Molecular heterogeneity and immunosuppressive microenvironment in Glioblastoma

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Abstract:

Glioblastoma (GBM) is the most aggressive primary brain tumour in adults, with a poor prognosis, despite surgical resection combined with radio- and chemotherapy. The major clinical obstacles contributing to poor GBM prognosis are late diagnosis, diffuse infiltration, pseudo-palisading necrosis, microvascular proliferation and resistance to conventional therapy. These challenges are further compounded by extensive inter- and intra-tumour heterogeneity and the dynamic plasticity of GBM cells. The complex heterogeneous nature of GBM cells is facilitated by the local inflammatory tumour microenvironment, which mostly induces tumour aggressiveness and drug resistance. An immunosuppressive tumour micro-environment of GBM provides multiple pathways for tumour immune evasion. Infiltrating immune cells, mostly tumour-associated macrophages, comprise much of the non-neoplastic population in GBM. Further understanding of the immunological microenvironment of GBM is essential to make advances in the development of immunotherapeutics. Recently, whole-genome sequencing, epigenomics and transcriptional profiling have significantly improved the prognostic and therapeutic outcomes of GBM patients. Here, we discuss these recent genomic advances, the role of innate and adaptive immune mechanisms, and the presence of an established immunosuppressive GBM microenvironment that suppresses and/or prevents the anti-tumour host response.

INTRODUCTION

Glioblastoma (GBM) is the most common primary brain tumour with an annual incidence of 3.19 per 100,000 population (1). GBM is a Grade IV astrocytoma, characterised by uncontrolled cellular proliferation, local infiltration, extensive genomic instability, tendency for necrosis, angiogenesis, and resistance to therapy. Histopathologically, GBM is composed of a heterogeneous cell population, consisting of differentiated and undifferentiated tumour cells, along with differences in morphology and capacity for self-renewal and proliferation (2, 3). Despite aggressive treatment including surgical resection and radiotherapy with concomitant chemotherapy, prognosis remains poor due to GBM recurrence, with a median survival of 14.6 months (4). In molecular terms, this poor prognosis is mostly characterised by deregulation of many key signalling pathways involving cell survival, growth, proliferation and apoptosis due to genomic mutations (5). GBM is a robust malignant tumour, distinguished by its local invasion pattern (6, 7). Generally, GBM do not metastasize extracranially; however there have been rare cases in which 0.44% of GBM have spread to other parts of the body usually when patients have undergone craniotomy (8, 9).

GBM is highly invasive, lack clear margins, and therefore, pose a challenge for complete surgical resection and almost inevitably recur in patients who have been treated. Despite recent advances in genomics, chemotherapy, immunotherapy, and technological approaches to cancer models, the treatment outcome for GBM patients has remained consistently poor. Clinical symptoms vary and depend on size and location of tumour; it may include headache, nausea, dizziness, confusion, speech difficulties, and change in personality, new onset of seizures and focal neurological deficit. The tumour is generally located in the frontal and temporal lobes of the brain and can also rarely occur in the brainstem, cerebellum and spinal cord (10, 11). GBM is most often *de novo* i.e. primary GBM, which account for approximately 90% of GBM cases and are predominately found in patients older than 45 years (5). The remaining 10% of GBM cases develop from a lower-grade tumour progressing to a higher-grade malignancy (secondary GBM) over a 5-10 year period, and is primarily present in patients younger than 45 years. These subtypes have distinct genetic aberrations but are histologically indistinguishable (5, 12, 13).

Despite advances in our understanding of cancer biology, managing GBM remains a challenge. It is important to understand why treatment for GBM has largely been ineffective; it is mainly

due to the heterogeneous nature of the tumour microenvironment. It has not been possible to produce appropriate cancer models for GBM that would help us study the properties by which GBM is promoted and sustained. Therefore, it is vital to study the role of the immune system in the GBM microenvironment. This review aims to analyse the recent genomic advances in dissecting the considerable molecular and cellular heterogeneity in GBM and the innate and adaptive immune mechanisms that are suppressed, which ultimately contribute towards tumorigenesis.

GENOMIC LANDSCAPE OF THE GBM MICROENVIRONMENT

GBM has considerable cellular and molecular heterogeneity, both between patients and within the tumour microenvironment itself. GBM subtyping via histological examinations is a poor prognostic indicator for gliomas. Glioma is an overarching term used for brain tumours of glial cells: astrocytes: glioblastoma; oligodendrocytes: oligodendroglioma; ependymal cells: ependymoma and was improved by combining histology with molecular genotyping of key markers (e.g. iso-citrate dehydrogenase (IDH), ATP-dependent helicase (ATRX), Lys-27-Met mutations in histone 3 (H3K27M), p53 mutations, and 1p/19q chromosomal deletion (Louis et al., 2016; Bent, 2010). However, the era of genomics and next generation sequencing (NGS) has led to a greater understanding of the formation and pathogenesis of these tumours by identifying core molecular pathways affected, facilitating the design of novel treatment regimens. The Cancer Genome Atlas (TCGA) network was among the first to conduct a major genomic study on cancer interrogating 33 different types, with particular emphasis on GBM, leading to the whole genome characterisation and molecular genotyping of 600 GBM and 516 other low-grade gliomas (Wang et al. 2017). Novel genomic variations were identified, e.g. deletions of neurofibromin gene (NF1) and parkin RBR E3 ubiquitin protein ligase (PARK2) as well as copy number variations (CNVs) of AKT serine/threonine kinase 3 (AKT3) and other single nucleotide variations (SNVs). Furthermore, patients who had undergone treatment were shown to have higher genetic variability in their recurrent tumours than untreated patients, showing additional layers of complexity in the pathogenesis and progression of GBM. These data allowed the TCGA to characterise GBM into distinct molecular subtypes (14). Subsequent studies further refined this classification using additional genomic and transcriptomic data to give the following three most clinically relevant molecular subtypes of GBM: proneural (PN), mesenchymal (MSC) and classical (CL) (Table 1). This classification was

based on platelet-derived growth factor receptor A (PDGFRA) gene/IDH mutation, NF1 mutation and epidermal growth factor receptor (EGFR) expression, respectively (15,16). EGFR is also an important marker for proliferation and MSC subtype (17).

These GBM classifications have been key in trying to associate genomic/molecular variation to clinical phenotypes, particularly in recurrent episodes and treatment failures, such as the PN-MSC subtype-switch in the tumour aggressiveness and resistance. In line with this, a recent study (where glioma cells were treated with varying concentrations of cytokines) revealed that cytokine storm in the GBM tumour microenvironment enforces PN-subtype switch to MES-subtype by transcriptional networking and induces radiation-resistance properties (18). Similarly, another study shows that post-translational modification of oncogenic transcription factors (TF) such as OLIG2, switches the proliferative nature of glioma cells into a highly invasive phenotype by controlling the inflammatory cytokine, TGF-β (19). Prognostically, GBM patients with the MSC subtype tend to have a poor survival and resistance to therapy in comparison to other subtypes. Inevitably, NF1 drives mutations and a characteristic NF-κB transcriptome profile, an important inflammatory TF that seems to be very specific to MSC subtype-specificity (20). Moreover, NF1 is an RAS-GTPase and an important tumour suppressor gene. Its disruption, through mutation or deletion, is associated with enhanced tumour aggression and invasiveness (21). Deficiency in NF1 is also key in macrophage/microglia recruitment (22-24).

Most of the early TCGA studies have utilised tissue from one single random location in the tumour, but as mentioned above, GBM has high levels of cellular heterogeneity, with several factors affecting the molecular subtype, including anatomical location. Using RNA-Seq, a single GBM sample was shown to contain cells from 3 different subtypes (25). Approximately 8% of the GBM samples contain more than one subtype. Therefore, there needs to be a refinement of these genomic approaches to characterise genetic and protein changes to both single cell and specific cell populations within the tumour (26). Understanding the nature and consequences of cellular and molecular heterogeneity in GBM is crucial in identifying new biomarkers and therapeutic interventions. To date, there has been little evidence of significant association between molecular subtype and prognosis, although recently poorer prognosis has been observed in the MSC subtype, compared to other subtypes (20).

Furthermore, enhanced survival was observed in GBM samples of low heterogeneity, in 20% of the total GBM samples analysed (16).

Further sub-classification and refinement of subtypes has also required an epigenetic approach. In gliomas, the mutational status of IDH is an important marker, and interestingly, gliomas with mutated IDH also have a particular cytosine-phosphate-guanine (CpG) island methylator phenotype (G-CIMP). The G-CIMP of DNA methylation seems to identify a distinct subgroup of glioma, with G-CIMP 'high' subgroup of tumours in younger patients at diagnosis that having better overall prognosis. The G-CIMP 'high' phenotype is also more commonly observed in lower-grade gliomas than GBM and tends to have the PN molecular subtype (27, 28). Furthermore, in patients treated with temozolomide (TMZ), those that had recurrences and had lost methylation of the O(6)-methylguanine-DNA methyl transferase (MGMT) promoter, had increased genetic mutations compared to untreated patients, indicating that this methylation phenotype could contribute to the chemotherapeutic resistance of the tumour (27, 28). However, MGMT methylation status is also predictive of treatment response in IDH wild-type GBM patients (29) and abnormal methylation of MGMT has increased prognosis in some GBM patients after TMZ treatment (30). Recently, small non-coding RNA molecules (ncRNAs or miRNAs) have been suggested to be involved in a number of cancers. Five miRNAs were found to be involved in MGMT alterations and tumour suppressor functions of TP53 (miR-21, miR-125b, miR-34a, miR-181d, and miR-648) in GBM progression (31). In particular, miR-21 and miR-181d were associated with GBM tumorigenesis (32-35), as have a number of other miRNAs, miR-144 and miR-29a (36-38). These miRNAs may prove to be important biomarkers for GBM, but their specificity needs to be further validated.

IDH mutation has been linked with chromosomal abnormalities and prognosis in low-grade gliomas. Correlations have been observed in 3 subtypes: IDH mutant with 1p/19q co-deletion correlating to increase survival (39, 40), whilst IDH mutant without 1p/19q co-deletion and IDH wild-type was correlated with poor prognosis that is similar to GBM (14). Furthermore, in patients with oligodendroglioma (which often contain the 1p/19q deletion), they tended to respond better to chemo- and radiotherapy, with an enhanced prognosis overall (41, 42). EGFR-TACC fusion via a chromosomal translocation has been described in a small number of GBM patients, but its clinical significance is unclear (26), but may have strong sensitivity to some tyrosine kinase inhibitors (43).

Further studies have identified known oncogenic pathways in GBM such as RB, p53, RTK/RAS/P13K (14); a putative attempt at linking GBM molecular subtypes to cell types of the central nervous system has also been suggested based on gene expression signature: PN subtype - oligondendrocytic, CL subtype-astrocytic and MSC subtype–astrocytic (cultured cells) (15,44). This remains to be fully substantiated. However, the MSC subtype generally is the most heterogeneous, showing its complexity compared to other non-MSC tumours (15). A few studies have also reported a switch between molecular subtypes in recurrent tumours that may be driven by the accumulation of new genetic mutations (17, 45, 46). It has been suggested that recurrent tumours may acquire extra mutations and evolve along two distinct molecular pathways governed by p53 mutation (Type 1 GBM) or EGFR amplification (Type 2 GBM) (45). Although the MSC subtype is the most common subtype in GBM, the shift from PN to MSC has not been clearly shown to occur (16).

Comparative studies between initial and recurrent GBM have been conducted using specific known markers and genome-wide analysis to further understand tumorigenesis and progression. Immunohistochemistry has been used to study proteins thought to be involved in DNA repair and tumour growth such as MutL homolog 1 (MLH1), MutS homolog2 (MSH2) and tumour suppressor protein p53 (47). These were found to be expressed significantly lower in recurrent GBM. Furthermore, reduction of MLH1 and post-meiotic segregation increased 2 (PMS2) proteins conferred TMZ resistance and is associated with recurrent TMZ (48). Genomic, transcriptomic and epigenetic approaches have been utilised in a number of longitudinal studies using whole epigenome sequencing (WES), targeted genome sequencing (TES), loss of heterozygosity (LOS), quantitative PCR, RNA-Seq, transcriptome profiling and whole genome sequencing (WGS). These studies have identified numerous additional pathways, biomarkers and deciphered the mutational behaviour of the tumour with and without treatment. Genetic differences in tumour evolution were observed in primary and recurrent tumours, sharing relatively few initial mutations (49). Subtype switching was also found to be common (66%) in primary GBM and may be a result of accumulation of additional mutations in highly expressed genes (50). A new mutation in latent TGF-β-binding protein 4 (LTBP4) gene was found in 10% of recurrent GBM, whilst the TGF-β pathway was also found to be involved in tumour pathogenesis (50). Primary GBM tumours without p53 and EGFR mutations gain novel EGFR amplification during recurrence and can follow two distinct

pathways, depending on the genetic type of the original tumour (45). In another study, using WES, considerable tumour heterogeneity, mediated by EGRF overexpression was observed in GBM, as well as a deletion on chromosome 10, losing phosphatase and tensin homolog (PTEN) and cyclin-dependant kinase inhibitor 2A (CDKN2A) genes (51). A further study analysed the evolution of mutations in GBM by using paired samples and found that 67.9% were clonal in nature, whilst 29.8% were sub-clonal (52). Of these, 90% of p53 and PIK3CA/PIK3R1 mutations were also clonal, suggesting that the nature of p53 mutations in GBM has implications for tumorigenesis (52). TMZ treatment also influences the nature and rate of mutations in recurrent GBM tumours (53). Transcriptomic profiling revealed that a macrophage/microglia-rich tumour microenvironment is key for the development of the MSC molecular subtype, which is further facilitated by NF1 depletion (16).

Epigenomic analysis has offered important insights into molecular mechanisms, such as methylation, underpinning clinical phenotypes. Promoter methylation of the DNA-repair gene MGMT results in gene silencing which was associated with significantly better prognosis in patients treated with TMZ, than those that did not have a methylated MGMT promoter (54). In this study, 45% of 206 GBM cases were found to have MGMT promoter methylation (54). In a recent study, a comprehensive DNA methylation analysis of 200 tumours from 77 GBM patients identified biomarkers which, at the time of diagnosis, were found to be predictive of GBM recurrence and prognosis. Patients in the G-CIMP 'high' subgroup, with IDH mutation and intact 1p19q were found to have a good clinical outcome upon recurrence compared to patients with altered and lowered methylation (G-CIMP 'low'), at the time of diagnosis, with the latter having an increased risk of recurrence and significantly poorer clinical outcome (55). Another important recent study conducted a detailed survey of DNA methylation in GBM tumours using the reduced representation bisulfite sequencing (RRBS) technique and RNA-Seq, and made significantly findings in dissecting out tumour heterogeneity based on DNA methylation profile (56). Transcriptional subtypes of tumour were identified as well as DNA methylation profiles, predictive of immune cell infiltration, necrosis and tumour cell morphology. Furthermore, de-methylation of Wnt signalling promoters upon recurrence and progression was also associated with worse clinical outcome (56).

These promising studies showing genomic variations, transcriptional profiles, molecular abnormalities of G-CIMP and other global DNA methylation profiles, along with the changes in the local tumour microenvironment, will lead to a greater understanding of the complex tumour-immune heterogeneity, and enable interventions to prevent GBM tumorigenesis and progression in the future. One such key player is the complement system, the most potent and versatile humoral innate immune system.

COMPLEMENT SYSTEM AND GBM

The complement system is one of the first lines of defence of innate immunity in the brain and is comprised of more than 30 different glycoproteins which are soluble proteins, cell associated regulators or receptors (57). Complement can be activated by pathogens and altered-self cells or indirectly by pathogen-bound antibodies. Activation of complement opsonises target pathogens or altered-self cells for phagocytic uptake, inducing an inflammatory response and enabling cell lysis. Complement is activated through 3 different pathways which are the Alternative, Classical and Lectin pathways (Figure 1) (58). All activated pathways result in covalent attachment of C3b to the target cell, where each pathway can finally assemble pores in the lipid bilayer of the cell under attack and cause cell lysis (59). The alternative pathway is auto-activated by a process termed 'tick-over', where C3 (the most abundant complement protein) is spontaneously hydrolysed, designated C3(H_2O). Complement protein Factor B associates with C3(H_2O) and in-turn is cleaved by Factor D generating Ba and Bb. The larger cleaved product Bb remains associated and forms the protease complex C3(H_2O)Bb which cleaves additional C3 to form the cleaved products C3a and C3b. The cleaved anaphylatoxin C3a can elicit inflammation whereas C3b can bind to and opsonise pathogens and also bind to C3 convertase (C3bBb) to form C5 convertase (C3bBbC3b). An amplification loop can also be initiated when C3b generated from the Classical and Lectin pathway bind with Factor B from the alternative pathway allowing Factor D to cleave it similarly to 'tick-over' (59,60). The activation of the Classical pathway is through the binding of C1q directly to pathogens, altered-self cells or to antibody antigen complexes. This triggers the C1r to activate C1s which cleaves C4 and C2 to generate C4a anaphylatoxin, C4b opsonin, C2a and C2b. C4b and C2b bind to form C3 convertase (C4b2b) (61). Similarly, in the Lectin pathway both C4 and C2 are also cleaved producing the same products that generate C3 convertase (C4b2b). The lectin pathway is activated by mannose binding lectin

(MBL) binding to oligosaccharides on pathogens. The associated enzyme mannan-binding lectin serine protease (MASP) 1 and 2 are responsible for the cleavage of C4 and C2 (62, 63). All 3 pathways converge at C3 convertase enabling the cleavage of the central complement component C3 to form C3a and C3b. The opsonin C3b binds to C3 convertase and generate C5 convertase (C3bBbC3b) (C4b2Bc3b), which enables the cleavage of C5 to form anaphylatoxin C5a, and opsonin C5b. C5b binds to the pathogen and also to C6, C7, C8, and C9, to produce a membrane attack complex (MAC) which generates pores through the pathogen's cell membrane, leading its destruction by osmotic cell lysis (57).

The complement system plays an important role in defence against pathogens, angiogenesis, neuroinflammation and neurodegeneration, as well as regulation of adaptive immunity. Apart from these functions, complement system also has a key role to play in cancer immunotherapy, cytotoxicity and tumorigenesis (64). Over the years, studies have shown that GBM is resistant to complement-mediated killing and this is facilitated by membrane-bound and soluble complement inhibitors. These regulators include Factor H (FH), FH-like protein 1 (FHL-1), C1 inactivator (C1-IA), protectin (CD59), membrane co-factor protein (CD46) and decay accelerating factor (CD55) (65-67). FH is an important soluble regulator of the Alternative pathway, as it competes with factor B for C3b binding, to prevent the formation of C3 convertases and thus accelerates the decay of C3 convertase (C3bBb) to dissemble the enzyme (Figure 1). FH also acts as a co-factor for factor I to inactivate C3b by cleaving the α-C3b chain into 2 fragments (68, 69). FH is composed of 20 complement control proteins (CCPs) of which CCPS 1-4 facilitate the functional activity of FH. FHL-1 represents the truncated form of FH as its 7 CCPs are identical to the N-terminal of FH, and therefore elicit the same inhibitory ability (69, 70). In the presence of glycosaminoglycans and sialic acid, which are present on self-cells, the affinity of FH increases for surface bound C3b via the 3 binding sites at CCPs 1-4, 7-15, and 19-20. The polyanions are only present on self-cells, thus enabling FH to differentiate between self and nonself-cells (68, 71).

Complement regulators

Complement regulatory proteins are important in protecting healthy self-cells from complement attack by exerting tight regulatory functions. Regulation is required at all major checkpoints of complement activation and amplification to prevent a deleterious effect on

self-cells from an over-reactive complement system. Healthy cells express soluble regulators such as FH and membrane bound regulators including CD59, CD55 and CD46 (Table 2), which all use different mechanisms to provide protection (72, 73). Soluble regulators inactivate complement as they are attracted to self-structure over foreign surfaces (74, 75). However, soluble and membrane-bound complement regulators can act as double-edged swords by overregulating the complement system to the point it is unable to eliminate tumour cells. Studies suggests that the expression of complement regulators by tumours including GBM allows these cells to proliferate unchecked. This highlights the significance that complement regulators play in the tumour cells' avoidance of complement attack. As knowledge of the relationship between complement regulatory proteins and tumours evolve, it is possible that their therapeutic blockade can have an important role in tumour treatment (76, 77).

Factor H

Factor H is secreted by GBM cell lines such as H2, U138, U118 and U87 (78). In another study by Junnikkala *et al.*, expression of RNA and protein production of FHL-1 in the malignant cells was found to exceed that of FH, in contrast to normal serum where the concentration of FH is greater than FHL-1 (66) (Table 2). It appears that endogenously synthesised and fluid phase FH and FHL-1 from plasma can successfully bind to the GBM cell membrane, efficiently regulating complement activation and promoting the cleavage of membrane deposited C3b into its inactive form iC3b. Ultimately, this mechanism prevents activation of the late stages of complement activity, to elicit cell lysis via MAC formation because there is reduced C5b-9 deposition. The inhibitory effect of secreted FH and FHL-1 can be overcome through neutralisation of FH and FHL-1 with antibodies that target the C3b binding site and by the removal of sialic acid to sensitise GBM cells to complement lysis. FH and FHL-1 play a crucial role in GBM tumorigenesis by enabling the acquisition of GBM cells' exceptional resistance to complement mediated killing (66). In a more recent study on primary tumour cells derived from 3 GBM patients, secretion of complement Factor H related protein 5 (FHR5) was also reported (79). It was found that the cells secreted FHR5, but not FH, and that FHR5 inhibited complement-mediated lysis and decayed acceleration of C3 convertase (79).

Complement 1 inactivator A

GBM resistance to complement-mediated lysis can be acquired by the production of Complement 1 inactivator (C1-IA) or C1 inhibitor (C1-inh) (Table 2). C1-IA, a serine protease, is able to regulate classical pathway activation by irreversibly binding to C1r and C1s proteases, which along with C1q, form the multiprotein complex C1, which is the first component in the initiation of the classical pathway (80, 81). The ability of C1-Inh to bind to C1r and C1s protease subsequently prevents C1r autoactivation and C1s activation, which in turn, prevents the cleavage of C4 and C2. This ultimately stops the formation of the Classical pathway's C3 convertase (C4b2a) (82). Gene expression and mRNA analysis in human GBM tissues showed an upregulation of C1-inh, a potent inhibitor of the classical pathway (65). Inhibition of C1-inh in rats with GBM, using appropriate antibodies, was found to increase survival but also led to decreased levels of cytokines IL-1β and GM-CSF, which are associated with an immunosuppressive tumour microenvironment (65, 83).

Membrane-bound complement regulators

The ability of GBM cells to avoid complement attack is not only determined by soluble inhibitors but also by membrane bound regulators such as CD59, CD55 and CD46 (76, 77) (Table 2). CD59 is a major protective element against complement mediated lysis. It binds to C5b-8 complex and blocks the sites to which C9 can attach, thus, preventing the insertion and polymerisation of C9. As a result, the final step of MAC assembly on the cell membrane is prevented (84). CD55 is an anchored membrane regulator that inhibits the formation and accelerates the decay of C3 and C5 convertase of the alternative and classical pathway to prevent complement activation (85). The complement cascade is also regulated by CD46, which serves as a co-factor of factor I inactivation of C3b and C4b, deposited on the membrane (86).

CD59 is considered one of the most important membrane regulators. In a study by Maenpaa et al., it was shown that CD59 was expressed in 14 human glioma tissues as well as 7 glioma cell lines (67). In normal astrocytes, the expression of CD59 is weak as the need to protect these cells from complement is reduced due to the blood-brain barrier, which restricts entry of many pathogens into the brain (77). Successful binding of CD59 to C5b-8 complex inhibits the formation of MAC at the point of insertion of C9 into GBM cell membrane, thus protecting

the cell from complement mediated killing (66). The inhibition of CD59 by neutralising antibodies enables the cells to overcome the resistance of GBM to complement mediated cytolysis (66). In the same study, CD55 and CD46 were also shown to be moderately expressed in GBM cell lines, and neutralising them with respective antibodies showed moderate complement-mediated cytolysis, although CD59 was considered to be the most important complement regulator on GBM cells (66).

Role of microglia and macrophages in GBM

The central nervous system (CNS) had historically been considered an immune privileged site. This is primarily because it lacks a traditional lymphatic system, containing only a few antigen presenting cells which would mount an extremely weak immune response (87). Considering recent data, the characteristics of immune privilege have been redefined and are no longer considered absolute (87). The concept of immune privilege had stemmed from the ability of antigens within the brain to avoid systemic immunological recognition (88). It is now evident that immune privilege is specific to brain parenchyma which is imperative for damage limitation during inflammation. The brain parenchyma is an extremely sensitive part of the organ with poor regenerative capacity and is protected by the blood brain barrier, a semi-permeable membrane consisting of endothelial cells that separate the blood from the cerebrospinal (88).

The CNS is able to coordinate a robust immune response involving both the innate and adaptive immune systems (89). During inflammation immune cells are able to migrate to perivascular spaces following chemotaxis (90). Studies have shown that antigens can enter the cervical lymph nodes by passing through the Virchow Robin Perivascular Space within the walls of the cerebral arteries (91). It is also possible for immunoglobulins to cross the blood-brain barrier via carrier mediated transporters by attaching to FcRn receptor (92). Antigen presentation occurs as dendritic cells can travel outside of the brain and present antigens to T-cells located in the cervical lymph nodes (93). However, inflammation and disease in the CNS can compromise the integrity of the blood-brain barrier, thereby enabling circulating immune cells to migrate past the it and infiltrate the parenchyma (94).

Microglia are the resident macrophage of the CNS comprising 5-20% of the total glial cell population. In the brain, microglia are an important immune cell involved in immune

surveillance and are a crucial component of the first line of defence provided by innate immunity (95). Originally discovered over a century ago by Pio Del Rio Hortega, it is now clear that resident microglia originate from haematopoietic precursor cells of immature yolk sac during early embryogenesis (96). Microglia are usually found in a 'resting' state, although it means that the shape of microglia is such that there are branched extensions or processes that actively patrol and perform surveillance of local areas. Upon receipt of inflammatory stimuli, circulating microglia change into 'amoeboid' shape, and additional recruitment of macrophage from infiltrating circulating monocytes takes place (97, 98). Apart from surveillance, microglia actively contribute to brain development and CNS homeostasis by apoptotic cell removal, maintenance and pruning of synapses, and regulation of neuronal activity (98, 99). In GBM, a second group of macrophages derived from peripheral bone marrow, are present (100). In the brain, macrophages are restricted to the perivascular, choroid and meningeal locations. However, disruption to the blood-brain barrier by disease or inflammation allows macrophage to gain entry to the parenchyma (101). These mononuclear cells are difficult to differentiate from microglia as they intermingle in GBM (102).

Traditional approaches to distinguish macrophage and microglia involved CD45 antibody as microglia are defined as $CD45^{low}$, whereas macrophages were defined as $CD45^{high}$ (102,103). Despite this, it is still unclear as to whether microglia or macrophage make up most of the mononuclear density in GBM. Parney et al. suggested that gliomas contained more recruited macrophages than resident microglia (104). However, Muller et al. challenged this concept as they demonstrated resident microglia were the main source of mononuclear cells in gliomas and that the microglia present had increased their expression of CD45 (105). Together, microglia and macrophages in GBM are generally referred to as tumour-associated macrophages (TAM) (Figure 3) (106).

It has also been reported that in the MES subtype, deficiency of NF1 leads to increased infiltration of TAM (16). This may explain why GBM subtype-specific cell autonomous functions drive tumour aggressiveness and therapy resistance and have poorer prognosis. Furthermore, this study also highlighted that the tumour microenvironment in recurrent GBM showed the presence of more resident microglia/macrophages as compared to peripherally-derived monocytes, indicating that treatment (such as radiotherapy) may have an impact on

monocytes, and thus in recurrent GBM, more efforts need to be made to address resident cells in the brain. This elegant study also showed increased $CD8^+$ T cells in TMZ-induced hypermutated recurrent GBM (16).

Microglial cells have been known to enhance infiltration leading to increased invasiveness of the tumour. A murine microglial cell study on mouse glioma cells found that tumour cell migration occurred sooner and was higher when compared to tumour cells without microglia (107). Another study using murine brain slices found that microglial cells stimulated the extracellular matrix metalloprotease (MMP)-2, which led to increased invasiveness of the tumour (108). Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, secreted by microglia, have been shown to increase tumour invasiveness *in vitro* (109). By specifically targeting microglia, using propentofylline which blocks secretion of IL-1β, IL-6 and TNF-α, tumour growth was found to regress (110).

GBM cells secrete a range of chemo-attractants such as CCL2, CXCL12 and SDF-1, which actively recruit microglia and macrophages (111,112). Various CC and CXC chemokines are secreted including CCL2, CXCL12 and their receptors (113,114). CCL2 is one of the most important CC chemokines commonly expressed by GBM as it plays a key role in regulating the penetrative migration of TAM to the GBM microenvironment (115). It was the first TAM chemo-attractant identified in GBM; the extent of CCL2 expression is associated with glioma grade (116). CCL2 is highly expressed in GBM at mRNA and protein levels, thus contributing to a high influx of TAM (117). Inhibiting CCL2 activity in mice studies (GL261 glioma and xenograft of human U87 models) with relevant antibodies has been shown to reduce infiltration and ultimately prolong survival (118). The receptor for CCL2 is CCR2 which are also present on microglia (119). In addition, microglia from the GBM tumour microenvironment have the capacity to secrete CCL2, thereby stimulating more microglia recruitment to the tumour (114).

CXCL12, also known as stromal derived factor 1 (SDF-1), a chemokine, promotes TAM recruitment in high-grade gliomas. A murine high-grade model, ALTS1C1, demonstrated the chemo-attractant ability of SDF-1 for microglia and macrophages. High expression of SDF-1 promoted the accumulation of TAM to areas of hypoxia in brain and tumour invasion (120). GBM cells also express colony stimulating factor-1 (CSF-1) which functions as TAM chemo-attractant (121,122). CSF-1 is overexpressed in GBM, thus contributing to the high influx of

456 microglia/macrophages, promoting tumour invasion (121,122). High glucose has been shown
457 to increase proliferation and inhibit apoptosis in a study on human GBM U87 cell line, by
458 upregulation of vascular endothelial growth factor (VEGF) and is mediated by increased
459 expression of chemotactic receptors including EGFR (123). A recent murine study showed that
460 osteopontin is an important chemokine that attracts TAM to the GBM site, via integrin $\alpha_v\beta_5$
461 (124). Further, $\alpha_v\beta_5$ deficiency was found to lead to a direct $CD8^+$ T cell cytotoxic effect at the
462 tumour site (124).

463 Majority of newly recruited TAMs acquire an alternatively activated M2 phenotype under the
464 direct influence of tumour cells to produce a pro-tumour microenvironment. M2 polarised
465 TAMs produce mediators that contribute to the immunosuppressive microenvironment
466 established by the tumour cells (125). TAMs are known to secrete anti-inflammatory
467 cytokines such as IL-6, IL-10 and TGF-β, thereby enhancing immunosuppression in tumour
468 microenvironment, leading to promotion of GBM cell growth and angiogenesis (126). Studies
469 have shown that these anti-inflammatory cytokines supress M1 phenotypes as TGF-β inhibits
470 pro-inflammatory cytokine expression and microglia proliferation whilst IL-10 polarises
471 microglia to a M2 phenotype (127). TAMs are also known to express Fas ligand (FasL) which
472 act as an immunosuppressant in GBM, as it contributes to the reduced presence of tumour
473 infiltrating leukocytes (128).

474 The pro-tumour microenvironment of GBM is supported by the expression of MMPs by TAM,
475 including MMP-2 and MMP-9, which are involved in tumour growth by having an impact on
476 angiogenesis, apoptosis and cell proliferation (129). Subsequent inhibition of MMPs derived
477 from TAM have shown a reduction in tumour growth and angiogenesis (130,131). A study has
478 shown that membrane type 1 (MT1) MMP is enhanced in TAM, which in turn, activates MMP-
479 2 in GBM, via microglial cells, thus increasing tumour invasion (132). TGF-β1 derived from
480 microglia in GBM plays an important role in TAM-mediated promotion of tumorigenesis (133).
481 It has been shown that TGF-β1, released by TAM, induces EMT and enhanced invasion of
482 $CD133^+$ GSCs which led to a pro-tumorigenic environment (134). Moreover, TAMs also
483 contribute to tumorigenesis in GBM by providing proliferation promoting factors such as
484 epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) (121).

485 IL-10 from TAM in GBM have the ability to promote tumour growth i*n vitro* via JAK2/STAT3
486 pathway (135). Activation of STAT3 co-ordinates the expression of immunosuppressive

molecules by decreasing expression of major histocompatibility complex (MHC) class II and co-stimulatory molecule, CD40 (136). An activation loop is formed as the stimulation of STAT3 by IL-10 enables activation of this transcription factor in nearby immune cells (136). These cells include macrophage, natural killer (NK) cells and dendritic cells. As a result, the anti-tumour activity of these immune cells is supressed (137). IL-10 derived by TAM also supresses MHC class II expression on monocytes and down-regulates the production of IFN-γ and TNF-α in GBM, thus preventing anti-tumour activity (138). The overall effect of IL-10, secreted by TAM, on GBM, is immunosuppression which ultimately promotes a pro-tumour milieu (139).

Dendritic cells (DCs) are antigen-presenting cells, involved in surveillance against pathogens and tumorigenic cells, and present these to T-cells, thereby serving as an important link between innate and adaptive immunity. This is utilised in anti-tumour therapies, to help induce a cytotoxic response against the tumour cells. In GBM, DCs are considered to present tumour cell peptides, leading to cytotoxic T cells response, and secretion of pro-inflammatory cytokines. Pre-clinical studies on murine glioma models have found DCs to be effective in inducing an effective tumour-response and increasing survival (140,141). Phase I clinical trials have found DC vaccination therapy to be safe and to elicit cytotoxic T cell responses (142,143). Early results from a subsequent Phase III clinical trial involving an autologous tumour-lysate pulsed DC vaccine was shown to be feasible and safe and may extend survival in GMB (144).

Microglia in GBM are a major source of TGF-β, which plays a key role in contributing to the immunosuppressive GBM microenvironment (145). TGF-β enhances immunosuppression in GBM through a range of mechanisms including blocking T-cell activation and proliferation, inhibiting the activation of NK cells, down regulating IL-2 production, and promoting T_{regs} (146). Blocking T-cell activation can be achieved by the ability of TGF-β2 to supress HLA-DR antigen expression which is essential for tumour associated antigen presentation to $CD4^+$ T-cells (147). TGF-β is also capable of facilitating immune escape by inhibiting NKG2D (an activating receptor responsible for host-response to pathogen and tumour cells) on $CD8^+$ T cells and NK cells ultimately rendering the cells less effective at cytotoxic destruction of GBM (148). Strategies which inhibit TGF-β expression can restore anti-tumour immunity in GBM. Transient silencing of TGF-β, using siRNA has been shown to prevent NKG2D expression and increase GBM susceptibility to destruction by immune cells (149). Murine glioma models have also shown that blocking TGF-β1 receptor increased the number of long-term survivors by

33% as opposed to the 6% observed in the control group. The level of $CD8^+$ T cells were also increased, demonstrating a reversal of the immunosuppressive effect when TGF-β1 is inhibited (150).

NK cells are a type of cytotoxic lymphocyte, which are known for its anti-viral and anti-tumour response, and secrete cytokines such as interferon-γ and TNF-α. Pre-clinical models of GBM have shown NK cells to be effective in HLA class I-mediated tumour lysis (151); IL-2 activated NK cells' ability to kill GBM cells (152), and NK cells' effectiveness in preventing metastasis in the GBM xenograft mouse model (153).

ADAPTIVE IMMUNITY AND T_{reg} CELLS

T_{reg} cells play a major role in mediating immune suppression of anti-tumour immune cells. In non-tumorigenic environments, T_{regs} usually are involved in preventing autoimmunity during an immune response (154). T_{regs} are a sub-population of $CD4^+$ T-cells and can be categorised into two groups based upon their developmental origin. Thymus derived T_{regs} develop after antigen presentation by thymic epithelial cells and are characterised by high level expression of the transcription factor Forkhead Fox P3 (FoxP3) (155). By contrast, peripherally induced T_{regs} differentiate in the periphery upon antigen presentation and recognition by naive conventional $CD4^+$ T-cells. IL-10 and TGF-β signalling are key contributors in supporting the induction of peripherally induced T_{regs} which have negligible FoxP3 expression (156). Studies have shown that there is a high influx of T_{regs} predominately of thymus origin, accounting for 25% of tumour infiltrating lymphocytes. (157,158). The abundance of T_{regs} is associated with poor prognosis, as they shift the tumour cytokine milieu towards immunosuppression, preventing immune destruction of tumour cells (159). This enhanced immunosuppression is achieved by T_{regs} ability to restrict the function of infiltrating T cells by preventing production of IL-12 (160). The high influx of T_{regs} in GBM is likely due to CCL22 and CCL2 secreted by GBM, as they bind to CCR4 commonly expressed by T_{regs} (161,162).

Immune checkpoint

Immune checkpoints are co-stimulatory and co-inhibitory pathways that restrict the function of the immune system. These regulatory pathways supress T-cell activation and proliferation ensuring that immune responses are limited to maintaining self-tolerance which prevents the immune system attacking self-cells (163). An immune checkpoint involved in GBM immune

548 evasion is programmed cell death protein 1 ligand (PD-L1), which is a transmembrane
549 glycoprotein of the B7 family co-stimulatory molecules (164). PD-L1 is not usually expressed
550 in the CNS, therefore, its presence in this location is associated with a pathological or
551 tumorigenic environment (165). PD-L1 is activated by binding to the receptor programmed
552 cell death protein 1 (PD-1) to exert its inhibitory effect (166). In GBM, activation of PD-L1
553 suppresses the proliferation and function of tumour derived cytotoxic T-cells, which would
554 otherwise destroy the tumour cells. PD-L1 can also enhance T_{reg} activity which will promote a
555 pro-tumorigenic microenvironment (166) (Figure 3).

556 Various immune cells express PD-L1 in GBM, such as $CD4^+$ and $CD8^+$ T cells (167). TAM express
557 PD-L1 on their surfaces, whilst promoting PD-L1 expression on GBM cells (164). Genetic
558 alterations have also been shown to contribute to PD-L1 expression as the loss of PTEN
559 tumour suppressor gene enhances the expression of PD-L1 on glioma cells (168). The
560 expression pattern of PD-L1 is positively correlated with glioma grade and is also associated
561 with poor survival of GBM patients (167). A study in mouse glioma cell-line has shown that
562 inhibiting PD-L1 with antibodies on glioma cells in combination with radiotherapy has clear
563 survival benefits (169). PD-L1 expression was found to be dependent on IL-6; inhibition of IL-
564 6 signalling diminished expression of PD-L1, leading to increased survival and reduced tumour
565 growth in orthotopic murine glioma model (170).

566 Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) is another immune checkpoint molecule which
567 plays a role in GBM immune evasion, as it modulates the early stages of T lymphocyte
568 activation. CTLA-4 is expressed on activated T-cell and T-regs in a tumour microenvironment
569 (171). Targeting CTLA-4 in glioma models with anti CTLA-4 antibodies proved useful in
570 reversing immune evasion. This study showed an increase in long term survival, increased
571 resistance to T_{reg} mediated suppression and enhanced proliferation of $CD4^+CD25^-$ T-cells
572 (171).

573 Despite several biological and clinical approaches, including the 2018 Nobel Prize for immune
574 checkpoint blockade in cancer immunotherapy, no specific immune therapy treatment for
575 GBM has been successful in phase III or randomised controlled trials due to either lack of
576 positive response, or due to side-effects (172). Some of the clinical trials that did not show
577 significant survival benefit include nivolumab (anti-PD-1) and ipilimubab (anti-CTLA-4) in
578 recurrent GBM (173); nivolumab versus TMZ and radiation therapy in newly-diagnosed GBM

(174); and nivolumab in combination with TMZ and radiation therapy in newly-diagnosed GBM (175).

Other emerging themes in cancer immunotherapy include inhibition of VEGF to reduce angiogenesis and vascular permeability, and cancer vaccine-based therapy such as use of DCs to activate T cells (172). The overall survival and progression-free survival was found to be increased in newly diagnosed GBM patients who received temozolamide, GM-CSF, and targeted cytomegalovirus (CMV) with DCs (176). The rationale for this being that CMV proteins have been found to be expressed in GBM but not normal brain tissue and this has been utilised to generate specific T-cell immune response to lyse GBM tumour cells (177). A follow-on randomised trial in GBM patients showed significant progression-free and overall survival in patients who received CMV-specific DC vaccination (178). Another exciting theme involves use of CART-cell therapy (chimeric-antigen receptor T-cell therapy), in which immune receptors are specifically engineered to generate an immune response when they face tumour proteins (179). A study in recurrent GBM patients, targeting a type of epidermal growth factor, using CART-cell therapy, was found to kick-start an immune response at the site of the glioma including infiltration by T_{reg} cells (180). This small study is the first in humans and involved 10 patients with recurrent GBM. They were treated with a single peripheral dose of autologous T-cells targeted to EGFR variant III, which is found in about 30% of GBM patients and associated with poorer prognosis (181). This particular CART-cell therapy was found to be safe, the infused product reached tumour site in the brain, and also found to assert anti-tumour activity by decreasing EGFR variant III expression (Figure 3).

Glioma stem-like cells (GSCs)

Cancer stem cell hypothesis relates to presence of cells with stem-cell like properties in the tumour microenvironment (i.e. cells that possess ability to differentiate into different cell lineage or generate new tumour or resistance to treatment) (182). The GBM microenvironment too is thought to contain such cells called as GCS that possess properties of self-renewal, pluripotency or ability to give rise to differentiated cell types, and resistance to multiple drug and radiation therapy. The presence of GSCs in GBM was first discovered by Singh et al., and since then numerous studies on GBM microenvironment have established their role in therapeutic resistance, tumour migration and invasion, capability to metastasise, as well as continued maintenance of stem cell-like state of cells (183, 184).

GSCs are considered to have the ability to escape immune response by down-regulating expression of MHC class I, thereby leading to failure of activation of cytotoxic T cells (185). One of the important mechanisms involves PD-L1 present on extracellular vesicles (lipid membrane-bound vesicles secreted by cells; also called exosomes and microvesicles) secreted by GBM cells, which block T-cell receptor by anti-CD3, thereby reducing activation and proliferation of $CD4^+$ and $CD8^+$ T cells (186). GCS have also been shown to evade immune response by increasing production and infiltration of T_{reg} cells (148), and by increasing levels of TGF-β produced by TAM, which in turn, increase levels of TGF-β which in turn down regulates MHC II and subsequent antigen processing mechanism, causing T-cell anergy (188). GSCs are known to attract TAM *in vitro* via chemo-attractants, CCL2 and periostin (188) and by secretion of cytokines TGF-β and CSF, which are known to polarise TAM to immunosuppressive mode (127).

Myeloid-derived suppressor cells (MDSCs) in the GBM microenvironment

One of the major characteristics of GBM is the abundance of Myeloid-derived suppressor cells (MDSCs) in the tumour microenvironment, which largely determines disease prognosis by immune suppressive functions. MDSCs are the key components of innate immune system which essentially originate from the bone marrow derived cells. Significantly, infiltrations of MDSCs in GBM tumour microenvironment were markedly associated with cytotoxic T cells suppression (189,190). A recent study showed that MDSCs substantially paralyze CD4+ T cell memory functions in GBM patients (191). Moreover, findings in GBM murine models showed that pharmacological targeting of MDSCs by Sunitinib resulted in significantly increased $CD3^+CD4^+$ T cell count in the tumour microenvironment (189,190). Moreover, the authors showed that MDSCs depletion led to improved animal survival as well as increased T cell activation in the in GBM patients' PBMCs (189,190). Notably, GBM is characterized by a complex intra-tumour heterogeneity, which underlies a highly immunosuppressive environment and is indicative of remarkable resistance against conventional immunotherapies. Within GBM, GSCs are the major neoplastic compartment, which substantially modulates immune suppressive functions by recruitment of non-neoplastic components such as MDSCs, TAMs and T_{regs} in the tumour microenvironment (192-195). Previous studies have reported that GSC produce intrinsic factors such as IL-10, IL4Rα, and TGF-β to program M2 macrophages and activation of T_{reg} cells for an effective

immunosuppressive function (188,192,194-196). In solid tumours, cell-intrinsic factors of the neoplastic compartment play a key role in recruiting TAMs and MDSCs for disease progression. For instance, recent study in GBM reported that CC chemokine CCL2 (MCP1) is the most abundant chemokine significantly correlated with poor prognosis in GBM patients (197,198). It has been shown that genetic depletion of CCL2 in the murine model, is associated with reduced infiltrations of MDSCs in the GBM microenvironment (199). The authors also showed that CCL2 depletion leads to a significant recruitment of cytotoxic T cell in the tumour microenvironment, which resulted in glioma growth suppression (199). The immunosuppressive functions of CCL2 is mediated through its binding on CCR2 and CCR4 receptors, which mainly expressed on T_{regs} and MDSCs in GBM, respectively. Moreover, high expression of CCL2 in the GBM microenvironment leads to infiltration of T_{reg} cells, MDSCs, and TAMS, which subsequently is associated with poor GBM prognosis (161,197,199). Another study reported that GSCs produce macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine that recruits MDSCs for immunosuppressive functions and GSC proliferation (195). In addition, TAMs and MDSCs account for up to 50% in the immune compartment of GBM microenvironment; in particular, MDSCs are the main source of TGF-β and PD-L1 that induces immunosuppressive environment (191,200,201). Hence, from a clinical viewpoint, targeting the CCL2-CCR axis, MIF, and PD-L1 could potentially offer effective therapies for GBM patients.

Unfortunately, the outcome of recent clinical trials of immunotherapies in GBM did not show any promising results. Therefore, personalized immunotherapy in combination with chemo-radiotherapy strategies for GBM patients are currently in consideration. In line with this, findings from the most recent preclinical study confirmed that combining immuno-radiation therapy exclusively targeting MDSCs and TAMs, did result in improved survival, compared to the monotherapy cohort (194, 202). Collectively, interfering with both cell-intrinsic factors of neoplastic compartments and immunosuppressive components (e.g. MDSCs) of the tumour microenvironment might offer an effective strategy to block GBM progression and overcome resistance to conventional therapies.

CONCLUSIONS

This review highlights the molecular determinants of the complex heterogeneous tumour-immune environment observed in GBM and the mechanisms and interactions of various genetic pathways, transcriptional programming, immune cells and the role of the immune suppressive microenvironment in Glioblastoma. Each aspect of metabolic pathways, adaptive and immune system responses (including complement system) have a key role to play in the initiation, progression, infiltration, maintenance and suppression of tumour cells, thereby continuing to provide hope for potential effective therapies in future. The multi-dimensional interactions of glioma cells along with immune cells and other metabolic pathways add to the complexity of finding successful treatment avenues. Further research into this interplay of the immune response in GBM, along with the genomic processes underlying this, together with parallel progress in clinical trials, is required to overcome this lethal disease.

DEDICATION

The authors would like to dedicate this article to the loving memory of *George Antoni Tsolaki* who died of Glioblastoma multiforme in February 2010.

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REFERENCES

(1) Ostrom QT, Gittleman H, Farah P, Ondracek A, Chen Y, Wolinsky Y, et al. CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006-2010. Neuro Oncol 2013 Nov;15 Suppl 2:ii1-56.

(2) Bonavia R, Inda MM, Cavenee WK, Furnari FB. Heterogeneity maintenance in glioblastoma: a social network. Cancer Res 2011 Jun 15;71(12):4055-4060.

(3) Soeda A, Hara A, Kunisada T, Yoshimura S, Iwama T, Park DM. The evidence of glioblastoma heterogeneity. Sci Rep. 2015 Jan 27;5:7979.

(4) Strupp R, Mason W, van den Bent M, Weller M, Fisher B, Taphoorn, Belanger K, Brandes A, Marosi C, Bogdahn U, et. al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, New England Journal of Medicine 2005, 352:987-996.

(5) Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 2007 Nov 1;21(21):2683-2710.

(6) Gabrusiewicz K, Liu D, Cortes-Santiago N, Hossain MB, Conrad CA, Aldape KD, et al. Anti-vascular endothelial growth factor therapy-induced glioma invasion is associated with accumulation of Tie2-expressing monocytes. Oncotarget 2014 Apr 30;5(8):2208-2220.

(7) Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin CH, Westermark B, et al. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res 1992 Jun 1;52(11):3213-3219.

(8) Anzil AP. Glioblastoma multiforme with extracranial metastases in the absence of previous craniotomy. Case report. J Neurosurg 1970 Jul;33(1):88-94.

(9) Robert M and Wastie M. Glioblastoma multiforme: a rare manifestation of extensive liver and bone metastases, *Biomedical imaging and intervention journal* 2008*,* 4(1), pp. e3.

(10) Kanu OO, Mehta A, Di C, Lin N, Bortoff K, Bigner DD, et al. Glioblastoma multiforme: a review of therapeutic targets. Expert Opin Ther Targets 2009 Jun;13(6):701-718

(11) Holland EC. Glioblastoma multiforme: the terminator. Proc Natl Acad Sci U S A 2000 Jun 6;97(12):6242-6244.

(12) Fujisawa H, Reis RM, Nakamura M, Colella S, Yonekawa Y, Kleihues P, et al. Loss of heterozygosity on chromosome 10 is more extensive in primary (de novo) than in secondary glioblastomas. Lab Invest 2000 Jan;80(1):65-72.

(13) Maher EA, Brennan C, Wen PY, Durso L, et al. Marked genomic differences characterize primary and secondary glioblastoma subtypes and identify two distinct molecular and clinical secondary glioblastoma entities, Cancer research 2006*,* 66(23), pp. 11502-11513.

(14) Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008 Oct 23;455(7216):1061-1068.

(15) Verhaak RG, Hoadley KA, Purdom E. et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1, *Cancer cell* 2010, 17(1). pp.98-110

(16) Wang Q, Hu B, Hu X,Kim H, et al. Tumor evolution of glioma-intrinsic gene expression subtypes associates with immunological changes in the microenvironment. Cancer Cell 2017 32(1):42–56.

(17) Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 2006; 9: 157–73.

(18) Bhat KPL, Balasubramaniyan V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F, et al. Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. Cancer Cell 2013 Sep 9;24(3):331-346.

(19) Singh, SK, Fiorelli R, Kupp R, Rajan S, Szeto E, Lo Cascio C, et al. Post-translational modifications of OLIG2 regulate glioma invasion through TGF-β pathway. Cell Reports 2016 16, 950-966.

(20) Behnan J, Finocchiaro G, Hanna G. The landscape of the mesenchymal signature in brain tumours. Brain 2019 Apr 1;142(4):847-866.

(21) Fadhlullah SFB, Halim NBA, Yeo JYT, Ho RLY, Um P, Ang BT, et al. Pathogenic mutations in neurofibromin identifies a leucine-rich domain regulating glioma cell invasiveness. Oncogene 2019 Jul;38(27):5367-5380.
(22) Daginakatte GC, Gutmann DH. Neurofibromatosis-1 (Nf1) heterozygous brain microglia elaborate paracrine factors that promote Nf1-deficient astrocyte and glioma growth. Hum Mol Genet 2007 May 1;16(9):1098-1112.
(23) Rutledge WC, Kong J, Gao J et al. Tumor-infiltrating lymphocytes in glioblastoma are associated with specific genomic alterations and related to transcriptional class. Clin Cancer Res. 2013 Sep 15;19(18):4951-60.
(24) Solga AC, Pong WW, Kim KY, Cimino PJ, Toonen JA, Walker J, Wylie T, Magrini V, Griffith M, Griffith OL, Ly A, Ellisman MH, Mardis ER, Gutmann DH. RNA Sequencing of Tumor-Associated Microglia Reveals Ccl5 as a Stromal Chemokine Critical for Neurofibromatosis-1 Glioma Growth. Neoplasia. 2015 Oct;17(10):776-88.
(25) Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. Science 2014; 344: 1396–401.
(26) Singh D, Chan JM, Zoppoli P, Niola F, Sullivan R, Castano A, Liu EM, et al. Transforming fusions of FGFR and TACC genes in human glioblastoma. Science. 2012 Sep 7;337(6099):1231-5.
(27) Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. Cell 2013 Oct 10;155(2):462-477.
(28) Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. Cancer Cell 2010; 17: 510–22.
(29) Radke J, Koch A, Pritsch F, Schumann E, Misch M, Hempt C, Lenz K, Löbel F, Paschereit F, Heppner FL, Vajkoczy P, Koll R, Onken J. Predictive MGMT status in a homogeneous cohort of IDH wildtype glioblastoma patients. Acta Neuropathol Commun. 2019 Jun 5;7(1):89.
(30) Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000 Nov 9;343(19):1350-1354.
(31) Jesionek-Kupnicka D, Braun M, Trabska-Kluch B, Czech J, Szybka M, Szymanska B, et al. MiR-21, miR-34a, miR-125b, miR-181d and miR-648 levels inversely correlate with MGMT and TP53 expression in primary glioblastoma patients. Arch Med Sci 2019 Mar;15(2):504-512.
(32) ParvizHamidi M; Haddad G; Ostadrahimi S, Ostadrahimi, N; Sadeghi, S; Fayaz S; Fard-Esfahani P. Circulating miR-26a and miR-21 as biomarkers for glioblastoma multiform. Biotechnol. Appl. Biochem. 2019, 66, 261–265.
(33) Seo YE; Suh HW; Bahal R; Josowitz A; Zhang J; Song E; Cui J; Noorbakhsh S; Jackson C; Bu T, et al. Nanoparticle-mediated intratumoral inhibition of miR-21 for improved survival in glioblastoma. Biomaterials 2019, 201, 87–98.
(34) Sippl C; Ketter R; Bohr L; Kim YJ; List, M; Oertel, J; Urbschat S. MiRNA-181d Expression Significantly Affects Treatment Responses to Carmustine Wafer Implantation. Neurosurgery 2019, 85, 147–155.
(35) Chen YY, Ho HL, Lin SC, Ho TD, Hsu CY. Upregulation of miR-125b, miR-181d, and miR-221 Predicts Poor Prognosis in MGMT Promoter-Unmethylated Glioblastoma Patients. Am J Clin Pathol 2018 Mar 29;149(5):412-417.
(36) Cardoso AMS, Sousa M, Morais CM, Oancea-Castillo LR, Regnier-Vigouroux A, Rebelo O, et al. MiR-144 overexpression as a promising therapeutic strategy to overcome glioblastoma cell invasiveness and resistance to chemotherapy. Hum Mol Genet 2019 Aug 15;28(16):2738-2751.
(37) Yang L; Li, N; Yan Z; Li C; Zhao Z. MiR-29a-Mediated CD133 Expression Contributes to Cisplatin Resistance in CD133+ Glioblastoma Stem Cells. J. Mol. Neurosci. 2018, 66, 369–377.
(38) Zhao Y; Huang W; Kim TM; Jung Y; Menon LG; Xing, H et al. MicroRNA-29a activates a multi-component growth and invasion program in glioblastoma. J. Exp. Clin. Cancer Res. 2019, 38, 36.
(39) Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008 Sep 26;321(5897):1807-12.
(40) Jiao Y, Killela PJ, Reitman ZJ, Rasheed AB, Heaphy CM, de Wilde RF, et al. Frequent ATRX, CIC, FUBP1 and IDH1 mutations refine the classification of malignant gliomas. Oncotarget 2012 Jul;3(7):709-722.
(41) van den Bent MJ. Interobserver variation of the histopathological diagnosis in clinical trials on glioma: a clinician's perspective. Acta Neuropathol 2010 Sep;120(3):297-304.

(42) Cairncross JG, Wang M, Jenkins RB, Shaw EG, Giannini C, Brachman DG, et al. Benefit from procarbazine, lomustine, and vincristine in oligodendroglial tumors is associated with mutation of IDH. J Clin Oncol 2014 Mar 10;32(8):783-790.
(43) Lasorella A, Sanson M, Iavarone A. FGFR-TACC gene fusions in human glioma. Neuro Oncol 2017 Apr 1;19(4):475-483.
(44) Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, et al. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci 2008 Jan 2;28(1):264-278.
(45) Martinez R, Rohde V, Schackert G. Different molecular patterns in glioblastoma multiforme subtypes upon recurrence. J Neurooncol. 2010 Feb;96(3):321-9.
(46) Halliday J, Helmy K, Pattwell SS, Pitter KL, LaPlant Q, Ozawa T, et al. In vivo radiation response of proneural glioma characterized by protective p53 transcriptional program and proneural-mesenchymal shift. Proc Natl Acad Sci U S A 2014 Apr 8;111(14):5248-5253.
(47) Stark AM, Witzel P, Strege RJ, Hugo HH, Mehdorn HM. p53, mdm2, EGFR, and msh2 expression in paired initial and recurrent glioblastoma multiforme. J Neurol Neurosurg Psychiatry 2003, 74(6):779–783.
(48) Shinsato Y, Furukawa T, Yunoue S, Yonezawa H, Minami K, Nishizawa Y et al. Reduction of MLH1 and PMS2 confers temozolomide resistance and is associated with recurrence of glioblastoma. Oncotarget 2013 4(12):2261.
(49) Kim J, Lee IH, Cho HJ, Park CK, Jung YS, Kim Y, et al. Spatiotemporal Evolution of the Primary Glioblastoma Genome. Cancer Cell 2015 Sep 14;28(3):318-328.
(50) Wang J, Cazzato E, Ladewig E, Frattini V, Rosenbloom DI, Zairis S et al. Clonal evolution of glioblastoma under therapy. Nat Genet 2016, 48(7):768–776.
(51) Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. Proc Natl Acad Sci U.S.A 2013, 110(10):4009–4014.
(52) Kim J, Lee IH, Cho HJ, Park CK, Jung YS, Kim Y, Nam SH, Kim BS etl. Spatiotemporal evolution of the primary glioblastoma genome. Cancer Cell 2015 28(3):318–328.
(53) Muscat AM, Wong NC, Drummond KJ, Algar EM, Khasraw M, Verhaak R et al The evolutionary pattern of mutations in glioblastoma reveals therapy-mediated selection. Oncotarget 2018 9(8): 7844.
(54) Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005 Mar 10;352(10):997-1003.
(55) de Souza CF, Sabedot TS, Malta TM, Stetson L, Morozova O, Sokolov A, et al. A Distinct DNA Methylation Shift in a Subset of Glioma CpG Island Methylator Phenotypes during Tumor Recurrence. Cell Rep 2018 Apr 10;23(2):637-651.
(56) Klughammer J, Kiesel B, Roetzer T, Fortelny N, Nemc A, Nenning KH, et al. The DNA methylation landscape of glioblastoma disease progression shows extensive heterogeneity in time and space. Nat Med 2018 Oct;24(10):1611-1624.
(57) Carroll MV, Sim RB. Complement in health and disease. Adv Drug Deliv Rev. 2011;63(12):965–975.
(58) Whaley K, Schwaeble W. Complement and complement deficiencies. Semin Liver Dis. 1997;17(4):297–310.
(59) Thurman, JM., Holers, VM. (2006). The central role of the alternative complement pathway in human disease, *J Immunol.* 176: pp.1305-1310.
(60) Daha MR, Fearon DT, Austen KF. C3 requirements for formation of alternative pathway C5 convertase. J Immunol 1976 Aug;117(2):630-634.
(61) Gigli I, Fujita T, Nussenzweig V. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. Proc Natl Acad Sci U S A 1979 Dec;76(12):6596-6600.
(62) Wallis R, Dodds AW, Mitchell DA, Sim RB, Reid KB, Schwaeble WJ. Molecular interactions between MASP-2, C4, and C2 and their activation fragments leading to complement activation via the lectin pathway. J Biol Chem. 2007;282(11):7844–7851.
(63) Heja D, Kocsis A, Dobo J, Szilagyi K, Szasz R, Zavodszky P, et al. Revised mechanism of complement lectin-pathway activation revealing the role of serine protease MASP-1 as the exclusive activator of MASP-2. Proc Natl Acad Sci U S A 2012 Jun 26;109(26):10498-10503.
(64) Afshar-Kharghan V. The role of the complement system in cancer. J Clin Invest 2017 Mar 1;127(3):780-789.
(65) (66) Fornvik K, Maddahi A, Persson O, Osther K, Salford LG, Nittby Redebrandt H. C1-inactivator is upregulated in glioblastoma. PLoS One 2017 Sep 7;12(9):e0183086.

(66) Junnikkala S, Jokiranta TS, Friese MA, Jarva H, Zipfel PF, Meri S. Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1. J Immunol 2000 Jun 1;164(11):6075-6081.
(67) Maenpaa, A., Junnikkala, S., Hakulinen, J., Timonen, T. and Meri, S. (1996). Expression of complement membrane regulators membrane cofactor protein (CD46), decay accelerating factor (CD55), and protectin (CD59). in human malignant gliomas, *The American journal of pathology,* 148(4), pp. 1139-1152.
(68) Kopp, A., Hebecker, M., Svobodova, E. and Jozsi, M. (2012). Factor H: a complement regulator in health and disease, and a mediator of cellular interactions, *Biomolecules,* 2(1), pp. 46-75.
(69) Rodríguez de Córdoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sánchez-Corral P. The human complement factor H: functional roles, genetic variations and disease association. Mol Immunol. 2004 Jun;41(4):355-67.
(70) Hellwage J, Jokiranta TS, Koistinen V, Vaarala O, Meri S, Zipfel PF. Functional properties of complement factor H-related proteins FHR-3 and FHR-4: binding to the C3d region of C3b and differential regulation by heparin. FEBS Lett 1999 Dec 3;462(3):345-352.
(71) Ferreira VP, Pangburn MK, Cortes C. Complement control protein factor H: the good, the bad, and the inadequate. Mol Immunol 2010 Aug;47(13):2187-2197.
(72) Pangburn, M.K., Fereira, V.P., Cortes C. Discrimination between Host and Pathogens by the complement system, *Vaccine,* 26(8) pp.15-21
(73) Schmidt, C.Q., Lambris, J.D., Rickline, D. (2017). Protection of host cells by complement regulators, *Immunol Rev,* 274(1):pp.152-171
(74) Cho H. Complement regulation: physiology and disease relevance. Korean J Pediatr 2015 Jul;58(7):239-244.
(75) Tegla, C, A., Cudrici, C., Patel, S., Trippe, R., Rus, V., Noculescu., Rus, H. (2013). Membrane attack by complement: The assembly and biology of terminal complement complexes, *Immunol Res,* 52 (1): pp. 45-60.
(76) Junnikkala S, Jokiranta TS, Friese MA, Jarva H, Zipfel PF, Meri S. Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1. J Immunol 2000 Jun 1;164(11):6075-6081.
(77) Maenpaa, A., Junnikkala, S., Hakulinen, J., Timonen, T. and Meri, S. (1996). Expression of complement membrane regulators membrane cofactor protein (CD46), decay accelerating factor (CD55), and protectin (CD59). in human malignant gliomas, *The American journal of pathology,* 148(4), pp. 1139-1152.
(78) Junnikkala S, Hakulinen J, Jarva H, Manuelian T, Bjorge L, Butzow R, et al. Secretion of soluble complement inhibitors factor H and factor H-like protein (FHL-1) by ovarian tumour cells. Br J Cancer 2002 Nov 4;87(10):1119-1127.
(79) DeCordova S, Abdelgany A, Murugaiah V, Pathan AA, Nayak A, Walker T, et al. Secretion of functionally active complement factor H related protein 5 (FHR5) by primary tumour cells derived from Glioblastoma Multiforme patients. Immunobiology 2019 Sep;224(5):625-631.
(80) Ratnoff OD, Lepow IH. Some properties of an esterase derived from preparations of the first component of complement. J Exp Med. 1957;106(2):327–343.
(81) Sim RB, Reboul A, Arlaud GJ, Villiers CL, Colomb MG. Interaction of 125I-labelled complement subcomponents C-1r and C-1s with protease inhibitors in plasma. FEBS Lett. 1979;97(1):111–115.
(82) Sim RB, Arlaud GJ, Colomb MG. Kinetics of reaction of human C1-inhibitor with the human complement system proteases C1r and C1s. Biochim Biophys Acta. 1980;612(2):433–449.
(83) Fornvik K, Ahlstedt J, Osther K, Salford LG, Redebrandt HN. Anti-C1-inactivator treatment of glioblastoma. Oncotarget 2018 Dec 21;9(100):37421-37428.
(84) Davies A, Simmons DL, Hale G, Harrison RA, Tighe H, Lachmann PJ, et al. CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. J Exp Med 1989 Sep 1;170(3):637-654.
(85) Fodor WL, Rollins SA, Guilmette ER, Setter E, Squinto SP. A novel bifunctional chimeric complement inhibitor that regulates C3 convertase and formation of the membrane attack complex. J Immunol 1995 Nov 1;155(9):4135-4138.
(86) Liszewski MK, Post TW, Atkinson JP. Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu Rev Immunol*. 1991;9:431-455.
(87) Louveau A, Harris TH, Kipnis J. Revisiting the Mechanisms of CNS Immune Privilege. Trends Immunol 2015 Oct;36(10):569-577.

(88) Galea I, Bechmann I, Perry VH. What is immune privilege (not)? Trends Immunol 2007 Jan;28(1):12-18.
(89) Bailey SL, Carpentier PA, McMahon EJ, Begolka WS, Miller SD. Innate and adaptive immune responses of the central nervous system. Crit Rev Immunol 2006;26(2):149-188.
(90) Russo, M.V. and McGavern, D.B. (2015). Immune Surveillance of the CNS following Infection and Injury, *Trends in immunology,* 36(10), pp. 637-650.
(91) Cserr HF, Harling-Berg CJ, Knopf PM. Drainage of brain extracellular fluid into blood and deep cervical lymph and its immunological significance. Brain Pathol 1992 Oct;2(4):269-276.
(92) Roopenian, D.C. and Akilesh, S. (2007). FcRn: the neonatal Fc receptor comes of age, *Nature reviews.Immunology,* 7(9), pp. 715-725.
(93) Mohammad MG, Tsai VW, Ruitenberg MJ, Hassanpour M., Li H, Hart PH, Breit SN, Sawchenko PE and Brown DA. Immune cell trafficking from the brain maintains CNS immune tolerance, *The Journal of clinical investigation,* 2014, 124(3), pp. 1228-1241.
(94) Engelhardt B, Ransohoff RM. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. Trends Immunol 2012 Dec;33(12):579-589.
(95) Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. Nat Immunol 2013 Oct;14(10):986-995.
(96) Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 2010 Nov 5;330(6005):841-845.
(97) Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science*. 2005;308(5726):1314–1318.
(98) Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat Neurosci 2011 Jul 31;14(9):1142-1149.
(99) Casano AM, Peri F. Microglia: multitasking specialists of the brain. Dev Cell 2015 Feb 23;32(4):469-477.
(100) Hickey WF, Kimura H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science 1988 Jan 15;239(4837):290-292.
(101) Perng, P. and Lim, M. Immunosuppressive Mechanisms of Malignant Gliomas: Parallels at Non-CNS Sites, *Frontiers in oncology,* 2015, 5, pp. 153.
(102) Badie B, Schartner JM. Flow cytometric characterization of tumor-associated macrophages in experimental gliomas. Neurosurgery 2000 Apr;46(4):957-61; discussion 961-2.
(103) Dick AD, Ford AL, Forrester JV, Sedgwick JD. Flow cytometric identification of a minority population of MHC class II positive cells in the normal rat retina distinct from CD45lowCD11b/c+CD4low parenchymal microglia. Br J Ophthalmol 1995 Sep;79(9):834-840.
(104) Parney, I.F., Waldron, J.S. and Parsa, A.T. (2009). Flow cytometry and in vitro analysis of human glioma-associated macrophages. Laboratory investigation, *Journal of neurosurgery,* 110(3), pp. 572-582.
(105) Muller, A., Brandenburg, S., Turkowski, K., Muller, S. and Vajkoczy, P. (2015). Resident microglia, and not peripheral macrophages, are the main source of brain tumor mononuclear cells, *International journal of cancer,* 137(2), pp. 278-288.
(106) Morantz RA, Wood GW, Foster M, Clark M, Gollahon K. Macrophages in experimental and human brain tumors. Part 2: studies of the macrophage content of human brain tumors. J Neurosurg. 1979;50(3):305–311.
(107) Bettinger I, Thanos S, Paulus W. Microglia promote glioma migration. Acta Neuropathol 2002 Apr;103(4):351-355.
(108) Markovic DS, Glass R, Synowitz M, Rooijen Nv, Kettenmann H. Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2. *J Neuropathol Exp Neurol*. 2005;64(9):754–762.
(109) Yeung YT, Bryce NS, Adams S, et al. p38 MAPK inhibitors attenuate pro-inflammatory cytokine production and the invasiveness of human U251 glioblastoma cells. *J Neurooncol*. 2012;109(1):35–44.
(110) Jacobs VL, Landry RP, Liu Y, Romero-Sandoval EA, De Leo JA. Propentofylline decreases tumor growth in a rodent model of glioblastoma multiforme by a direct mechanism on microglia. Neuro Oncol 2012 Feb;14(2):119-131.
(111) Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, et al. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. J Exp Med 2006 Sep 4;203(9):2201-2213.

(112) Rempel SA, Dudas S, Ge S, Gutiérrez JA. Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. Clin Cancer Res. 2000;6(1):102–111.
(113) Salmaggi, A., Gelati, M., Pollo, B., Frigerio, S., Eoli, M., Silvani, A., Broggi, G., Ciusani, E., Croci, D., Boiardi, A. and De Rossi, M. (2004). CXCL12 in malignant glial tumors: a possible role in angiogenesis and cross-talk between endothelial and tumoral cells, *Journal of neuro-oncology,* 67(3), pp. 305-317.
(114) Chang AL, Miska J, Wainwright DA, Dey M, Rivetta CV, Yu D, et al. CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. Cancer Res 2016 Oct 1;76(19):5671-5682.
(115) Bowman RL, Joyce JA. Therapeutic targeting of tumor-associated macrophages and microglia in glioblastoma. Immunotherapy 2014;6(6):663-666.
(116) Hambardzumyan D, Gutmann DH, Kettenmann H. The role of microglia and macrophages in glioma maintenance and progression. Nat Neurosci 2016 Jan;19(1):20-27.
(117) Takeshima, H., Kuratsu, J., Takeya, M., Yoshimura, T. and Ushio, Y. (1994). Expression and localization of messenger RNA and protein for monocyte chemoattractant protein-1 in human malignant glioma, *Journal of neurosurgery,* 80(6), pp. 1056-1062.
(118) Zhu, X., Fujita, M., Snyder, L.A. and Okada, H. (2011). Systemic delivery of neutralizing antibody targeting CCL2 for glioma therapy, *Journal of neuro-oncology,* 104(1), pp. 83-92.
(119) Platten, M., Wick, W. and Weller, M. (2001). Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape, *Microscopy research and technique,* 52(4), pp. 401-410.
(120) Wang, S.C., Hong, J.H., Hsueh, C. and Chiang, C.S. (2012). Tumor-secreted SDF-1 promotes glioma invasiveness and TAM tropism toward hypoxia in a murine astrocytoma model, *Laboratory investigation,* 92(1), pp. 151-162.
(121) Coniglio SJ, Eugenin E, Dobrenis K, Stanley ER, West BL, Symons MH, et al. Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. Mol Med 2012 May 9;18:519-527.
(122) De I, Steffen MD, Clark PA, Patros CJ, Sokn E, Bishop SM, et al. CSF1 Overexpression Promotes High-Grade Glioma Formation without Impacting the Polarization Status of Glioma-Associated Microglia and Macrophages. Cancer Res 2016 May 1;76(9):2552-2560.
(123) Bao Z, Chen K, Krepel S, Tang P, Gong W, Zhang M, et al. High Glucose Promotes Human Glioblastoma Cell Growth by Increasing the Expression and Function of Chemoattractant and Growth Factor Receptors. Transl Oncol 2019 Sep;12(9):1155-1163.
(124) Wei J, Marisetty A, Schrand B, et al. Osteopontin mediates glioblastoma-associated macrophage infiltration and is a potential therapeutic target. *J Clin Invest*. 2019;129(1):137–149.
(125) Charles NA, Holland EC, Gilbertson R, Glass R, Kettenmann H. The brain tumor microenvironment. Glia 2011 Aug;59(8):1169-1180.
(126) Liu Q, Li G, Li R, Shen J, He Q, Deng L, et al. IL-6 promotion of glioblastoma cell invasion and angiogenesis in U251 and T98G cell lines. J Neurooncol 2010 Nov;100(2):165-176.
(127) Wu, A., Wei, J., Kong, L.Y., Wang, Y., Priebe, W., Qiao, W., Sawaya, R. and Heimberger, A.B. (2010). Glioma cancer stem cells induce immunosuppressive macrophages/microglia, *Neuro-oncology,* 12(11), pp. 1113-1125.
(128) Badie B, Schartner J, Prabakaran S, Paul J, Vorpahl J. Expression of Fas ligand by microglia: possible role in glioma immune evasion. J Neuroimmunol 2001 Nov 1;120(1-2):19-24.
(129) Friedberg MH, Glantz MJ, Klempner MS, Cole BF, Perides G. Specific matrix metalloproteinase profiles in the cerebrospinal fluid correlated with the presence of malignant astrocytomas, brain metastases, and carcinomatous meningitis. *Cancer*. 1998;82(5):923-930.
(130) Belien AT, Paganetti PA, Schwab ME. Membrane-type 1 matrix metalloprotease (MT1-MMP) enables invasive migration of glioma cells in central nervous system white matter. J Cell Biol 1999 Jan 25;144(2):373-384.
(131) Du R, Petritsch C, Lu K, Liu P, Haller A, Ganss R, et al. Matrix metalloproteinase-2 regulates vascular patterning and growth affecting tumor cell survival and invasion in GBM. Neuro Oncol 2008 Jun;10(3):254-264.
(132) Markovic, D.S., Vinnakota, K., Chirasani, S., Synowitz, M., Raguet, H., Stock, K., Sliwa, M., Lehmann, S., Kalin, R., van Rooijen, N., Holmbeck, K., Heppner, F.L., Kiwit, J., Matyash, V., Lehnardt, S., Kaminska, B., Glass, R. and Kettenmann, H. (2009). Gliomas induce and exploit microglial MT1-MMP

expression for tumor expansion, *Proceedings of the National Academy of Sciences of the United States of America,* 106(30), pp. 12530-12535.
(133) Wesolowska, A., Kwiatkowska, A., Slomnicki, L., Dembinski, M., Master, A., Sliwa, M., Franciszkiewicz, K., Chouaib, S. and Kaminska, B. (2008). Microglia-derived TGF-beta as an important regulator of glioblastoma invasion--an inhibition of TGF-beta-dependent effects by shRNA against human TGF-β type II receptor, *Oncogene,* 27(7), pp. 918-930.
(134) Ye, X.Z., Xu, S.L., Xin, Y.H., Yu, S.C., Ping, Y.F., Chen, L., Xiao, H.L., Wang, B., Yi, L., Wang, Q.L., Jiang, X.F., Yang, L., Zhang, P., Qian, C., Cui, Y.H., Zhang, X. and Bian, X.W. (2012). Tumor-associated microglia/macrophages enhance the invasion of glioma stem-like cells via TGF-beta1 signaling pathway, *Journal of immunology (Baltimore, Md.: 1950),* 189(1), pp. 444-453.
(135) Qi, L., Yu, H., Zhang, Y., Zhao, D., Lv, P., Zhong, Y. and Xu, Y. (2016). IL-10 secreted by M2 macrophage promoted tumorigenesis through interaction with JAK2 in glioma, *Oncotarget,* 7(44), pp. 71673-71685.
(136) Wang, T., Niu, G., Kortylewski, M., Burdelya, L., Shain, K., Zhang, S., Bhattacharya, R., Gabrilovich, D., Heller, R., Coppola, D., Dalton, W., Jove, R., Pardoll, D. and Yu, H. (2004). Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells, *Nature medicine,* 10(1), pp. 48-54.
(137) Matsumura, Y., Kobayashi, T., Ichiyama, K., Yoshida, R., Hashimoto, M., Takimoto, T., Tanaka, K., Chinen, T., Shichita, T., Wyss-Coray, T., Sato, K. and Yoshimura, A. (2007). Selective expansion of foxp3-positive regulatory T cells and immunosuppression by suppressors of cytokine signaling 3-deficient dendritic cells, *Journal of immunology,* 179(4), pp. 2170-2179.
(138) Van Meir, E.G. (1995). Cytokines and tumors of the central nervous system, *Glia,* 15(3), pp. 264-288.
(139) Nduom, E.K., Weller, M. and Heimberger, A.B. (2015). Immunosuppressive mechanisms in glioblastoma, *Neuro-oncology,* 17 Suppl 7, pp. vii9-vii14.
(140) Liau LM, Black KL, Prins RM, Sykes SN, DiPatre PL, Cloughesy TF, et al. Treatment of intracranial gliomas with bone marrow-derived dendritic cells pulsed with tumor antigens. J Neurosurg 1999 Jun;90(6):1115-1124.
(141) Pellegatta S, Poliani PL, Corno D, et al. Dendritic cells pulsed with glioma lysates induce immunity against syngeneic intracranial gliomas and increase survival of tumor-bearing mice. Neurol Res. 2006;28(5):527–531.
(142) (109) Liau LM, Prins RM, Kiertscher SM, Odesa SK, Kremen TJ, Giovannone AJ, et al. Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T-cell responses modulated by the local central nervous system tumor microenvironment. Clin Cancer Res 2005 Aug 1;11(15):5515-5525.
(143) Yu JS, Wheeler CJ, Zeltzer PM, et al. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. Cancer Res. 2001;61(3):842–847.
(144) Liau LM, Ashkan K, Tran DD, Campian JL, Trusheim JE, Cobbs CS, et al. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. J Transl Med 2018 May 29;16(1):142-018-1507-6.
(145) Platten, M., Wick, W. and Weller, M. (2001). Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape, *Microscopy research and technique,* 52(4), pp. 401-410.
(146) Fontana A, Bodmer S, Frei K, Malipiero U, Siepl C. Expression of TGF-beta 2 in human glioblastoma: a role in resistance to immune rejection? Ciba Found Symp 1991 ;157:232-8;157:232-8; discussion 238-41.
(147) Matsushita, K., Takenouchi, T., Shimada, H., Tomonaga, T., Hayashi, H., Shioya, A., Komatsu, A., Matsubara, H. and Ochiai, T. (2006). Strong HLA-DR antigen expression on cancer cells relates to better prognosis of colorectal cancer patients: Possible involvement of c-myc suppression by interferon-gamma in situ, *Cancer science,* 97(1), pp. 57-63.
(148) Crane CA, Ahn BJ, Han SJ, Parsa AT. Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy. Neuro Oncol 2012 May;14(5):584-595.
(149) Friese MA, Wischhusen J, Wick W, et al. RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. *Cancer Res*. 2004;64(20):7596-7603.

(150) Tran, T.T., Uhl, M., Ma, J.Y., Janssen, L., Sriram, V., Aulwurm, S., Kerr, I., Lam, A., Webb, H.K., Kapoun, A.M., Kizer, D.E., McEnroe, G., Hart, B., Axon, J., Murphy, A., Chakravarty, S., Dugar, S., Protter, A.A., Higgins, L.S., Wick, W., Weller, M. and Wong, D.H. (2007). Inhibiting TGF-beta signaling restores immune surveillance in the SMA-560 glioma model, *Neuro-oncology,* 9(3), pp. 259-270.

(151) Sivori S, Parolini S, Marcenaro E, et al. Involvement of natural cytotoxicity receptors in human natural killer cell-mediated lysis of neuroblastoma and glioblastoma cell lines. J Neuroimmunol. 2000;107(2):220–225.

(152) Castriconi R, Daga A, Dondero A, Zona G, Poliani PL, Melotti A, et al. NK cells recognize and kill human glioblastoma cells with stem cell-like properties. J Immunol 2009 Mar 15;182(6):3530-3539.

(153) Lee SJ, Kang WY, Yoon Y, Jin JY, Song HJ, Her JH, et al. Natural killer (NK) cells inhibit systemic metastasis of glioblastoma cells and have therapeutic effects against glioblastomas in the brain. BMC Cancer 2015 Dec 24;15:1011-015-2034-y.

(154) Ooi, Y.C., Tran, P., Ung, N., Thill, K., Trang, A., Fong, B.M., Nagasawa, D.T., Lim, M. and Yang, I. (2014). The role of regulatory T-cells in glioma immunology, *Clinical neurology and neurosurgery,* 119, pp. 125-132.

(155) Allan SE, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, et al. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. J Clin Invest 2005 Nov;115(11):3276-3284.

(156) Colombo MP, Piconese S. Regulatory-T-cell inhibition versus depletion: the right choice in cancer immunotherapy. Nat Rev Cancer 2007 Nov;7(11):880-887.

(157) Jacobs JF, Idema AJ, Bol KF, Grotenhuis JA, de Vries IJ, Wesseling P, et al. Prognostic significance and mechanism of Treg infiltration in human brain tumors. J Neuroimmunol 2010 Aug 25;225(1-2):195-199.

(158) Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, Yang DS, Sun W, Qiao W, et al. Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas. Clin Cancer Res 2008 Aug 15;14(16):5166-5172.

(159) Elliott LH, Brooks WH, Roszman TL. Activation of immunoregulatory lymphocytes obtained from patients with malignant gliomas. J Neurosurg 1987 Aug;67(2):231-236.

(160) Humphries W, Wei J, Sampson JH, Heimberger AB. The role of tregs in glioma-mediated immunosuppression: potential target for intervention. Neurosurg Clin N Am 2010 Jan;21(1):125-137.

(161) Jordan JT, Sun W, Hussain SF, DeAngulo G, Prabhu SS, Heimberger AB. Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy. Cancer Immunol Immunother 2008 Jan;57(1):123-131.

(162) Desbaillets I, Tada M, de Tribolet N, Diserens AC, Hamou MF, Van Meir EG. Human astrocytomas and glioblastomas express monocyte chemoattractant protein-1 (MCP-1) in vivo and in vitro. Int J Cancer 1994 Jul 15;58(2):240-247.

(163) Topalian, S.L., Drake, C.G. and Pardoll, D.M. (2015). Immune checkpoint blockade: a common denominator approach to cancer therapy, *Cancer cell,* 27(4), pp. 450-461.

(164) Bloch O, Crane CA, Kaur R, Safaee M, Rutkowski MJ, Parsa AT. Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages. Clin Cancer Res 2013 Jun 15;19(12):3165-3175.

(165) Liang SC, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, et al. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. Eur J Immunol 2003 Oct;33(10):2706-2716.

(166) Pardoll, D.M. (2012). The blockade of immune checkpoints in cancer immunotherapy, *Nature reviews.Cancer,* 12(4), pp. 252-264.

(167) Wei, B., Wang, L., Zhao, X., Du, C., Guo, Y. and Sun, Z. (2014). The upregulation of programmed death 1 on peripheral blood T cells of glioma is correlated with disease progression, *Tumour biology,* 35(4), pp. 2923-2929.

(168) Parsa, A.T., Waldron, J.S., Panner, A., Crane, C.A., Parney, I.F., Barry, J.J., Cachola, K.E., Murray, J.C., Tihan, T., Jensen, M.C., Mischel, P.S., Stokoe, D. and Pieper, R.O. (2007). Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma, *Nature medicine,* 13(1), pp. 84-88.

(169) Zeng, J., See, A.P., Phallen, J., Jackson, C.M., Belcaid, Z., Ruzevick, J., Durham, N., Meyer, C., Harris, T.J., Albesiano, E., Pradilla, G., Ford, E., Wong, J., Hammers, H.J., Mathios, D., Tyler, B., Brem, H., Tran, P.T., Pardoll, D., Drake, C.G. and Lim, M. (2013). Anti-PD-1 blockade and stereotactic

radiation produce long-term survival in mice with intracranial gliomas, *International journal of radiation oncology, biology, physics,* 86(2), pp. 343-349.
(170) Lamano JB, Lamano JB, Li YD, DiDomenico JD, Choy W, Veliceasa D, et al. Glioblastoma-Derived IL6 Induces Immunosuppressive Peripheral Myeloid Cell PD-L1 and Promotes Tumor Growth. Clin Cancer Res 2019 Jun 15;25(12):3643-3657.
(171) Fecci PE, Ochiai H, Mitchell DA, Grossi PM, Sweeney AE, Archer GE, et al. Systemic CTLA-4 blockade ameliorates glioma-induced changes to the CD4+ T cell compartment without affecting regulatory T-cell function. Clin Cancer Res 2007 Apr 1;13(7):2158-2167.
(172) McGranahan, T., Therkelsen, K.E., Ahmad, S., Nagpal, S. (2019). Current state of immunotherapy for treatment of glioblastoma, *Current treatment options in oncology*, 20(3). 24.
(173) Caccese M, Indraccolo S, Zagonel V, Lombardi G. PD-1/PD-L1 immune-checkpoint inhibitors in glioblastoma: A concise review. Crit Rev Oncol Hematol 2019 Mar;135:128-134.
(174) Arrieta VA, Iwamoto F, Lukas RV, Sachdev S, Rabadan R, Sonabend AM. Can patient selection and neoadjuvant administration resuscitate PD-1 inhibitors for glioblastoma? J Neurosurg 2019 Dec 6:1-6.
(175) Adhikaree J, Moreno-Vicente J, Kaur AP, Jackson AM, Patel PM. Resistance Mechanisms and Barriers to Successful Immunotherapy for Treating Glioblastoma. Cells 2020 Jan 21;9(2):10.3390/cells9020263.
(176) Batich KA, Reap EA, Archer GE, Sanchez-Perez L, Nair SK, Schmittling RJ, et al. Long-term Survival in Glioblastoma with Cytomegalovirus pp65-Targeted Vaccination. Clin Cancer Res 2017 Apr 15;23(8):1898-1909.
(177) Nair SK, De Leon G, Boczkowski D, et al. Recognition and killing of autologous, primary glioblastoma tumor cells by human cytomegalovirus pp65-specific cytotoxic T cells. Clin Cancer Res. 2014;20(10):2684–2694.
(178) Mitchell DA, Batich KA, Gunn MD, et al. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. Nature. 2015;519(7543):366–369.
(179) Eisenstein M. Immunotherapy offers a promising bet against brain cancer. Nature 2018 Sep;561(7724):S42-S44.
(180) ORourke, D.M., Nasrallah, M.P., Desai, A., et al., (2017). A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma, *Science translational medicine* 9(399): eaaa0984.
(181) Johnson LA, Scholler J, Ohkuri T, Kosaka A, Patel PR, McGettigan SE, et al. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. Sci Transl Med 2015 Feb 18;7(275):275ra22.
(182) Jackson M, Hassiotou F, Nowak A. Glioblastoma stem-like cells: at the root of tumor recurrence and a therapeutic target. Carcinogenesis 2015 Feb;36(2):177-185.
(183) Singh D, Chan JM, Zoppoli P, Niola F, Sullivan R, Castano A, Liu EM, Reichel J, Porrati P, Pellegatta S, Qiu K, Gao Z, Ceccarelli M, Riccardi R, Brat DJ, Guha A, Aldape K, Golfinos JG, Zagzag D, Mikkelsen T, Finocchiaro G, Lasorella A, Rabadan R, Iavarone A. Transforming fusions of FGFR and TACC genes in human glioblastoma. Science. 2012 Sep 7;337(6099):1231-5.
(184) Audia A, Conroy S, Glass R, Bhat KPL. The Impact of the Tumor Microenvironment on the Properties of Glioma Stem-Like Cells. Front Oncol 2017 Jul 10;7:143.
(185) Yang W, Li Y, Gao R, Xiu Z, Sun T. MHC class I dysfunction of glioma stem cells escapes from CTL-mediated immune response via activation of Wnt/β-catenin signaling pathway. Oncogene. 2020;39(5):1098–1111.
(186) Ricklefs, F., Alayo, Q., Krenzlin, H., Mahmoud, A., Speranza, M., Nakashima., H et al. (2018). Immune evasion mediated by PD-L1 on glioblastoma-derived extracellular vesicles Science Advances 4(3), eaar2766.
(187) Facoetti A, Nano R, Zelini P, Morbini P, Benericetti E, Ceroni M, et al. Human leukocyte antigen and antigen processing machinery component defects in astrocytic tumors. Clin Cancer Res 2005 Dec 1;11(23):8304-8311.
(188) Zhou W, Ke SQ, Huang Z, et al. Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. Nat Cell Biol. 2015;17(2):170–182.
(189) Raychaudhuri B, Rayman P, Ireland J, Ko J, Rini B, Borden EC, et al (2011) Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. Neuro Oncol; 13 (6):591–9.

(190) Raychaudhuri, B, Rayman P, Huang P, Grabowski M, Hambardzumyan D, Finke JH, Vogelbaum MA (2015). Myeloid derived suppressor cell infiltration of murine and human gliomas is associated with reduction of tumor infiltrating lymphocytes. J. Neurooncol; 122, 293–301.
(191) Dubinski D, Wolfer J, Hasselblatt M, Schneider-Hohendorf T, Bogdahn U, Stummer W, et al (2016) CD4+ T effector memory cell dysfunction is associated with the accumulation of granulocytic myeloid-derived suppressor cells in glioblastoma patients. Neuro Oncol; 18:807–18. 10.1093
(192) Di Tomaso T, Mazzoleni S, Wang E, Sovena G, Clavenna D, Fran-zin A, Mortini P, Ferrone S, Doglioni C, Marincola FM, et al (2010) Immunobiological characterization of cancer stem cells isolated from glioblastoma patients. Clin Cancer Res; 16: 800–813
(193) Justin D Lathia, Stephen C. Mack, Erin E. Mulkearns-Hubert, Claudia L.L. Valentim, Jeremy N. Rich (2015) Cancer stem cells in glioblastoma. Genes Dev; 15; 29(12): 1203–1217.
(194) Kamran N, Kadiyala P, Saxena M, et al. Immunosuppressive Myeloid Cells' Blockade in the Glioma Microenvironment Enhances the Efficacy of Immune-Stimulatory Gene Therapy. *Mol Ther*. 2017;25(1):232-248.
(195) Otvos B, Silver DJ, Mulkearns-Hubert EE, Alvarado AG, Turaga SM, Sorensen MD, et al (2016) Cancer stem cell-secreted macrophage migration inhibitory factor stimulates myeloid derived suppressor cell function and facilitates glioblastoma immune evasion. Stem Cells; 34(8):2026–39.
(196) Wei J, Barr J, Kong LY, Wang Y, Wu A, Sharma AK, Gumin J, Hen-ry V, Colman H, Sawaya R, et al (2010) Glioma-associated cancer-initiating cells induce immunosuppression. Clin Cancer Res; 16: 461–473.
(197) Chang AL, Miska J, Wainwright DA, et al. CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. *Cancer Res*. 2016;76(19):5671-5682.
(198) Chen Z, Feng X, Herting CJ, Garcia VA, Nie K, Pong WW, et al (2017) Cellular and molecular identity of tumor-associated macrophages in glioblastoma. Cancer Res; 77:2266–78.
(199) Fujita M, Kohanbash G, Fellows-Mayle W, Hamilton RL, Komohara Y, Decker SA, et al (2011) COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. Cancer Res; 71:2664–74.
(200) Alban TJ, Alvarado AG, Sorensen MD, et al (2018) Global immune fingerprinting in glioblastoma patient peripheral blood reveals immune-suppression signatures associated with prognosis. JCI Insight; 3 (21):e122264.
(201) Umemura N, Saio M, Suwa T, Kitoh Y, Bai J, Nonaka K, et al (2008) Tumor- infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. J Leukoc Biol; 83:1136–44. 10.1189
(202) Zhang P, Miska J, Lee-Chang C, et al. Therapeutic targeting of tumor-associated myeloid cells synergizes with radiation therapy for glioblastoma. *Proc Natl Acad Sci U S A*. 2019;116(47):23714-23723.
(203) Behnan J, Stangeland B, Hosainey SA, Joel M, Olsen TK, Micci F, et al. Differential propagation of stroma and cancer stem cells dictates tumorigenesis and multipotency. Oncogene 2017a; 36: 570–84.
(204) Sturm D, Witt H, Hovestadt V, Khuong-Quang DA, Jones DT, Konermann C, et al. Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. Cancer Cell 2012; 22: 425–37.
(205) Gill BJ, Pisapia DJ, Malone HR, Goldstein H, Lei L, Sonabend A, et al. MRI-localized biopsies reveal subtype-specific differences in molecular and cellular composition at the margins of glioblastoma. Proc Natl Acad Sci USA 2014; 111: 12550–5.
(206) Reifenberger G, Wirsching HG, Knobbe-Thomsen CB, Weller M. Advances in the molecular genetics of gliomas - implications for classification and therapy. Nat Rev Clin Oncol. 2017 Jul;14(7):434-452.
(207) Waker CA, Lober RM. Brain Tumors of Glial Origin. Adv Exp Med Biol. 2019;1190:281-297.

Figure legends:

Figure 1: **Regulation of complement pathways in Glioblastoma: A**) C1 inactivator (C1-IA), also called C1 inhibitor (C1-Inh), binds covalently to the active site of C1r and C1s, blocking their function. It also dissociates $C1r_2C1s_2$ from C1, releasing the C1q. This inactivation subsequently prevents the cleavage of C4 and C2 mediated classical pathway. C1-IA can also inhibit the function of MASP-1 and MASP-2 and in turn prevents cleavage of C4 and C2 of the lectin pathway. **B)** Endogenous or GBM synthesized Factor H (FH) and FH-like protein 1 (FHL-1) can successfully bind to GBM cell membrane. FH is a decay accelerating factor for C3 convertase. This plasma alternative pathway regulator FH binds with C3b in the convertase, displacing Factor Bb to inactivate the convertase. This FH-C3b also acts as a cofactor for cleavage of C3b by Factor I (FI) to yield the inactive product iC3b. CR1 allows FI to perform the second cleavage generating C3c and C3dg. Complement factor H related protein 5 (FHR5) secreted from GBM also exhibits functional activity similar to factor H. FHR5 functions as a co-factor for factor I mediated cleavage of C3b, and decay acceleration of C3 convertase, thus inhibiting complement mediated lysis. **C)** The membrane bound regulators such as CD59, CD55 and CD46 are also found to be important for resisting complement attack on GBM cells. CD59 binds to C5b-8 complex and blocks the sites for C9 attachment, thus, preventing polymerization of C9 and inhibition of MAC formation. CD55 inhibits the formation and accelerates the decay of C3 and C5 convertase of alternative and classical pathway to prevent complement activation. CD46 causes inactivation of C3b and C4b deposited on the membrane.

Figure 2. Dissection of Mutational and Epigenetic GBM Subtype Classifications. Glioblastoma multiforme (GBM) is a highly heterogeneous disease with distinct, recurring molecular subtypes that differ in their associated expression profile, mutational signature and epigenetic modifications. GBM can be classified into three main subtypes: the proneural (PN), mesenchymal (MSC) and classical subtype. PN gliomas tend to display an expression profile resembling oligodendrocytes, high levels of PDGFRα (due to amplifications or mutations) as well as characteristic mutations in IDH1. The latter leads to an epigenetic CpG island methylator phenotype (C-GIMP), which is associated with younger patients and a better prognosis. MSC subtype tumors, on the other hand, show a high rate of NF1 mutations which, in turn, promote NF-κB activation and, thereby, aggressiveness, invasiveness and myeloid recruitment. This translates into a therapy resistant phenotype for MSC gliomas with poorer survival compared to the other subtypes. The third subtype is the classical subtype, which preserves wild-type p53 expression, but shows over-expression and/or mutation of EGFR. Both MSC and CL tumor cells resemble (cultured) astrocytic gene expression profiles as well as epigenetically a G-CIMP low phenotype. The distinction between G-CIMP high and low is not only prognostically relevant (as G-CIMP high shows improved prognosis), but also predictively. Methylation of MGMT, which is observed in G-CIMP high tumors, in conjunction with 1p/19q deletion, has been shown to sensitize cells to TMZ treatment, leading to significantly improved survival.

Figure 3. Inflammatory Tumor Microenvironment of GBM and its Therapeutic Implications. Illustration of the interplay of innate and adaptive immune components within the glioma microenvironment. On the side of the innate immune system, tumour-associated macrophages (TAMs), mainly comprised of microglia and peripheral monocytes, are attracted by tumour cells and, in turn, release pro-inflammatory cytokines, matrix remodelers and growth factors to aid tumorigenesis. Myeloid-derived suppressor cells (MDSCs) are also recruited by the tumor and potently suppress anti-tumor immunity. Alternative pathway molecules factor H (FH) and FH-like protein 1 of the complement system enhance immunosuppression and prevent complement-mediated lysis of the tumor cells. The adaptive immune system, on the other hand, is largely suppressed in its function through the recruitment of regulatory T cells (T_{reg}). These inhibit the action of cytotoxic T cells and dendritic cells, disturbing a competent anti-tumor immune response. Tumor cells also exert direct suppression of adaptive immunity through immune checkpoint expression, e.g. PD-L1 or CTLA-4. Therapeutically, this tumor-immune crosstalk can be targeted by inhibiting chemoattractants of pro-tumor immune cells, such as anti-CCL2 monoclonal antibody, by immune checkpoint inhibition, dendritic cell vaccination approaches or adoptive transfer of chimeric antigen receptor (CAR) T cells that target the glioma cells (see red indicators).

Table 1: Adult (WHO Grade IV) Glioblastoma multiforme (GBM) subtypes defined by genomic, transcriptome and epigenomic markers.

GBM phenotype	Methylation status	Genotypic/phenotypic abnormality	
Proneural (PN)	G-CIMP+*	IDH1/IDH2 mutations	Ch10 deletion
	MGMT gene promoter (high)	ARTX mutation	MYC
		TP53 mutation	CDKN2A/CDKN2B deletion
	G-CIMP−*	IDH1 wildtype	RTKI
		TERT promoter mutation	CDK4 amplification
		PDGRFA amplification	DLL3, OLIG2 and NKX2-2
		Ch7 insertion/chr10 deletion	
Classic (CL)	Cluster M3*	EGFR amplification/mutation	CDKN2A/CDKN2B deletion
	MGMT gene promoter (moderate)	RTKII	PTEN deletion
		EGFRvIII	TERT promoter mutation
		Ch7 insertion/chr10 deletion	IDH1/IDH2 wildtype
Mesenchymal (MSC)	Cluster M1*	NF1 mutation	VEGRF2
		TP53 mutation	CD40, CD31, CD68
		S100A1, PTPRC	CHI3L1/YKL-40, MET
		TERT promoter mutation	EGFR amplification (MSC subtypes)
		Ch7 insertion/chr10 deletion	↑NF-κB driven inflammation

Note: Neural "subtype" not use in classification as no gene clustering observed in several studies (16,20,203-205). G-CIMP: Glioma CpG island methylator phenotype; MGMT: O^6-methylguanine-DNA methyltransferase; TERT: Telomerase reverse transcriptase; RTKI, RTKII: Receptor tyrosine kinase I and II; EGFR: Epidermal growth factor receptor; VEGRF2: vascular endothelial growth factor receptor 2; PTPRC: Protein Tyrosine Phosphatase Receptor Type C; S100A1: S100 Calcium Binding Protein A1; MET: MET-Proto-Oncogene, Receptor Tyrosine Kinase. *: Methylation cluster and G-CIMP phenotype defined by Brennan et al., 2013 (27). ↑: enhanced. Ch: Chromosome. Table compiled using data from the following: (14-18,25,28,55,206,207).

Table 2: Immune system components associated with Glioblastoma multiforme (GBM) microenvironment.

Immune system component	Source	Effect on GBM microenvironment	Reference
Cytokine			
IL-10	TAM	Enhances Immunosuppression, promotes tumorigenesis, decreases expression of MHC class II on monocytes, promotes Tregs, inhibits expression of TNF-α and IFN-γ, suppresses anti-tumor effect of immune cells	(135-137)
TGF-β	TAM & GSC (TGFB2; Singh et al, 2016)	Suppresses anti-tumor immune response, promotes tumorigenesis, blocks NK cells activity, Inhibits T-cells, promotes Tregs, downregulates IL-2, Inhibits NKG2D on CD8+ T-cells, upregulates CD133+	(133,134,146-148)
IL-6	TAM	Suppresses immune effector cells	(126,170)
CSF-1	TAM	Enhances immunosuppression	(121,122,127)
Complement system			
FH	GBM cells	Enhances immunosuppression, inactivates C3b, inhibits activation of the complement alternative pathway	(66)
C1-IA	GBM cells	Enhances immunosuppression, prevents activation of the complement classical pathway	(65)
CD59	GBM cells	Enhances immunosuppression, prohibits the formation of MAC, prevents activation of the complement pathway	(66)
CFH5	GBM cells	Inhibits complement-mediated lysis and decay acceleration of C3 convertase	(79)
TAM			
TAM	Microglia and macrophage/ monocyte	Polarized towards M2 phenotype, enhanced immunosuppression, promotes tumor invasion, secretes anti-tumor cytokines, Expresses FasL which act as an immunosuppressant, expresses MMPs which promote tumor invasion, promotes proliferation of growth factors	(121,128)

Note: IL: interleukin; TGF: transforming growth factor; CSF: colony stimulating factor; FH: factor H; C1-1A: complement 1-inactivator A; CFH5: complement factor H related protein 5; TAM: tumor-associated macrophage.

Fig.1

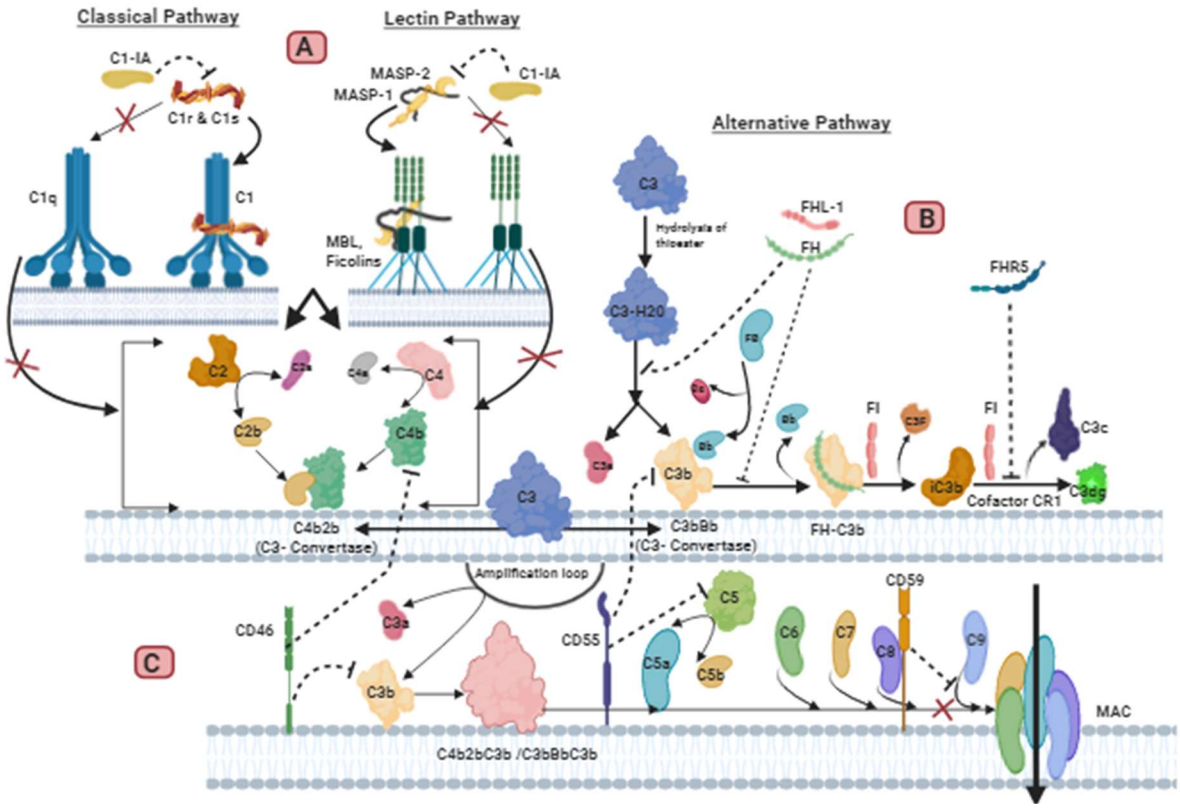

Classical Pathway
Lectin Pathway
A
C1-IA
C1r & C1s
C1q
C1
MASP-2
MASP-1
C1-IA
MBL,
Ficolins
Alternative Pathway
C3
Hydrolysis of thioester
C3-H2O
FHL-1
FH
B
FHR5
FB
Bb
C2
C2a
C4a
C4
C2b
C4b
C3a
C3b
FI
C3F
iC3b
C3c
C3dg
Cofactor CR1
C4b2b
(C3- Convertase)
C3bBb
(C3- Convertase)
FH-C3b
Amplification loop
C3
CD46
C3a
C3b
CD55
C5
C5a
C5b
C6
C7
C8
CD59
C9
MAC
C
C4b2bC3b /C3bBbC3b

Figure 2

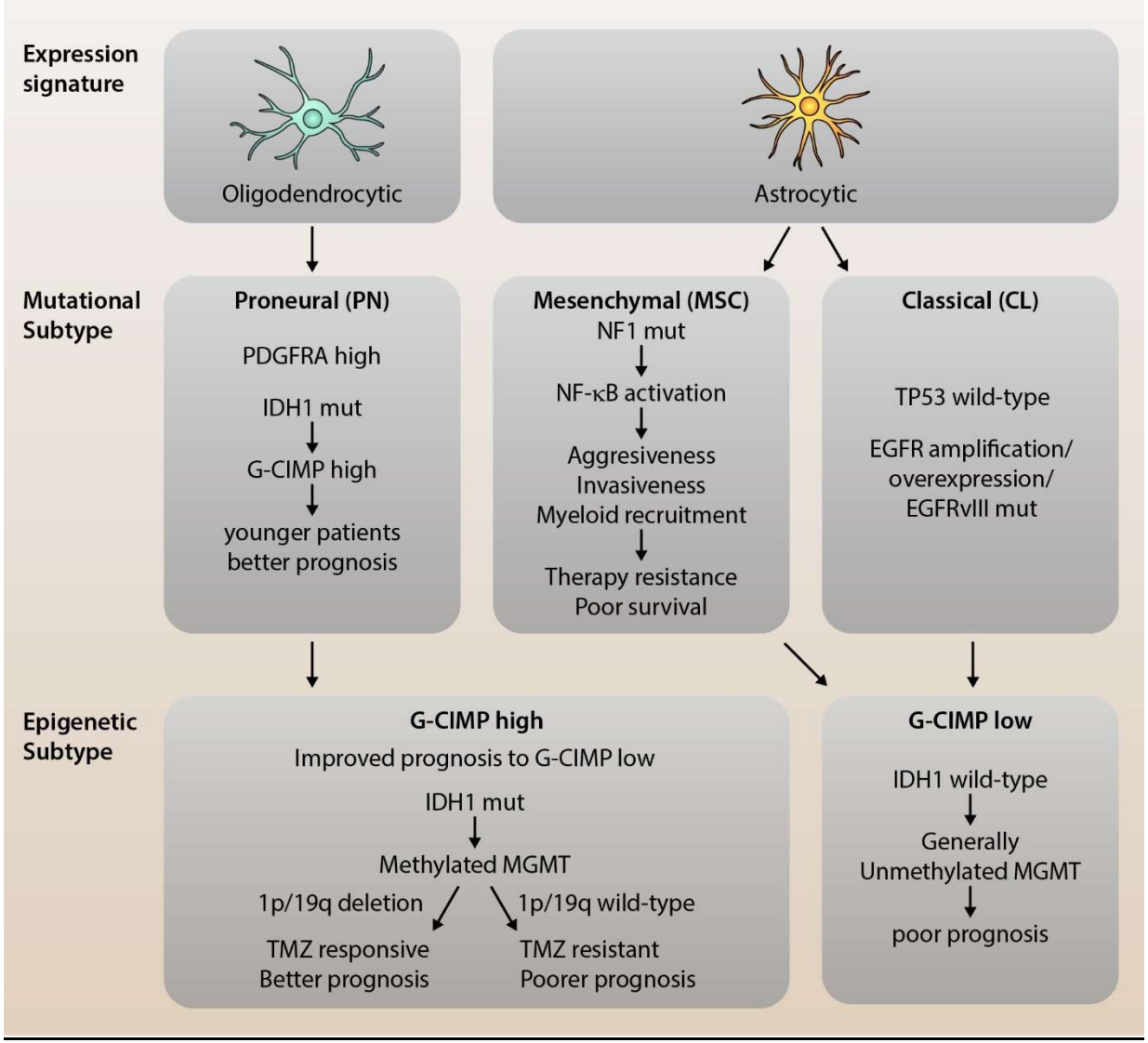
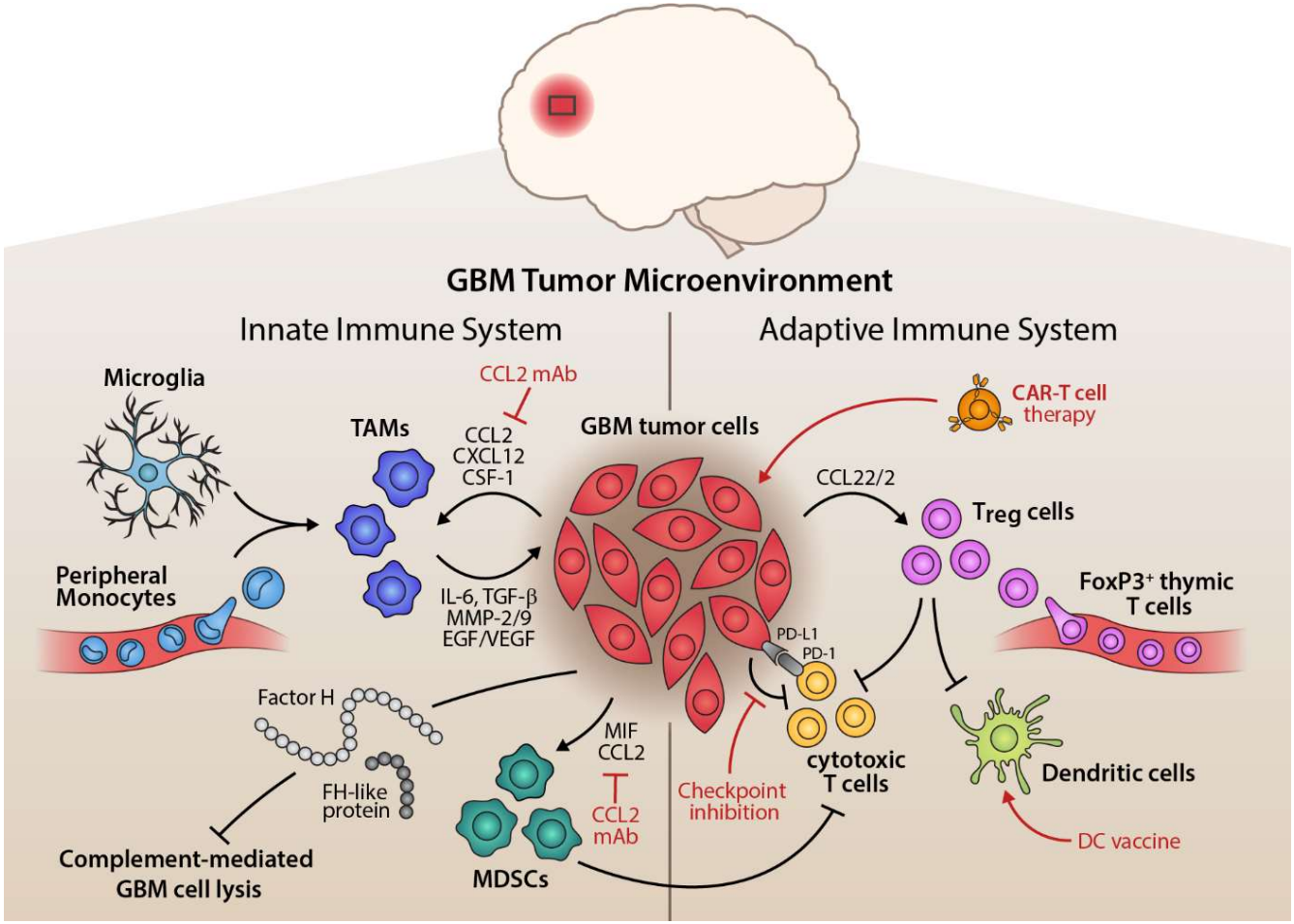

Expression signature
Oligodendrocytic
Astrocytic
Mutational Subtype
Proneural (PN)
PDGFRA high
IDH1 mut
G-CIMP high
younger patients
better prognosis
Mesenchymal (MSC)
NF1 mut
NF-κB activation
Aggresiveness
Invasiveness
Myeloid recruitment
Therapy resistance
Poor survival
Classical (CL)
TP53 wild-type
EGFR amplification/
overexpression/
EGFRvIII mut
Epigenetic Subtype
G-CIMP high
Improved prognosis to G-CIMP low
IDH1 mut
Methylated MGMT
1p/19q deletion
1p/19q wild-type
TMZ responsive
Better prognosis
TMZ resistant
Poorer prognosis
G-CIMP low
IDH1 wild-type
Generally
Unmethylated MGMT
poor prognosis

Figure 3


GBM Tumor Microenvironment
Innate Immune System
Adaptive Immune System
Microglia
TAMs
CCL2 mAb
CCL2
CXCL12
CSF-1
GBM tumor cells
CAR-T cell therapy
CCL22/2
Treg cells
FoxP3+ thymic T cells
Peripheral Monocytes
IL-6, TGF-β
MMP-2/9
EGF/VEGF
PD-L1
PD-1
Factor H
MIF
CCL2
FH-like protein
CCL2 mAb
Checkpoint inhibition
cytotoxic T cells
Dendritic cells
DC vaccine
Complement-mediated GBM cell lysis
MDSCs