïve GBM cells. This effect was not observed when radiation-naïve GBM cells were treated with irradiated GBM CM, thus indicating a specific heterotypic-heterocellular communication between microglia and GBM. We also confirm that irradiated microglia act as a promoter of GBM proliferation through the secretion of thermo-stable molecules, ruling out the hypothesis that such an effect is primarily induced by protein mediators release in the surrounding milieu.

The hypothesis of a potential influence of TAMs on GBM metabolism and energy state is currently a largely unexplored field. Recent evidence suggests that metabolic alterations following RT may be linked to potential tumor-permissive changes and may be related to recurrences [4]. Given the high impact of metabolism in tumor growth, migration and resistance to therapy, we sought to evaluate the metabolic state of GBM cells.

It is well established that RT promotes mitochondrial rearrangement, as a mechanism related to cell stress response [29]. Mitochondria-related morphology, metabolism, respiration and ROS production are largely involved in RT-mediated alterations [30]. Therefore, we assume that microglia prevent and protect directly RT-mediated effects, as bystander mechanisms promoted by RT on tumor mass. This assumption correlates with evidence that RT mediates direct effects on GBM cells, and also off-target bystander effects, modifying both TME cell composition and tumor [31]. Furthermore, we demonstrate that HMC3 CM induces GBM proliferation stimulating oxidative phosphorylation mechanisms. This effect was not observed in GBM CM-induced process. In fact, metformin reduces GBM clone formation in radiation-naïve tumor cells cultured with irradiated HMC3 CM as compared to controls. As such, we speculate that microglia act as a promoter of GBM proliferation, at least in vitro, through the stimulation of respiration mechanisms, known as “the reverse Warburg effect”. This mechanism may represent an adaptive response that modulates

tumor cell metabolism according to the composition and state of surrounding cell populations [25, 32].

Our study would benefit from future research on in vivo models of GBM and the effect of radiotherapy on either cancer cells or CNS-resident cell populations. Moreover, a focus on mitochondrial DNA and mitochondria transfer via connexins/gap junction, crucial for cell-to-cell interaction [33], or by subcellular transporting mechanisms, such as tunneling nanotubes and microvesicles or extravesicles, would add significant information on the underlying biological processes [25]. It is well documented that microvesicles are secreted by tumor and microglia cells, and they could mediate GBM-no-tumoral communication using TME as a medium. Released exosomes may serve as carriers for cell-to-cell communication, which affects brain tumor progression and malignancy and controls microglia activation and GBM cell development in an autocrine and paracrine fashion [34]. Furthermore, in recent years the involvement of immunometabolism have been acquiring an increasing scientific interest in re-orchestrating GBM TME. Recent studies show how GBM and immune system metabolism interplay may interact to drive immunosuppressive processes [35, 36].

We report evidence that could have important consequences for radioresistance mechanisms of GBM and for the molecular processes that increase the post-radiotherapy recurrence rate. There are many in vitro and pre-clinical studies evaluating the combinatorial approach of temozolomide and disulfiram, which can cross the blood-brain barrier (BBB) and metformin, which has been shown to inhibit GBM stem cells (GSCs) proliferation [37]. Metformin mediated effects as antitumoral agent has been reported for a number of human tumors, including GBM [38]. Indeed, it was demonstrated that anti-cancer metformin-related effect are not only indirect, related to antagonist role on protumoral effects induced by hyperglycemia, but also directly related to a decrease of tumor growth [39]. Pharmacological efficacy of metformin was confirmed in terms of reduction of proliferation, survival, clonogenicity and in vivo tumorigenicity of GSCs. In conclusion, metformin, due to the ability to cross BBB, reveals a valuable and promising therapeutic tool for GBM treatment [40].

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Data availability statement: The datasets used and/or analyzed in this study are reported within the manuscript and/or additional files are available from the corresponding authors.

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Patient consent statement: Not applicable

Permission to reproduce material from other sources: Not applicable

Clinical trial registration: Not applicable

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Supplementary Materials

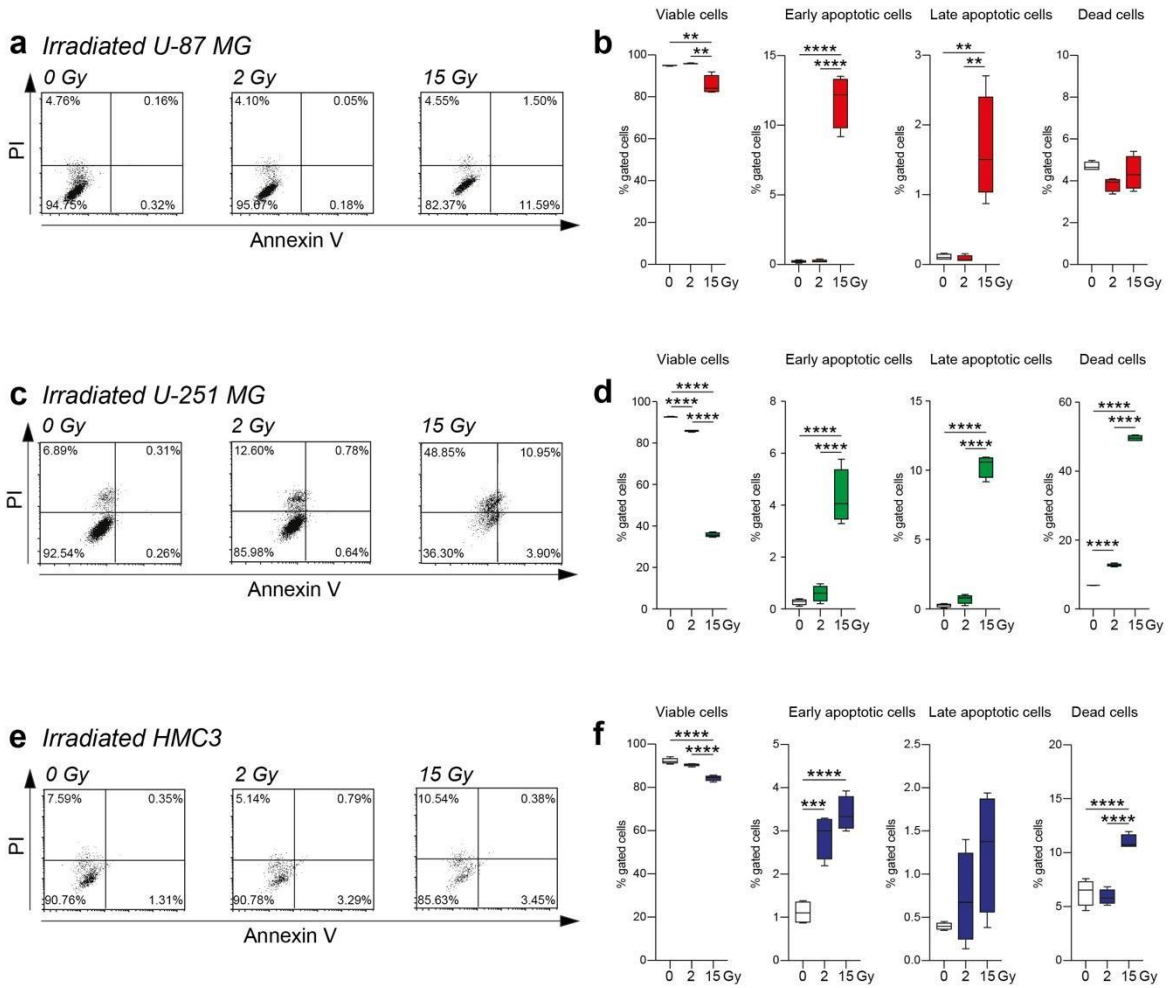

Figure S1. Direct radiation induces apoptosis and cell death in U-87 MG, U-251 MG and HMC3 cell lines. *a-f) Cytofluorimetric analysis of viability evaluated with Annexin V/PI assay on U-87 MG (a-b), U-251 MG (c-d) and HMC3 (e-f) cell lines. Data are shown as standard box and whiskers and viability is expressed as the percentage of gated cells, n = 4 independent replicates for each experimental condition. ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001.*

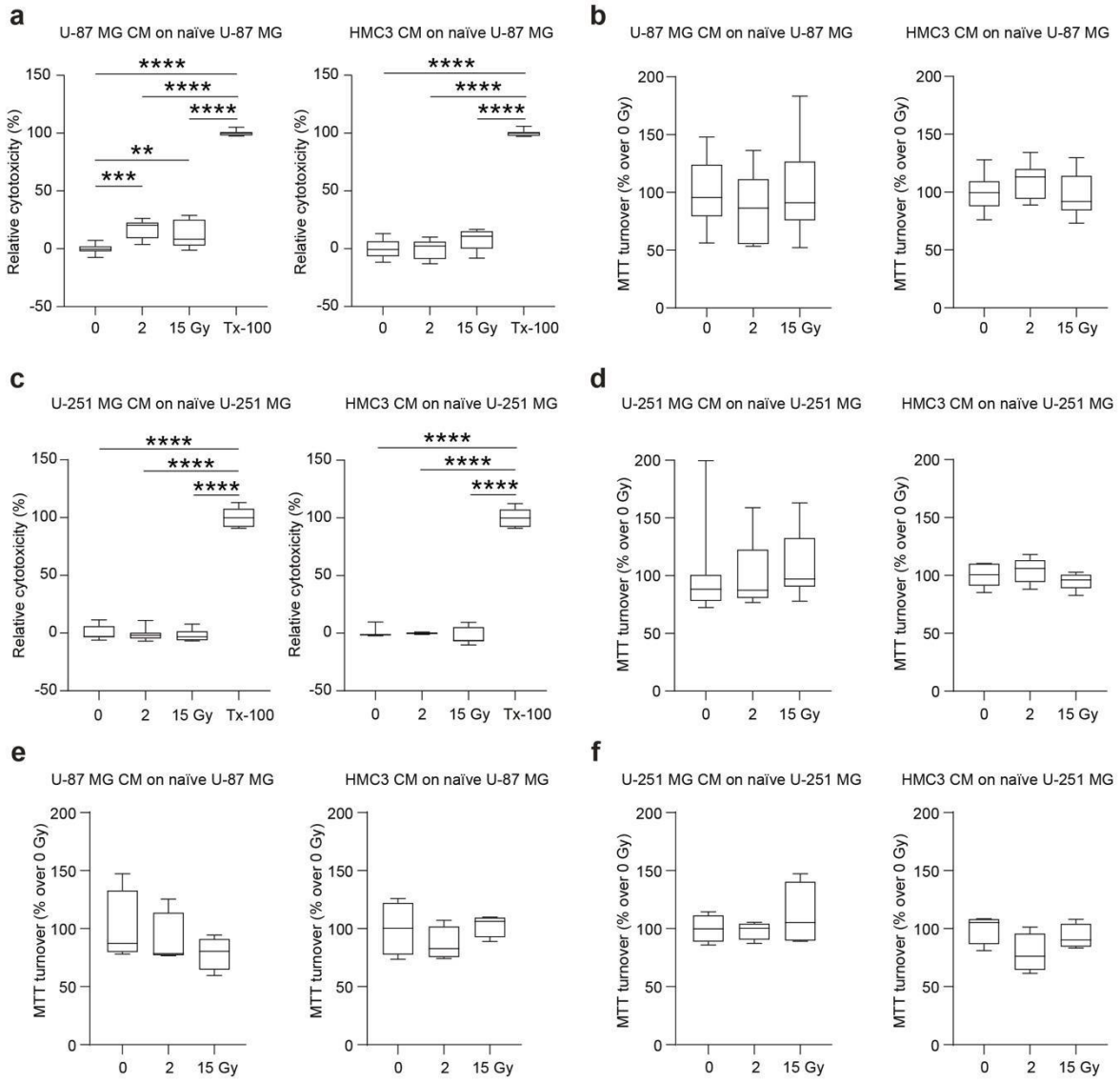

Figure S2. Irradiated HMC3 CM treatment on U-87 MG and U-251 MG cell lines maintains cell viability and preserves metabolic turnover. *a) LDH viability assay on U-87 MG cultures treated with 0 Gy, 2 Gy or 15 Gy U-87 MG CM or HMC3 CM. b) MTT turnover of U-87 MG treated with 0 Gy, 2 Gy or 15 Gy U-87 MG CM or HMC3 CM. c) LDH viability assay on U-251 MG cultures treated with 0 Gy, 2 Gy or 15 Gy U-251 MG CM or HMC3 CM. d) MTT turnover of U-251 MG treated with 0 Gy, 2 Gy or 15 Gy U-251 MG CM or HMC3 CM. e) MTT turnover of U-87 MG treated with 0 Gy, 2 Gy or 15 Gy U-87 MG CM or HMC3 CM for 72 hours. f) MTT turnover of U-251 MG treated with 0 Gy, 2 Gy or 15 Gy U-251 MG CM or HMC3 CM for 72 hours. Data are shown via standard box and whiskers of n ≥ 4 independent replicates for each experimental condition. **p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001.*

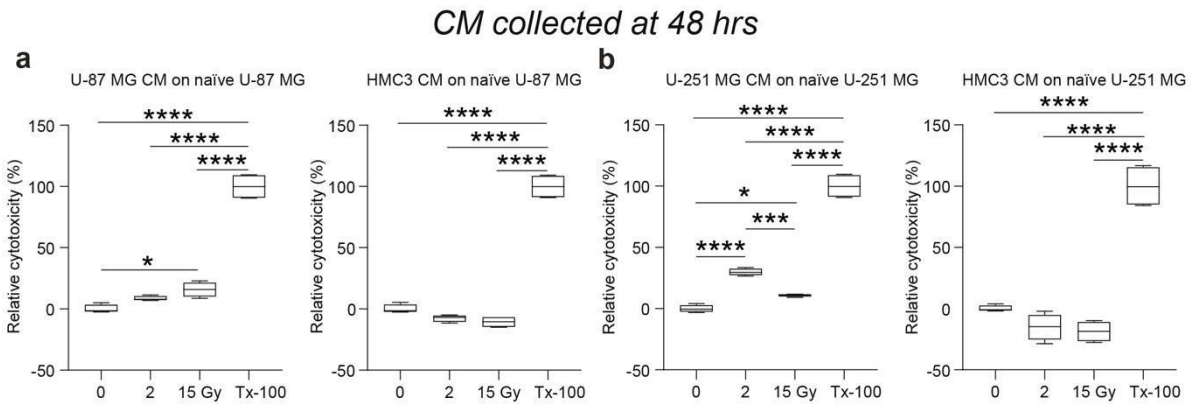

Figure S3. LDH assay on U-87 MG and U-251 MG cells exposed to homocellular or heterocellular CM collected at 48 hours post-irradiation*. a) LDH viability assay on U-87 MG cultures treated with 0 Gy, 2 Gy or 15 Gy U-87 MG CM or HMC3 CM, collected at 48 hours post-IR. b) LDH viability assay on U-251 MG cultures treated with 0 Gy, 2 Gy or 15 Gy U-251 MG CM or HMC3 CM, collected at 48 hours post-IR. Data are shown via standard box and whiskers of n ≥ 4 independent replicates for each experimental condition.*p-value < 0.05; ***p-value < 0.001; ****p-value < 0.0001.*

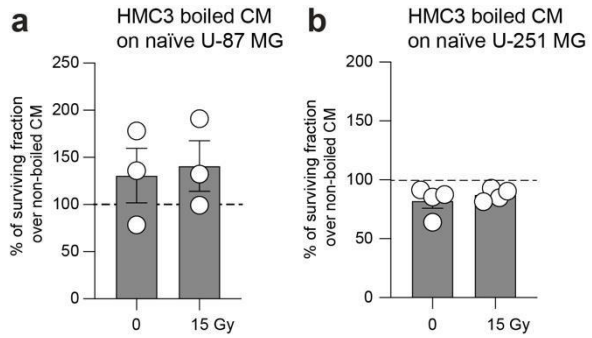

Figure S4. Boiled HMC3 CM retains its effects on U-87 MG and U-251 MG cell lines. *a-b) Surviving fraction and representative pictures of U-87 MG and U-251 MG cell lines exposed to 0 Gy and 15 Gy boiled HMC3 CM. Data are expressed via scattered dot-plot and mean ± SEM of n ≥ 3 independent replicates for each experimental condition.*

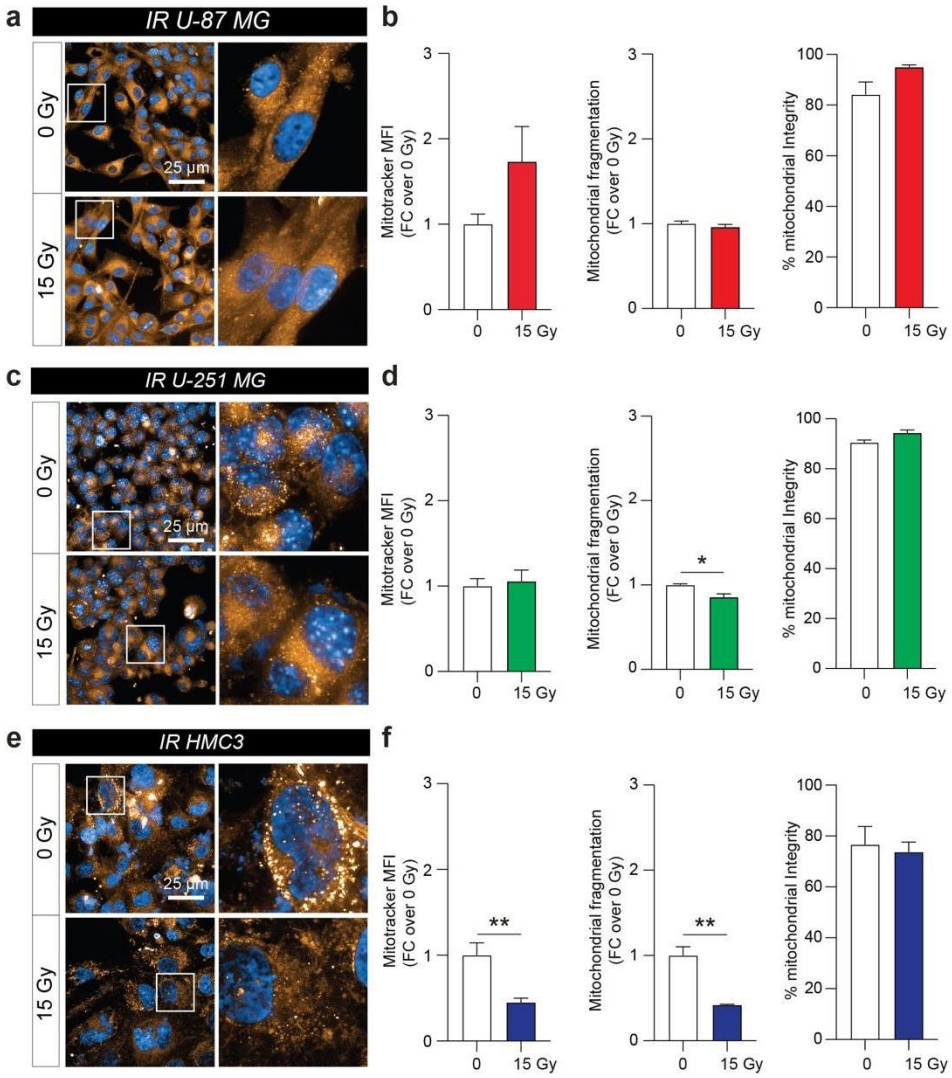

Figure S5. Effects of direct radiation on GBM and microglia mitochondrial fitness. *a-b) Representative pictures of immunofluorescence staining with Mitotracker of 0 Gy and 15 Gy irradiated U-87 MG (a) and high-content analysis of Mitotracker MFI, mitochondrial fragmentation and percentage of mitochondrial integrity (b). c-d) Representative pictures of immunofluorescence staining with Mitotracker of 0 Gy and 15 Gy irradiated U-251 MG (c) and high-content analysis of Mitotracker MFI, mitochondrial fragmentation and percentage of mitochondrial integrity (d). e-f) Representative pictures of immunofluorescence staining with Mitotracker of 0 Gy and 15 Gy irradiated HMC3 (e) and high-content analysis of Mitotracker MFI, mitochondrial fragmentation and percentage of mitochondrial integrity (f). Data are shown as bar plot and expressed as mean ± SEM of n = 4 independent replicates. *p-value < 0.05; **p-value < 0.01.*

Gene of interest	Forward primer (5’ -> 3’)	Reverse primer (5’-> 3’)
OPA1	AGGAGCTCATCTGTTTGGAGTC	GCTCACCAAGCAGACCCTTT
MNF2	GCGGAGACTCATAATGGCAGA	TCCGAGATAGCACCTCACCA
MNF1	ATGCAGTGGGAGTCCGAGC	CAGGGACATTGCGCTTCAC
FIS1	AAGAAAGATGGACTCGTGGGC	CCGCGTCTCCTTCAGGATTT
DNM1L	TGGGCGCCGACATCA	GCTCTGCGTTCCCACTACGA
ACTB	CCTTTGCCGATCCGCCG	AACATGATCTGGGTCATCTTCTCGC

Table S1. *List of primers’ sequences.*

Paper 3

antioxidants

MDPI

Review

Epigenetics and Metabolism Reprogramming Interplay into Glioblastoma: Novel Insights on Immunosuppressive Mechanisms

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Abstract: The central nervous system represents a complex environment in which glioblastoma adapts skilfully, unleashing a series of mechanisms suitable for its efficient development and diffusion. In particular, changes in gene expression and mutational events that fall within the domain of epigenetics interact complexly with metabolic reprogramming and stress responses enacted in the tumor microenvironment, which in turn fuel genomic instability by providing substrates for DNA modifications. The aim of this review is to analyse this complex interaction that consolidates several conditions that confer a state of immunosuppression and immunoevasion, making glioblastoma capable of escaping attack and elimination by immune cells and therefore invincible against current therapies. The progressive knowledge of the cellular mechanisms that underlie the resistance of the glioblastoma represents, in fact, the only weapon to unmask its weak points to be exploited to plan successful therapeutic strategies.

Keywords: glioblastoma; epigenetics; immunometabolism; tumor microenvironment; immunosuppression.

Introduction

Glioblastoma (GBM) is one of the main solid tumors that brings together the main hallmarks of cancer, conferring it a complexity and heterogeneity that make difficult the upmost therapeutical approaches [1]. The abnormal and dysregulated neovascularization associated with its typical pattern, consisting of necrotic foci with surrounding cellular pseudopalisades and microvascular hyperplasia, distinguishes GBM as a neoplasm with the lowest oxygenation levels. Such a characteristic makes GBM resistant to the current treatment protocol that includes surgical resection, radiotherapy and chemotherapy [2–5].

Metabolic rewiring and immune suppression in GBM are two closely related features, supporting pathogenesis and an aggressive pattern, limiting the efficacy of standard therapies and novel clinical approaches such as immunotherapy [6]. Indeed, the dynamic metabolic condition characterizing GBM profoundly reshapes the features of the tumor microenvironment (TME), thus creating hostile conditions for T cell proliferation and survival, negatively affecting the host immune response [6]. Several lines of evidence show that GBM progression is closely related to the interaction that tumor cells establish with other cell populations in the TME, which adopt mechanisms to avoid being detected and killed by immune cells [7]. In the same way that under physiological conditions, glial cells can influence stromal cells' behaviour, GBM cells can similarly mediate the homing, the recruitment and the differentiation of infiltrating cells in paracrine fashion or via a direct cell–cell contact [8–12].

In addition to glucose consumption and significant impairment in cytolytic activity and cytokine secretion due to TME acidification, several additional pathways in GBM drive immunosuppressive mechanisms [1]. In particular, aberrant lipid metabolism and specifically high free fatty acid levels closely modulate the immune response in the TME [13–15]. Moreover, like many other cancers, GBM also exhibits "glutamine addiction". Glutamine serves as the major contributor to cell growth and energy production after it is converted into glutamate via glutaminase, and then into αketoglutarate (α-KG) via glutamate dehydrogenase or several aminotransferases [16]. Mesenchymal GBM, which is the most aggressive molecular subtype, reported a significantly higher uptake of glutamine to generate glutamate-derived metabolites such as α-KG to produce ATP [17,18]. Glutamine deprivation in the TME determines the ideal conditions for immunosuppression due to the production of interleukin-23 (IL-23), interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), stimulating immune-suppressive regulatory T cells and suppressing cytotoxic

cells [19]. An additional detrimental factor to this dynamic metabolic condition is the onset of stress responses, such as hypoxia or anoxia, caused by the lack of an appropriate vascular system in the tumor core. A hypoxic TME dramatically reshapes the transcriptional landscape, which profoundly impacts metabolic networks [20,21]. Indeed, in hypoxic conditions, the dimerization of hypoxia inducible factors (HIFs) determines the transcriptional activation of several genes involved in metabolic reprogramming and immunosuppression [22,23]. Hence, immunometabolism can be considered an integrated hallmark of cancer cells that ensures their ability to escape immune system surveillance by masking DNA damage signaling factors for cellular defence activation or by releasing factors that prevent immune system aggression. Therefore, this condition fosters the path of neoplastic transformation [24,25].

Despite metabolic reprogramming being largely depicted as a prominent factor in restructuring the TME immune system of GBM, uncovered mechanisms between metabolism and immune evasion should be explored further, offering new research perspectives. In particular, the high heterogeneity and complexity of GBM suggest a critical influence of epigenetic factors including DNA methylation, histone modifications and microRNA (miRNA) post-transcriptional gene regulation, which remodel the genetic composition of the tumor and the interaction between immune cells and TME metabolic niches, thus favoring the escape from immune destruction [26–31].

In recent years, the epigenetic mechanisms influencing the TME in different cancers, including GBM, have acquired an expanding field of interest. Huo et al. examined glycolysis and gluconeogenesis enzymes by epigenetic modifications including miRNA, circular RNA and long non-coding RNA in HIFs regulation. They also reported how glucose metabolites, tricarboxylic acid (TCA) cycle, lipids and amino acids, as well as metabolites produced by gut microbiota, provide substrates for epigenetic modifications [32]. Wu et al. reported the impact of epigenetic modifications on the evolution of adaptive resistance to therapy in GBM [33]. The role of chromatin and epigenetic dysregulation have also been reported to promote glioblastoma stem cells (GSCs), which represent therapy-resistant reservoirs in GBM [26]. Epigenetic regulators in response to treatments were evaluated in shaping the phenotypic heterogeneity of GBM, reporting a variable balance between pre-existing and adaptive resistant cells, trying to clarify mutable adaptation to treatment [34]. Markouli et al. summarized main drugs targeting the epigenetic and metabolic interplay in gliomas from preclinical and clinical studies [35]. The crosstalk between epigenetics and metabolism was recently reviewed in GBM, showing its dual role in promoting and inhibiting the activity of metabolic

pathways [35]. However, despite these advanced investigations, the underlying epigenetic mechanisms have not yet been fully elucidated in driving the immunosuppressive state of GBM. In the intricate TME of GBM, a bidirectional linkage of metabolism and epigenetic modification is responsible for metabolic reshaping and histones/DNA modifications. Epigenetic regulation modulates the expression of metabolic enzymes, which ultimately affect overall tumor metabolism **(Figure 1**).

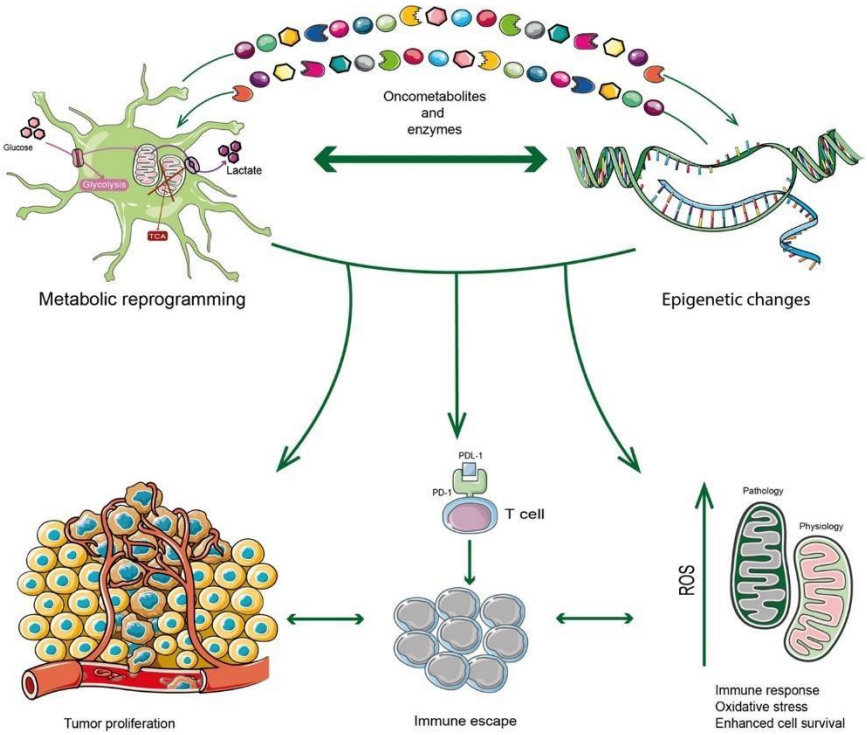

Figure 1. GBM tumor cells are highly inclined to find the metabolic adaptation to survive in hostile conditions including attacks by the immune system*. In this regard, their metabolic reprogramming is induced by epigenetic changes that determine the transcription of enzymes or factors belonging to several metabolic pathways. At the same time, enzymes or metabolites can also trigger epigenetic changes. The crosstalk between metabolism and epigenetics can be considered a bridge that leads to immunosuppression mechanisms, generating GBM tumor progression in a complex TME characterized by oxidative stress as well.*

This review aims to provide an insight into the epigenetic regulation and metabolism interaction in establishing an immune suppressive TME towards GBM aggressiveness, highlighting potential therapeutic targets.

Epigenetics and metabolism interplay

The epigenetic theories were firstly introduced in 1942 by C. Waddington, who coined the term and defined it as a "branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" [36]. Over time, evidence has increasingly converged to define epigenetics as the modifications of gene expressions mitotically and meiotically hereditable without involving modifications of gene sequences [37]. Inside the complex world of epigenetic regulations, the pre-eminent portion is assumed by the chromatin remodeling processes that modify the accessibility to the regions of the genome, also including the non-coding sequences [38]. Most of the DNA in eukaryotic cells is packed into nucleosomes, where it is wrapped around a core of eight histone proteins, which are the most abundant proteins associated with genomes. They consist of two dimers of H2A and H2B associated with a tetramer of H3 and H4, while H1 joins adjacent nucleosomes working as histone linker [39]. Histones are mostly composed of positively charged amino acids including up to 20% lysine, arginine and in minor quantities serin, which generate hydrogen bonds with the oxygen atoms of the phosphodiester of DNA. Nucleosome also contains amino-terminal tails which are not necessary for DNA winding, but represent the fulcrum of substantial modifications capable of changing the function of the nucleosome thanks to phosphorylation, acetylation and methylation of amino acid residues. Modifications of the histone tails can form a histone code that can be encoded by proteins involved in gene expression, repriming or activating specific genes [40].

Epigenetic regulation at the DNA level depends on dynamic interaction in nucleosomes through ATP-dependent remodeling of the histone octamer. Furthermore, the dynamism of histone modifications depends on the action of specific enzymes, including acetyltransferases and deacetylases, which catalyze the addition or removal of acetyl groups on histones, respectively, and methyltransferases and demethylases, which add or remove methyl groups. DNA methylation refers to the covalent bond of a methyl group supplied by S-adenosyl methionine (SAM) to the fifth position of the cytosine ring (5-methylcytosine) or the sixth position of adenine (6-methyladenine), catalyzed by the enzyme DNA methyltransferase (DNMT) [41]. 5-methylcytosine is the most prevalent form of DNA methylation in eukaryotes and occurs predominantly on the cytosines preceding the guanines, the so-called CpG sites or islands. Furthermore, methylated cytosines can be oxidized to 5-hydroxymethylcytosine by ten–eleven translocation enzymes (TET), even if these are less than 5-methylcytosine. However, CpG sites are not always associated with methylation site,

as many CpG islands remain constitutively protected from DNA methylation [42]. Other complex mechanisms of epigenetic regulation add to the structural modifications of the DNA–nucleosome interaction. For instance, the addition of acetyl groups changes the conformation of the DNA, making the binding of specific transcriptional complexes more effective, as in the case of bromodomain. Likewise, methylated sites can also be more easily recognized by proteins that act as repressors [43].

The accumulation of genomic instability and copy number alterations lead to genetic and epigenetic changes and transcription disorders, exerting a key role in the development of several tumors, including GBM [44,45]. A critical role in this process is represented by genomic changes induced by copy number variation and single nucleotide mutations and the following transcriptional dysregulation [45]. In this context, the methylation of O6-methylguanine DNA methyltransferase and the following repression of gene expression induce a greater chemoresistant ability to temozolomide, the elected therapy for GBM [46]. Moreover, ubiquitination of histone H2A, which interacts with epidermal growth factor receptor (EGFR), has also been described to control GBM resistance to senescence [47].

Within the complex TME of GBM, chromatin modifications can directly or indirectly be influenced by the activity of metabolic enzymes, metabolites and cofactors. Metabolites such as acetyl-CoA, ATP and SAM are precursors of acetylated, phosphorylated and methylated histones, respectively, while NAD+ acts as a cofactor for sirtuin (SIRT1) deacetylase [48]. In GBM, the activity of isocitrate dehydrogenase (IDH) represents a typical example of the relationship between epigenetics and metabolic reprogramming. Firstly, it is worth noticing that from the new 2021 WHO CNS tumor classification, IDH-mut term has been abolished for GBM; in fact, GBM IV grade was only considered as IDH-wt, whereas IDH-mut was limited to astrocytomas, including low-grade glioma [49–51]. However, epigenetic changes may drive the progression of IDH-mut low-grade gliomas to high-grade GBM; thus, the role of IDH-mut subtypes should be addressed in this scenario [52,53]. For this reason, here and after in this review we mention IDH-mut by implying that it refers to low-grade forms of glioma.

When mutated, IDH has been associated with tumorigenesis due to the change in enzymatic function. While IDH-wild type (IDH-wt) converts isocitrate and NADP+ to α-KG and NADPH, IDHmutated (IDH-mut) resides in the catalytic pocket and results in a neo-enzymatic activity: α-KG + NADPH → 2-hydroxyglutarate (2-HG) + NADP [54]. This neo-reaction in IDH-mut cells may also use

α-KG derived from glutamine, which is converted to glutamate by glutaminase and further metabolized to α-KG [55]. Innate metabolic dysregulation also occurs including L-2- or D-2hydroxyglutaric aciduria production due to non-functional enzymes, which metabolizes L- or D-2HG [56]. IDH represents the major pathway for cellular NADPH generation in most tissues, together with the TCA and pentose phosphate pathway (PPP). NADPH is an essential reducing factor that controls cellular defense mechanisms against oxidative damage through glutathione (GSH) reduction by GSH reductase, a crucial antioxidant that acts as a cofactor to reduce hydrogen peroxide. The classification of GBM tumor subtypes highlighted an interesting aspect related to the IDH mutated state and the methylation profile: the pro-neural subtype and the lower grade forms of astrocytoma share an IDH status with a high methylation profile defined glioma CpG island methylator phenotype [57,58]. The correlation between IDH-mut and methylated phenotype in GBM is a clear example of the mutual dependence between metabolism and epigenetics. In fact, the NADPH-dependent reduction of α-KG to the oncometabolite 2-HG leads to the inhibition of αKG-dependent dioxygenases due to structural similarities between α-KG and 2-HG. Specifically, TET demethylase activity is inhibited by the presence of 2-HG, blocking the demethylation process. D-2HG also inhibits histone demethylation by blocking the activity of demethylase such as lysinespecific demethylase (KDM) [54]. It was also reported that IDH-mut induced histone hypermethylation in genomic regions associated with DNA damage response pathways [59]. Likewise, it is possible to consider the other side of epigenetic regulation for IDH-wt, giving that IDH determines a lowering of the methylation profile due to the production of α-KG, which is essential for the demethylation functions of histone demethylase 1 (LSD1) and the lysine-specific JmjC domain containing histone demethylase (JHDM) on histones [60].

In addition to IDH activity, acetylation and succinylation, mediated by Acetyl-CoA and succinil-CoA, respectively, have been described as epigenetic processes driving resistance to targeted therapies [61,62]. Indeed, lysin succinylation leads to changes in many mitochondrial metabolic pathways, playing relevant roles in cell metabolism including glycolysis, fatty acid oxidation, urea cycle and glycolysis [63]. The aberration of these pathways is related to the occurrence of many tumors such as breast cancer, gastric cancer and gliomas. The latter is particularly sensitive to histone H3 succinyltransferase action, which promotes the proliferation and development of this tumor [62]. Moreover, other products of aerobic glycolysis include oncometabolites able to regulate epigenetic processes. Lactate released through monocarboxylate transporters (MCTs) represents an emerging oncometabolite which influences epigenetic mechanisms. Indeed, several lactylation sites on core

histones in humans have been identified [64]; radiolabelling carbon atoms, it has been observed that lactate represents a source for generating metabolites of the TCA cycle, converting citrate to acetyl-coA and increasing histone acetylation [65]. However, lactate also causes an increase in α-KG, triggering the previous epigenetic machinery, illustrating demethylated TET enzyme-dependent processes [66]. Lactate accumulation within cells induces histone lactylation, thus promoting cell proliferation and migration, modulating cell metabolism and promoting tumorigenesis [67–69]. In addition, lactic acidosis in the TME promotes a series of mechanisms that alter tumor metabolism and promote oncogenesis [70]. Thus, given the strong crosstalk among metabolism and epigenetic modifications in GBM, it becomes critical to explore the underlying mechanisms of immune escape.

Epigenetics and metabolism reprogramming interplay in promoting immunosuppression

Environmental and internal stressful stimuli, such as bacteria, virus, accumulation of metabolites, dead cells and uncontrolled proliferating cells could disrupt the constancy of steady states in an organism [71]. The innate and adaptive response of the immune system has a key role in maintaining tissue homeostasis, encompassing a plethora of effects beyond the gamut of "self" versus "nonself" interactions [72]. Cancer immunosurveillance, especially for extremely aggressive tumors such as GBM, is even more intricate given the complex network of interactions established between stromal, parenchymal and TME sites [7]. This blends the clear-cut boundary between innate and adaptive response, since physiologically the characters of the two responses closely cooperate in the various defense pathways. However, a distinctive feature of the immune system physiology of the CNS is represented by the tissue-resident microglia cells, that in GBM are known by the names tumor or glioma, associated microglia/macrophages (TAMs) and dendritic cells, which inhabit a major region of the brain parenchyma [73]. Cytokines, chemokines and nitric oxide (NO) are produced by microglia to initiate innate responses with phagocytic and cytotoxic functions, which can trigger additional responses due to the recruiting of soluble factors and peripheral immune cells, including natural killer cells, lymphocytes and macrophages. Moreover, CD4- and CD8-specific T cells for adaptive immune response are also mediated by the activation of microglia, which act as antigen presenting cells (APCs), upregulating MHC and co-stimulatory molecules [74]. A significant influence on the adaptive immune response comes from the meningeal spaces where the immune repertoire is mainly composed of cytokine-secreting CD4 T cells including IL-4, IFN-γ and IL-17 [75]. This evidence supports the change in the view of the CNS as an immune-privileged site; rather, the existence of a TME populated by immune cells dominates the processes of immunosuppression. Indeed, GBM is able to bypass the immunological response of several strategies which do not

depend only on the physiology and anatomical aspects of the CNS related to the presence of the protection offered by the BBB, limiting T cell trafficking and infiltration [7]. Certainly, as for most tumors, a reduced immune response is ascribable to the aggressive pharmacological and radiotherapy treatments which cause a depletion of the myeloid components [76]. T cell deficiencies can also result from a senescence process triggered by premature thymic involution in GBM patients [77]. In addition to quantitative defections, T cells have qualitative limitations determined by two interconnected processes, named anergy and exhaustion: the chronic exposure of T cells to antigens and inflammatory signals convoy them in a hyporesponsive or anergic state leading to exhaustion, which impairs T cell activation and downregulates cytokines [78]. T cell exhaustion is also supported by the immunosuppressive phenotype of APCs due to the expression of inhibitor-immune checkpoints, such as PD-1 and cytotoxic T-lymphocyte-associated proteins. PD1 is expressed in T regulatory cells (Tregs) which inhibit the proliferation and degranulation of T cells and APC, binding PD-L1 that is also upregulated in TAMs and in other immune cells. The expansion of Tregs is also promoted by immunosuppressive monocytes such as myeloid-derived suppressor cells that exert their effects locally, releasing immunomodulatory cytokines [79,80].

The pathogenic effects of oncometabolites in driving the immunosuppressive state of GBM are also mediated by complex mechanisms that definitely rely on epigenetic deregulation. The depletion of α-KG affects the canonical functioning of the prolyl hydroxylases (PHD) in the hydroxylation of HIF1α towards its ubiquitination [81]. Consequently, the production of 2-HG indirectly supports the dimerization of HIF-1α and the transcription of hypoxia response-element (HRE). Changes in chromatin structure, especially in histone methylation, acetylation and DNA methylation, are promoted by HRE transcription [82]. The downstream effects of this transcriptional regulation are related to the promotion of several cancer hallmarks including immune escape, such as the production of immunosuppressive cytokines and immunosuppressive myeloid-derived suppressive cells [23,79].

In a recent work by Friedrich and coworkers, it was highlighted that IDH-mut GBM reshapes immune populations, exploiting tryptophan metabolism-mediated immunosuppressive responses via 2-HG [83]. The authors identified subclasses of infiltrating myeloid cells in GBM IDH-mut that longitudinally change during tumor progression, generating an immunosuppressive TME. Surprisingly, infiltrated myeloid cells use 2-HG and tryptophan degradation via the kynurenine pathway and tryptophan 2,3-dioxygenase (TDO) activation leading to a metabolic reprogramming.

The main transporters involved in these mechanisms (i.e., LAT1-CD98) mediate tryptophan intake in a dose dependent fashion, leading to IL-10 and TGF-β production [83].

The involvement of tryptophan metabolism in mediating immunosuppression processes was evaluated in another study, which reported tumor-associated macrophage (TAM) recruitment and aryl hydrocarbon receptor (AHR) signaling, but no association with 2-HG production. Indeed, GBM cells have been reported to directly produce l-kynurenine by stimulating AHR-mediated recruitment of TAMs via CCR2 signaling triggered by CCL2 [84]. Furthermore, this study described epigenetic factors with low expression of miR-29b in TAMs able to suppress AHR levels [84]. It has been also shown that high levels of NAD+ are generated by the tryptophan and kynurenine metabolisms. Furthermore, a hypomethylation state in GBM has been associated with high NAP+ levels, leading to mesenchymal phenotypes and cancer progression [85].

The involvement of epigenetic processes in the immune evasion mechanism of GBM has been identified in another work revealing interesting perspectives linked to a process known as epigenetic immunoediting [86]. First, these findings were not linked to IDH-mut and metabolic alterations' relationship; engineered GSCs from neural stem cells were used to model the mesenchymal subtype with Nf1/Pten co-deletion and EGFRvIII overexpression. Immune cells have been observed to be the key responsible factor for the remodelling of GBM cell transcriptomics leading to an immunosuppressive TME. Therefore, the mutual connection between tumor and immune cells has been highlighted as, upon attack by the immune system, GBM modifies its transcriptomic profile to enhance an immunosuppressive myeloid-enriched TME [87]. Indeed, the most interesting result was related to the evidence that GBM cells, along with tumor progression, showed the upregulation of genes, such as interferon regulatory factor 8 (IRF-8) and chemokines belonging to the myeloid population, regardless of gene mutations and clonal selections. The maintenance of genomic stability, concomitantly with gene expression changes, has been attributed to epigenetic alterations that have been confirmed with the site-specific DNA methylation of GBM by interferon regulatory factor signaling [86]. Molecular subtyping of patient-derived GSCs revealed decreased levels of DNA methylation associated with a distinct transcriptional profile related to interferon gamma signaling as a dominant feature [86]. Moreover, induction of the mesenchymal phenotype in GBM by macrophages was associated with the production of specific ligands such as the pleiotropic cytokine of the interleukin-6 family Oncostatin M and leukemia inhibitory factor receptors. These are

associated with glycoprotein GP130 on GBM cells, in activating STAT3 signaling and a lower expression of colony stimulating factors [88].

It has been reported that glucose transporters were upregulated by hypermethylation and hypomethylation of CpG islands encoding for derlin-3 and caveolin-1, which are a glucose transporter (GLUT) inhibitor and GLUT stimulator, respectively [89]. More broadly, the upregulation of glycolytic enzymes such as pyruvate kinase M1/2 (PKM1/2) and hexokinase 2, mediated by epigenetic mechanisms, leads to an increase in glycolytic metabolism. PKM1/2 was also upregulated by c-myc expression after histone deacetylation, including the action of specific miRNA [90]. PKM2 also binds to histone H3 and phosphorylates histone H3 at T11 upon EGF receptor activation, determining histone H3 at lysine 9 with upregulation of cyclin D1 and c-myc expression [90]. MYC is a key oncogene belonging to transcription factors that coordinate the expression of several genes also involved in PD-L1 regulation [91]. The involvement of c-myc in GBM tumorigenesis, associated with epigenetics and metabolism reprogramming interplay, has been further demonstrated by recent reports [92]. In fact, it has been reported that specific mutations on the telomerase reverse transcriptase (TERT) gene enhance the trimethylation of histone H3 Lys4 (H3K4me3) and the recruitment of the multimeric GA-binding protein A (GABPA) in de novo binding motifs for the E-Twenty-Six transcription factor family members. This process is orchestrated by ERK1/2-dependent phosphorylation of arginino-succinate lyase at Ser417, facilitating the recruitment of GABPA and c-myc to TERT. In this context, H3K4me3 is driven by the inhibition of KDM α-KG-dependent due to fumarate production [92]; in this framework, it is worth noticing that telomere Repeat binding Factors have been implicated in immune escape [93].

GBM glycolytic activity causes low glucose availability in the TME, which determines the exhaustion phenotype in T cells; impaired lymphocyte proliferation, activation and degranulation have been associated with an anti-inflammatory phenotype after lactate exposure [94,95]. The enhancement of glycolytic metabolism is mediated by TGF-β, with TGF-β/Wnt inhibiting the expression of cytochrome C oxidase and resulting in increased glycolysis, which downregulates tumor surface antigens such as HLA-DR, NKG2DL and intercellular adhesion molecule 1, impairing immune cell infiltration and leading to tumor cells escaping from immune surveillance [96]. Acetyl-CoA from pyruvate dehydrogenase provides a precursor for fatty acid and cholesterol synthesis, but it can also modify histone proteins by direct acetylation [29]. In addition, acetyl-CoA is also a substrate with NADPH through the mevalonate pathway to the de novo synthesis of cholesterol. Farnesyl

diphosphate synthase belongs to the catalytic reactions to finally generate cholesterol, and it is involved in maintaining the stemness of GBM with prenylation, considered essential for nuclear remodelling [97,98]. Moreover, prenylation has also been associated with promoting immunosuppressive function on T cells and in the regulation of PD-L1 expression [99,100].

The formation, maintenance and recurrence of GBM are mainly governed by GSCs, which can be considered a subset of tumor cells with a significant ability to proliferate and a high self-renewal capacity [101]. GSCs are primarily localized in specific niches, including the so-called immune niche, where they promote signaling pathways that ensure the maintenance of a hypoxic TME, which stimulates the production of cytokines and immunosuppressive factors [102]. Their pre-eminent role in GBM pathogenesis has also been associated with immune suppression and metabolic reprogramming that can be classed under epigenetic regulation [101]. A recent study has shown how maintaining stemness and cell anaplasia in GBM is mediated by interconnected epigenetic and metabolic processes. In this study, Kosty and co-workers investigated the role of Serpine1 mRNA binding protein 1 (SERBP1), an oncogene RNA-binding protein, that has been found overexpressed in several tumors, including high-grade glioma [103]. Their study demonstrated that the expression of this protein is essential for maintaining the undifferentiated state of GBM cells by contributing to cell renewal and stemness. In particular, it was observed that SERBP1 regulates the metabolism of folate substrates by the interconnected serine biosynthesis and one-carbon (1C) cycle metabolic pathway, leading to additional metabolites such as cysteine and methionine. The first is involved in GSH production, whereas the second is associated with epigenetic processes via H3K27me3 levels, upregulating genes for neurogenesis and neuronal differentiation [104]. Although the authors analysed effects related to the maintenance of tumor progression, they did not evaluate the correlation to the immune evasion processes. However, the gene modulation-controlling cancer epigenome through methylation was also associated with PI3K/AKT signaling, which has been largely recognized as a key player in immunosuppression. Indeed, it has been reported that T cell apoptosis was induced under PI3K control by the co-stimulatory molecule B7-homologue 1 that is overexpressed in PTEN loss tumor subtypes [105].

In the epigenetic landscape, miRNAs certainly play a role of primary significance in GBM progression, involving several hallmarks of cancer [106]. However, there are few references in the literature connecting them to GBM immunometabolism. Rather, the role of miRNAs is often singly analyzed in metabolic reprogramming and immunosuppression processes, in which major effort is needed to

understand the touchpoints. Certainly, the direct gene expression modulation of miRNA-mediated metabolic enzymes has downstream consequences with immunosuppressive effects, as in the case of miR-326 targeting PKM2 [107]. In addition to the hypomethylation processes of genes encoding glycolytic enzymes, miRNAs have been described to act on c-MYC and on the PI3K/Akt pathway, leading to glucose transporters' accumulation, and glutamine levels as well, supporting the Warburg effect [108–110]. However, the expression of glycolytic transporters and enzymes in GBM were also indirectly regulated by miRNA, targeting receptors' tyrosine kinases [111,112]. As for immunosuppression, several key miRNAs may be regulating multiple mechanisms of both innate and adaptive immune responses [113].

A long list of miRNA expression profiles can be made in relation to the immunosuppression process they are controlling. However, this goes beyond the scope of this review which, among the epigenetic changes in relation to metabolism, wants to better highlight those related to chromatin remodelling rather than to non-coding sequences.

Ros contribution to immunosuppressive state

Mitochondrial dysfunction and oxidative stress are concomitant events in cancers, mainly attributed to the strong dependence on less energy-deriving pathways. Metabolic alterations in cancer contribute to the loss of redox balance between reactive oxygen species (ROS) elimination and production, creating a TME that supports tumor progression. Impaired mitochondrial metabolic capacity in GBM is strongly correlated with ROS production and antioxidant defense dysfunction [4]. During mitochondrial oxidative phosphorylation and electron transfer chain, the alteration of the flow rate of the respiratory chain increases the formation of superoxide radical and the consequent formation of the hydroxyl radical [114,115]. In this regard, cells may act with detoxification systems such as superoxide dismutase or glutathione peroxidase, converting GSH into its oxidised form to produce oxygen and water from hydrogen peroxide. In GBM IDH-mut, the dysregulation of TCA, towards the production of 2-HG, not only drives cells toward aerobic glycolysis but also interferes with the electron transport chain, altering mitochondrial physiology [116]. In addition to these effects, the NADPH consumption for IDH-mut activity affects GSH restoring and suppresses the chain of detoxification systems interfering with antigen presentation and T cell proliferations [117–119]. Therefore, the source of 2-HG increases oxidative stress and ROS in cancer cells, which encourages tumor cell growth [120]. These characteristics induce, on the one hand, the accumulation of ROS that might further stimulate tumor development due to the increase in genetic instability; on the

other hand, tumor cells' vulnerability to oxidative damage arises with decreasing GSH levels [121]. Increased oxidative stress in IDH-mut forms could explain the weakness of low-grade gliomas, although additional mechanisms related to IDH may be implicated in tumor viability. Indeed 2-HG has also been shown to function as an ATP synthase inhibitor and interfere with mTOR signaling, leading to a decrease in tumor growth and cell viability, whereas IDH-wt forms correlate to older patients with shorter than median survival, due to additional mutations such as higher EGFR amplification [122,123].

GBM response to mitochondria stress can also be examined in relation to the mitohormesis mechanism. More precisely, the term mitohormesis indicates a biological response to mitochondrial stress which can also lead to an increment in health and viability [124]. Indeed, mitohormesis enhances the effectiveness of adaptive metabolic strategies that produce acquired resilience leading to an enlarged healthspan, particularly improving metabolism and the immune system [125]. The positive benefits exerted by mitohormesis require a coordinated communication with the nuclear processes based on the redox-activated transcription factor [126]. Moreover, upon oxidative stress and hypoxia, which are common traits of GBM, the transcription factor nuclear factor erythroid-derived 2-like 2 (NRF2) is upregulated, linking mitochondrial dysregulation and epigenetic changes [127–129]. Indeed, oxidative stress-induced NRF2 nucleus translocation to bind antioxidant response elements (ARE) encodes proteins involved in response to stress, such as injury, inflammation and free radicals' production, including GSH synthesis [130,131]. Enzymes participating in the PPP are also expressed due to the epigenetic regulation of NRF2 [132]. The NRF2 involvement in epigenetic changes may be found in its cooperation with cobalamin metabolism and the D4 receptor on the regulation of the epigenetic state in both GBM and myeloid cells [133]. Furthermore, the lack of ubiquitination of NRF2 triggers the transcriptional cascade of the vitagene network, including redox sensitive genes, such as heme oxygenase-1 (HO-1), heat shock proteins (Hsps), thioredoxin (Trx) and SIRT1 [134,135]. In recent years, the vitagene system has emerged as a potential target, as it has been shown to have a high neuroprotective power. Therefore, the discovery of molecules capable of activating this system may represent a new therapeutic strategy to limit the consequences induced by oxidative stress, such as tumor progression [136]. HO-1 has been reported to maintain an immunosuppressive TME, this being expressed as monocyte and TAMs that suppress the antitumor CD8+ T cell effector [137–139]. Interestingly, HO-1 expression was demonstrated to educate myeloid transcriptional and epigenetic programs leading to the control of PD-L1, PD-L2 and MERTK expression [140]. Furthermore, the immunosuppressive activity

of bone marrow-derived macrophages in the glioma TME was abolished after HO-1 inhibition, restoring T cell proliferation. These results were both also associated with tryptophan metabolism, since myeloid HO-1 activity significantly increases IDO1 and to PD-L1 expression through the phosphorylation of STAT3 [141]. Hsps were involved in several mechanisms of proteostasis, including the host immune system evasion; indeed, T cell activation through T cell CD3-zeta downregulation is inhibited by HSP10 [142]. There are no direct correlations between SIRT1 and the epigenetic modifications that regulate immune escape in tumors. However, SIRT1 is involved in a multitude of effects related to histone and non-histone deacetylation that may include signal transduction and gene transcription suppressing the TME. It has been reported that in B cells, the activation-induced cytidine deaminase suppresses SIRT1 expression [143]. Moreover, IL-2 production was induced by c-Jun transcription factor inactivation mediated by SIRT1 [144]. Vice versa, it has also been reported that SIRT1 activates the adaptive response and differentiation of Th2 cells; there is also evidence indicating the enhancement of Treg acting as immune suppressive and inducing allograft tolerance [145].

In addition to ROS, reactive nitrogen species (RNS) also play a significant role in redox homeostasis alteration, contributing to further pathophysiological effects for TME supporting. RNS derive from nitric oxide (NO), a highly reactive molecule synthetized by NO synthase (NOS). Under physiological conditions, NO acts as a second messenger with the activation of cyclic guanosine monophosphate, generating multiple signaling pathways and downstream effects for neurotransmission [146]. NO plays an important role in the regulation of oxidative stress by providing neuroprotection with the production of antioxidants. However, when the homeostatic equilibrium is lacking, the effect of NO becomes cytotoxic and, in the presence of free radicals and inflammatory conditions, generates RNS leading to protein nitration and cell damage [147]. Most studies are limited to analysing the pro- or antitumor effects of NO in GBM rather than its role in relation to immunometabolism under epigenetic control [148]. The catalytic activity of JmjC-domain, containing histone demethylases, has been found to be inhibited by NO exposure in cancer cells, fostering an oncogenic phenotype [149]. Furthermore, the emerging correlations with epigenetic alterations have led to the definition of epigenetics as the third pillar of NO signaling, in addition to its role in soluble guanylate cyclase production and protein nitration [150,151]. The gap in mechanistic interpretation of transcriptional programs in response to oxidative stress, including ROS and RNS exposure, may be filled by the knowledge of epigenetic changes. Understanding these processes could provide further insights into GBM immunometabolism.

Taken together, we reported an integrated vision of immunometabolism that shows how immune mechanisms' and metabolic pathways' interplay can be the cause or the effect for epigenetic changes in a complex system where metabolites can be either the products or the source of posttranslational modifications driving the immunosuppressive state for GBM progression (**Figure 2**).

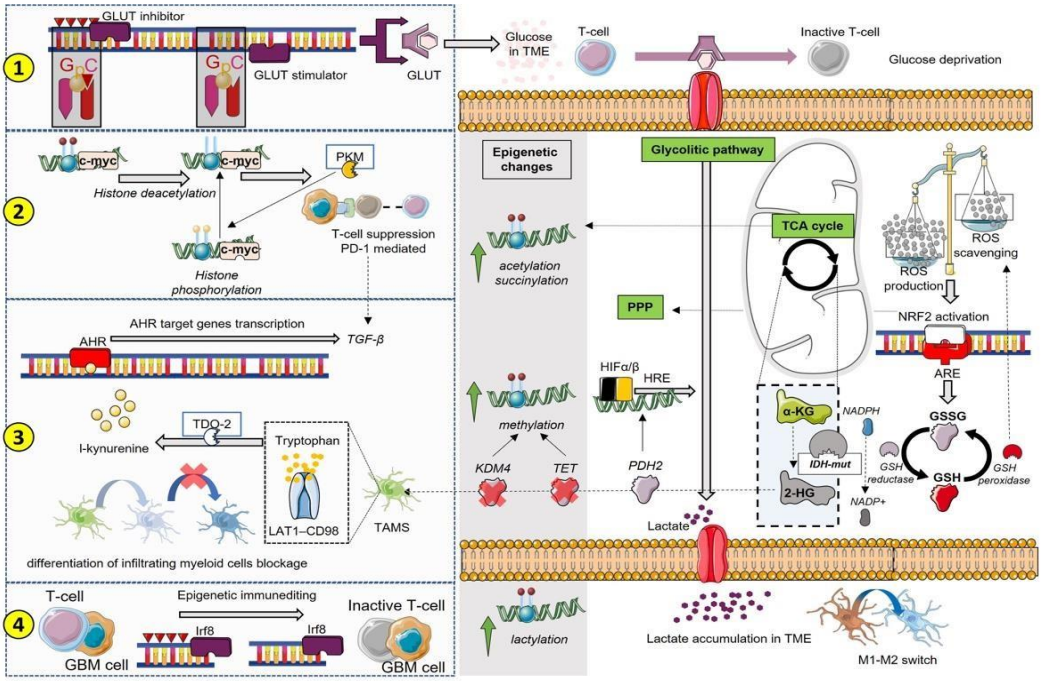

Figure 2. Several paths are involved in metabolism and epigenetics' interplay for immunosuppression. *We summarized four different ways that should be recognized as interconnected rather than separate from each other. Starting from point 1, the CpG islands control the expression of enzymes and factors involved in several metabolic pathways; therefore, the state of hypomethylation and hypermethylation can enhance or reduce specific metabolic pathways. For instance, methylation of GLUT inhibitors determines an increase in glucose intake, depleting the TME, where myeloid cells will not be able to perform their functions. TCA break determines an increase in lactate and a TME acidification that, besides increasing the processes of lactylation, determines mechanisms of immunosuppression including reshaping macrophages' phenotype. Point 2 shows the direct involvement of epigenetic-mediated c-myc gene expression in association with PKM production and PD-1/PD-L1 immunosuppression mechanisms. Point 3 describes the contribution of tryptophan metabolism in 2-HG-induced TAMs, which results in the activation of AHR signaling with the production of interleukins and immunosuppressive cytokines, such as TGF-β. The production of 2-HG determines methylation changes linked to the blockade of TET and KDM4, as well as to the modulation of HIF mediated by the lack of PHD activity. Point 4 illustrates the effects of immune and tumor cells' interplay inducing the so-called epigenetic immunoediting that re-educates and hijacks the transcriptional programs of the tumor towards an immunosuppressive phenotype. In this broad scenario, the alterations of the mitochondrial respiration chain must be taken into account. ROS production activates the response of genes to oxidative stress, which includes NRF2 signaling with the expression of genes and factors involved in metabolic reprogramming and immunosuppression. Among these, the production of GSH certainly plays an important role in detoxification processes that may be hindered by NADPH consumption for IDH-mut activity. Functional depletion of GSH has also an impact on antigen presentation and T cell proliferation.*

Limitations and challenges of immunotherapy

Phase II and III clinical trials have been performed for immune checkpoint inhibitors in both newly diagnosed and recurrent GBM. Nivolumab is one of the most common FDA-approved monoclonal IgG4 antibodies targeting the PD-1 receptor, which has been tested in combination with bevacizumab and with temozolomide. It was also tested comparing gene promoter profile methylation of O(6)-methylguanine DNA methyltransferase (MGMT) [152,153]. Pembrolizumab is also a PD1 receptor inhibitor, that was tested in phase I/II for recurrent GBM and phase III for neoadjuvant GBM, reporting both local and systemic antitumor immune response enhancement. Despite the local anti-tumor immune response increase observed in GBM patients treated with this anti-PD1 receptor, overall survival improvements have not been reported to declare the success of this drug [154–156]. Other FDA-approved immune checkpoint inhibitors, such as atezolizumab, were directed to target PD-L1. Atezolizumab efficacy for GBM is still under investigation, although promising results have been observed for peripheral CD4+ T cells and in evaluating the correlation of hypermutation phenotypes with tumor mutation analysis [157].

Immune checkpoint inhibitor limitations may lay in several potential issues that make challenging the current approaches for GBM. First, the genomic heterogeneity of GBM is crucial in determining the efficacy of immunotherapies, since specific mutations such as PTEN, MAPK pathway alterations, germline DNA polymerase epsilon and broad mutation, such as somatic mutations, microsatellite instability and tumor mutational burden, seem to acquire prognostic and predictive values [158]. If on the one hand, the stratification of GBM types can be beneficial in guiding the treatment personalization, on the other hand, the high tumor heterogeneity hinders the immunotherapy approaches. Some limitations, though, which can be broadly referred to as several pharmacological treatments, should be also mentioned in the case of immunotherapy. These are related to the pharmacological issues for achieving the adequate doses based on the CNS toxicity that may also be associated with immune response or a misdirected immune-mediated injury increasing intracranial pressure [159]. In conclusion, the Immunotherapy Response Assessment in Neuro-Oncology criteria have a key role in interpreting radiographic endpoints that, after immunotherapy, may be critical to confirm the effectiveness of the treatment, especially to discriminate the radiologic features of tumor progression from the inflammatory response induced by an effective anti-tumor immune response [160].

The transitions from preclinical evaluation to the clinical development of potential immunotherapy require appropriate preclinical models. The most commonly used over the years in the immunotherapy setting are represented by syngeneic mice, which show several advantages, including their logistically easy application, but also show limitations in matching directly with human clinical disease and outcomes [161]. This limitation is overcome by the use of transgenic mouse models or genetically engineered mouse models which reproduce a faithful stromal biology, although the few neo-antigen formations might hinder the immune oncology evaluation [162]. Cell line- and patient-derived xenografts have been currently applied for immune oncology studies, overcoming major limitations of syngeneic and genetically engineered mouse models; however, the poor predictive values, the heterogeneity loss and the difficulty in evaluating immune-mediated responses are critical points [163].

The issues listed in the field of immunotherapy, if retained within the epigenetics and metabolic reprogramming interplay, acquire further complexity. In this regard, preclinical studies should be addressed to the pathophysiological mechanisms' evaluation in response to the novel and currently targeted therapies for GBM, including antiangiogenic agents and kinase inhibitors combined with immune checkpoint therapy, considering the tumor subtype and biological profile.

Conclusions

Immunotherapy represents a promising strategy for the treatment of GBM. However, the different clinical approaches and attempts encounter significant obstacles represented by the processes of immunosuppression, which therefore need to be further investigated.

In the intricate TME of GBM, metabolic reprogramming, oncometabolite production and oxidative stress support immunosuppression. Epigenetic changes establish a complex interaction with metabolic rewrite actors orchestrating a condition promoting immunosuppression, including the production of specific metabolites that ultimately affect chromatin states. The correlation between epigenetics and metabolism in ensuring an immunosuppressive phenotype is strongly mediated by IDH activity with downstream and upstream processes and effects. However, many other immunosuppressive mechanisms are activated, including the prominent role of the stress response pathways, thus suggesting the need to explore additional factors underlying immunosuppression in the complex TME of GBM in an effort to highlight new drug targets. Metabolism, immunosuppression, and epigenetic regulation are undoubtedly macroscopic and complex areas.

Each field contains a multiplicity of cellular and molecular mechanisms whose relational analysis is challenging. Many processes are still unclear, but remarkable research perspectives have been progressively emerging. Among them, the role of GSCs in the interplay between immunosuppression and metabolic reprogramming may reveal interesting scenarios since they play a prominent role in the pathogenic processes of GBM. Further efforts will be needed to expand this research field which could provide better therapeutic strategies for GBM treatment.

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Abbreviations

α-KG: α-ketoglutarate

AHR: Aryl hydrocarbon receptor

APCs: Antigen presenting cells

ARE: Antioxidant response elements

DNMT: DNA methyltransferase

EGFR: Epidermal growth factor receptor

GABPA: GA-binding protein A

GLUT: Glucose transporter

GBM: Glioblastoma

GSCs: Glioblastoma stem cells

GSH: Glutathione

H3K4me3: Trimethylation of histone H3 Lys4

HIFs: Hypoxia inducible factors

HO-1: Heme oxygenase-1

HRE: Hypoxia response-element

Hsps: Heat shock proteins

IDH: Isocitrate dehydrogenase

IDH-mut: IDH-mutated

IDH-wt: IDH-wild type

IL-10: Interleukin-10

IL-23: Interleukin-23

IRF-8: Interferon regulatory factor 8

JHDM: Jumonji-domain histone demethylase

KDM: Lysine-specific demethylase

LSD1: Demethylation functions of histone demethylase 1

miRNA: MicroRNA

NO: Nitric oxide

NOS: NO synthase

NRF2: Nuclear factor erythroid-derived 2-like 2

PHD: Prolyl hydroxylases

PKM: Pyruvate kinase M1/2

PPP: Pentose phosphate pathway

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SAM: S-adenosyl methionine

SIRT1: Sirtuin

TAMs: Tumor-associated macrophages

TCA: Tricarboxylic acid

TDO: Tryptophan 2,3-dioxygenase activation

TERT: Telomerase reverse transcriptase

TET: Ten-eleven translocation enzymes

TGF-β: Transforming growth factor-beta

TME: Tumor microenvironment

Tregs: Regulatory T cells

Trx: Thioredoxin

2-HG: 2-hydroxyglutarate

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10. List of published papers

- Denaro S, Pasquinucci L, Turnaturi R, Alberghina C, Longhitano L, Giallongo S, Costanzo G, Spoto S, Grasso M, Zappalà A, Li Volti G, Tibullo D, Vicario N, Parenti R, Parenti C. Sigma-1 Receptor Inhibition Reduces Mechanical Allodynia and Modulate Neuroinflammation in Chronic Neuropathic Pain. Mol Neurobiol. 2023 Nov 3. doi: 10.1007/s12035-023-03717-w. Epub ahead of print. PMID: 37922065.

- Torrisi F, D'Aprile S, Denaro S, Pavone AM, Alberghina C, Zappalà A, Giuffrida R, Salvatorelli L, Broggi G, Magro GG, Calabrese V, Vicario N, Parenti R. Epigenetics and Metabolism Reprogramming Interplay into Glioblastoma: Novel Insights on Immunosuppressive Mechanisms. Antioxidants (Basel). 2023 Jan 18;12(2):220. doi: 10.3390/antiox12020220. PMID: 36829778; PMCID: PMC9952003.

- Denaro S, D'Aprile S, Alberghina C, Pavone AM, Torrisi F, Giallongo S, Longhitano L, Mannino G, Lo Furno D, Zappalà A, Giuffrida R, Tibullo D, Li Volti G, Vicario N, Parenti R. Neurotrophic and immunomodulatory effects of olfactory ensheathing cells as a strategy for neuroprotection and regeneration. Front Immunol. 2022 Dec 19; 13:1098212. doi: 10.3389/fimmu.2022.1098212. PMID: 36601122; PMCID: PMC9806219.

- D'Aprile S, Denaro S, Pavone AM, Alberghina C, D'aprile S, Torrisi F, Li Volti G, Zappalà A. FROM PHYSIOLOGICAL TO NEOPLASTIC TRANSFORMATION: THE CRITICAL ROLES OF CONNEXINS AND WT1. EuroMediterranean Biomedical Journal. 2022 Apr 29; 17 (12) 48-58; doi: 10.3269/1970-5492.2022.17.12

- Torrisi F, Alberghina C, D'Aprile S, Pavone AM, Longhitano L, Giallongo S, Tibullo D, Di Rosa M, Zappalà A, Cammarata FP, Russo G, Ippolito M, Cuttone G, Li Volti G, Vicario N, Parenti R. The Hallmarks of Glioblastoma: Heterogeneity, Intercellular Crosstalk and Molecular Signature of Invasiveness and Progression. Biomedicines. 2022 Mar 30;10(4):806. doi: 10.3390/biomedicines10040806. PMID: 35453557; PMCID: PMC9031586.

- Torrisi F, Alberghina C, Lo Furno D, Zappalà A, Valable S, Li Volti G, Tibullo D, Vicario N, Parenti R. Connexin 43 and Sonic Hedgehog Pathway Interplay in Glioblastoma Cell Proliferation and Migration. Biology (Basel). 2021 Aug 12;10(8):767. doi: 10.3390/biology10080767. PMID: 34439999; PMCID: PMC8389699.

- Vicario N, Spitale FM, Tibullo D, Giallongo C, Amorini AM, Scandura G, Spoto G, Saab MW, D'Aprile S, Alberghina C, Mangione R, Bernstock JD, Botta C, Gulisano M, Buratti E, Leanza G, Zorec R, Vecchio M, Di Rosa M, Li Volti G, Lazzarino G, Parenti R, Gulino R. Clobetasol promotes neuromuscular plasticity in mice after motoneuronal loss via sonic hedgehog signaling, immunomodulation and metabolic rebalancing. Cell Death Dis. 2021 Jun 16;12(7):625. doi: 10.1038/s41419-021-03907-1. PMID: 34135312; PMCID: PMC8209072.

11. List of congress contribution

- 73rd SIF National Congress- The Italian society of Physiology [Pisa, Toscana, Italy, 06/09/2023 –08/09/2022] - Poster session
  Abstract title: Microglia promotes glioblastoma proliferation via heterocellular communication as off-target effects of radiotherapy
- 47th ERRS Annual Meeting of the European Radiation Research Society (ERRS 2022) [Catania, Sicily, Italy, 21/09/2022 – 24/09/2022] - Poster session
  Abstract title: Tumor microenvironment alteration induced by radiotherapy modifies glioblastoma cells proliferation and mitochondrial fitness
- 72nd SIF National Congress- The Italian society of Physiology [Bari, Puglia, Italy, 14/09/2022 – 17/09/2022]
  Abstract title: Heterocellular communication mediated by irradiated microglia sustains glioblastoma growth and proliferation - Poster session
- 71st SIF National Congress- The Italian society of Physiology [Milano, Lombardia, Italy (online), 07/09/2021 – 09/09/2021] - Poster session
  Abstract title: Radiotherapy-induced effects on microglia support Glioblastoma growth and malignance by tumor microenvironment alteration

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