Tissue Transglutaminase 2 Expression and Function in Glioblastoma

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Abstract

Glioblastoma is the most common and aggressive type of adult brain tumour. It is currently incurable and requires more effective treatments. Tissue transglutaminase 2 (TGM2) has previously been suggested to have a role in glioblastoma. Previous studies focused on TGM2 expression and inhibition in glioblastoma cells. Here we were interested in TGM2 expression in glioblastoma-associated microglia/macrophages and in identifying the role it plays in the tumor microenvironment. Based on data from bioinformatics, cell culture experiments, immunohistochemistry and immunofluorescence on mouse samples and human samples, we have shown that glioblastoma-associated microglia/macrophages are the major source of TGM2 in the tumor microenvironment. We also identified a novel role for TGM2 in efferocytosis in glioblastoma; this suggests a role for TGM2 in the maintenance of an immunosuppressive environment in this cancer. With this, we hope that further studies will be designed to evaluate the use of TGM2 antagonists as therapeutic agents for glioblastoma.

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List of Abbreviations

GBM:	Glioblastoma Multiforme
WHO:	World Health Organization
MRI:	Magnetic resonance imaging
IDH:	Isocitrate Hydrogenase
GFAP:	Glial Fibrillary Acidic Protein
MGMT:	O6-Methylguanine-DNA Methyltransferase
EGFR:	Epidermal Growth Factor Receptor
G-CIMP:	Glioma-CpG Island Methylator Phenotype
NF1:	Neurofibromatosis type 1
TME:	Tumor Microenvironment
MHC:	Major Histocompatibility Complex
TGFβ:	Transforming Growth Factor Beta
IL:	Interleukin
PD-L1:	Programmed Death Ligand 1
GAMs:	Glioblastoma Associated Myeloid Cells
MDSCs:	Myeloid Derived Suppressor Cells
DAMPs:	Damage-Associated Molecular Patterns
TGM2:	Transglutaminase 2
PriGO:	Primary Glioblastoma
LPS:	Lipopolysaccharides
IFN- γ:	Interferon-Gamma
Th1:	T Helper Type 1 Cell
TNF- α:	Tumor Necrosis Factor-Alpha
CXCL9:	C-X-C Motif Ligand 9
NO:	Nitric Oxide

ROI:	Reactive Oxygen Intermediates
Th2:	T Helper Type 2 Cell
Arg-1:	Arginase-1
LPC:	Lysophosphotidylcholine
S1P:	Sphingosine 1-Phosphate
CX_3CL1:	Chemokine C-X_3-C Motif Ligand 1
ATP:	Adenosine Triphosphate
Gas6:	Growth Arrest Specific-6
ProS1:	Protein S1
PI3K:	Phosphatidylinositol 3 Kinase
Akt:	Protein Serine Threonine Kinase
CSC:	Cancer Stem Cell
EMT:	Epithelial Mesenchymal Transition
ECS:	Epidermal Cancer Stem Cells
TCGA:	Cancer Genome Atlas
CGGA:	Chinese Glioma Genome Atlas
PMA:	Phorbol 12-Myristate 13-Acetate
PVDF:	Polyvinylidene Difluoride
IHC:	Immunohistochemistry
scRNA seq:	Single Cell RNA Sequencing
tSNE:	T-Distributed Stochastic Neighbour Embedding

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1. Results

1- Introduction

1.1 Glioblastoma: Disease, Prognosis and Treatment

Glioblastoma, sometimes termed glioblastoma multiforme (GBM), is the most common and aggressive primary adult brain tumor that, as of today, is still incurable by conventional therapies (1). It is classified by the World Health Organization (WHO) as a grade IV astrocytoma based on pathology and morphology (1). Grade IV being the most severe, it represents approx. 50% of all primary brain gliomas, with poor prognosis for patients (2). Histopathological features of GBM are extremely variable. Some common features include thin cytoplasm, nuclear pleomorphism, brisk mitotic activity, increased cellularity and vascularization, tumor cell proliferation and necrosis (3, 4). Necrosis caused by thrombosis is a key characteristic feature for diagnosis in which there are two patterns. The first being a large necrotic region within the center of the tumor that results from insufficient blood supply, while the second consists of small, irregular regions of necrosis surrounded by palisading cells (3,4).

Common symptoms of GBM are presented as headaches, seizures and/or focal neurological deficits due to the tumour mass affecting normal brain functioning (3, 4, 5). Glioblastoma is diagnosed using magnetic resonance imaging (MRI) followed by pathological analysis of the biopsy (3, 4, 5). Prognosis of GBM will depend on mutation status which will predict the response to therapy. Biomarkers commonly tested for are isocitrate hydrogenase 1/2 (IDH 1/2) mutations, glial fibrillary acidic protein (GFAP) expression, O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation, or mutations in transmembrane epidermal growth factor receptors (*EGFR*s) (3, 4, 5, 6). IDH, a krebs cycle enzyme, is involved in the control of oxidative damage and DNA repair (2, 6, 7). Mutations in IDH 1/2 are associated with improved survival and response to therapy due to the lack of immune cells available to trigger an

immune response that will increase tumor growth and aggressiveness (2, 6, 7). GFAP plays a role in GBM maturation related to malignancy and proliferation (3). Loss of GFAP is associated with increased proliferation and malignancy (3). MGMT enzyme which is involved in DNA damage repair predict responses to chemotherapy based on methylation status (5, 6). Patients with methylated (not activated) MGMT had longer survival, and better responses to radiation and temozolomide treatments (5, 6). Finally, *EGFR* which is amplified in 40% of GBM tumors, is a receptor protein involved in cell signaling pathways related to cell survival and division (6, 8). Typically*, EGFR* is overexpressed in primary glioblastomas, increasing cancer cell survival, proliferation, and invasion, but when altered, it correlated with increased survival and response to therapy (6, 9).

Over the years, cancer immunotherapy has made remarkable advancements in the development of targeted treatments like immune checkpoint blocking antibodies, bispecific T-cell–engaging antibodies, and chimeric antigen receptor-transduced T cells (10). While these immunotherapies benefit many cancers, glioblastoma is still considered an exception with a median overall survival of one year (10). As a result, the only treatments available for glioblastoma are surgical resection with a combination of radiation and temozolomide treatment (11). Following treatment, an MRI is done to assess responsiveness. Glioblastoma is difficult to diagnose and treat due to the heterogeneity of the tumours, their invasive nature, and the challenges associated with the blood brain barrier (11). Thus, it is clear that we must better understand the unique mechanistic challenges underlying the resistance of glioblastoma tumors to immunotherapy, as well as acquiring insights for future strategies in the development of novel therapeutics.

1.1.1 Genetics

Traditionally, the WHO classified and diagnosed glioblastoma based solely off of histological features. As of today, diagnosis is based on the presence or absence of mutations in IDH 1/2 and histone 3 (12). Glioblastomas have been divided into two types, primary and secondary GBMs. Primary GBM has no signs of progression and is identified from onset as advanced stage glioma, while secondary GBM evolves from low grade gliomas and anaplastic astrocytomas to an advanced stage cancer (13, 14). Histologically, they are indistinguishable, but genetically/metabolically, they have different genetic/metabolic properties and alterations (13, 14). Most GBMs are primary tumors that can arise in the absence of prior disease, are highly invasive, and affect older patients (14, 15). Secondary GBMs on the other hand are less common, affect younger patients, and are associated with a better prognosis (14, 15). The most frequent genetic alterations seen in glioblastoma are loss of heterozygosity at 10q, *EGFR* and *PDGFR* amplifications, PTEN, TP53, and Rb alterations, and aberrations in signalling pathways like RTK/Ras/PI3K (14, 15).

Recent studies classified glioblastoma into 5 molecular subtypes based on transcriptional features analyzed by The Cancer Genome Atlas: classical, neural, proneural, mesenchymal, and only recently glioma-CpG island methylator phenotype (G-CIMP), each of which is defined by its own set of biomarkers (13, 14). The classical subtype is associated with greater EGFR amplification, decreased TP53 mutation, CDKN2A alterations, loss of chromosome 10, and amplification of mitogen activated protein kinase and chromosome 7 (13, 14). Proneural GBMs had IDH1 and TP53 mutations, PDGFR amplification, and PI3K pathway activation (13, 14). This subtype is more common in younger patients and had prolonged overall survival with no change in survival regardless of treatment (13, 14). The neural subtype was found to have

associations with a variety of neuron markers like NEFL, GABRA1, SYT1 and SLC12A5, and most closely resembled normal brain tissue (13, 14). The G-CIMP subtype was associated with IDH 1/2 mutations and was enriched in secondary GBMs and the proneural subtype (14, 16). It most commonly occurs in younger patients, in which G-CIMP+ patients had better prognosis and longer survival (14, 16). Finally, the mesenchymal subtype was associated with greater neurofibromatosis type 1 (NF1) mutations, PTEN alterations, and is histologically observed to have a greater degree of necrosis (13, 14). It was also discovered that they had a high degree of association with immunosuppressive macrophages (typically named M2 macrophage) and NF1 deactivation which might suggest a link between NF1 loss and microglia/macrophage recruitment and invasion into the tumor microenvironment (TME) (13, 14). As such, the mesenchymal subtype leads to poorer prognosis but shows a modest increase in survival with aggressive treatment (13). It is important to note that although these are the most common characteristics of each subtype, these events are not mutually exclusive as there tends to be a lot of overlap and variation from one patient's tumor to the next. This suggests that it may be beneficial to target tumors based on mutations/alterations that greatly promote tumor growth for future therapeutic approaches.

1.1.2 Heterogeneity

As previously mentioned, one of the major challenging factors for diagnosis and treatment of glioblastoma is tumor heterogeneity. Like many other cancers, glioblastoma has also been shown to have intertumoral heterogeneity where tumors from different patients respond to therapeutic treatments differently due to distinct differences in genetic and molecular alterations (17). Glioblastomas has also been found to display intratumoral heterogeneity where cells from

the same tumor exhibit different molecular and phenotypic profiles which has been linked to resistance to therapy and disease reoccurrence (17). Single cell RNA analysis done by Patel *et al* (18) categorized the variability of cancer cells based off of oncogenic signaling, proliferation, complement/immune response, and response to hypoxia. They found that this variability between tumors led to enhanced capacities for stemness and variable expression in glioblastoma subtype classifiers. Ultimately, intratumoral heterogeneity is associated with poor prognosis and survival, and is considered as the leading determinant for resistance to therapy and treatment failure (17). Given that many strategies for targeting cancerous mutations have had limited success (19, 20, 21, 22), it might be beneficial to thoroughly understand and characterize the determinants of tumor heterogeneity (both intertumoral and intratumoral) in order to develop effective treatments.

1.1.3 Immunology

Traditionally, the brain was considered an immune privileged organ, but it is now acknowledged that there is indeed an interaction between the brain and the immune system. It is however important to note that despite this active interaction, the immune cells cannot freely access the brain parenchyma (23). It has been found that patients with glioblastoma frequently exhibited systemic (blood/lymphopoietic systems) or local (innate/adaptive immune systems) immunosuppression which affected the efficacy of therapeutic treatments (24, 25, 26, 27). In order for tumours to proliferate and invade adjacent tissue, they must evade immune surveillance. Glioblastoma cells do this by sometimes downregulating major histocompatibility complex (MHC) expression (28, 29), secreting immunomodulatory factors such as transforming growth factor β (TGFβ) and interleukin 10 and 6 (IL-10, IL-6) (30, 31, 32, 33), and expressing

immunomodulatory ligands like programmed death ligand 1 (PD-L1) (34). TGFβ secreted by glioblastoma cells was found to help support the transition of microglia/macrophages infiltrating the TME into the immune suppressive phenotype (35, 36), while expression of PD-L1 promoted exhaustion of CD4+ T cells and immune evasion (34).

Increasing evidence suggests that the TME in glioblastoma patients had high myeloid cell infiltration, T cell dysfunction, and marked reductions in tumor infiltrating lymphocytes (37). The infiltrating myeloid cells termed glioblastoma associated myeloid cells (GAMs) are made up of a mixture of microglia/macrophages and myeloid derived suppressor cells (MDSCs) (37). GAMs are reprogrammed into the immunosuppressive phenotype by interacting with tumor cells in the TME to help support immune escape and promote tumor growth and progression (37, 38). They make up approximately 30-50% of the cells in GBM tumors (37, 38). In comparison to other glioblastoma subtypes, the mesenchymal subtype was found to have the highest degree of macrophage/microglia and T cell infiltration (39, 40, 41). Given this information, it might be beneficial to re-educate, reactivate, and reconstruct GAM functions in an immunosuppressive TME for effective immunotherapy in glioblastoma.

1.1.4 Regions of Necrosis

In the past, hypoxia and necrosis was thought to occur due to "cancer outgrowing its blood supply". Today, new lines of evidence suggest that necrosis caused by intravascular thrombosis leads to the restructuring of the TME which causes disruption of the blood brain barrier, accumulation of immunosuppressive immune cells, rapid microvascular proliferation, cancer stem cell enrichment, and outward migration of tumor cells, all of which drives glioblastoma progression and aggressiveness (42, 43, 44).

During necrosis, the cells that do not migrate will undergo apoptosis and release endogenous damage-associated molecular patterns (DAMPs) which will recruit GAMs into the TME, facilitating disease progression (42). Given the mesenchymal subtype's high association with necrosis, an enzyme called transglutaminase 2 (TGM2) was found to be a key molecular switch that regulates transcription factors associated with necrosis induced mesenchymal differentiation (41). Ultimately, it would be beneficial to understand the underlying mechanisms of necrosis in malignant GBMs since the necrotic TME could significantly modulate tumor development, is an indicator of poor prognosis, and has a negative overall impact on patient survival due to resistance to therapeutic interventions (43).

1.1.5 Primary Cultures

In this study, glioblastoma cell cultures referred to as primary glioblastoma (PriGO) cells were isolated from tumor samples that were surgically obtained from patients at the Ottawa Hospital. Cells were grown on laminin coated cell culture flasks with serum-free neural stem cell media that was supplemented with growth factors and vitamins at 37°C and 5% O2 to mimic the brain's environment (refer to methods). These in culture conditions preserve and retain the genetic and molecular profiles of the original tumor as well as its invasiveness in intracranial xenografts (45, 46, 47). Culturing primary glioblastoma cells in serum free media is better since it has been shown that serum induces aberrant differentiation leading to the expression of multiple lineage markers and increases rates of senescence (45, 48). In vivo and in vitro models showed that the use of serum causes primary glioblastoma cells to lose their invasive potential which leads to the formation of well-defined tumors (45). Other important features to note are that these primary cells have the potential to differentiate and mimic glioblastoma heterogeneity,

as well as express markers of neural stem cells like nestin, vimentin, and SOX2 (45, 46, 47). By culturing PriGO cells in this way thereby preserving their original heterogeneity and phenotypic characteristics, it provides a powerful in vitro and in vivo model to study disease mechanism, screen drugs, and develop novel therapeutic strategies.

1.2 Macrophages and Cancer

Observations of glioblastoma interacting with microglia/macrophages have been known since the 1920s. More recently there has been an increased interest in the role macrophages play in regulating tumor immunity, however there are still significant challenges that must be overcome in order to produce effective therapeutics. In the following subsections, relevant information on macrophages will be provided.

1.2.1 Macrophage Polarization

Traditionally, macrophages have been thought to have two subtypes: M1 classically activated (immune stimulatory) and M2 alternatively activated (immunosuppressive). It is however important to emphasize that macrophage activation exist on a spectrum based on type of stimulus (49, 50). M1 macrophages play a critical role in host protection against pathogens like viruses and intracellular bacteria, and fight against tumors (51, 52). They are induced/activated by lipopolysaccharides (LPS), interferon-gamma (IFN- γ), interleukin-1 beta (IL-1β), and are involved in T helper type 1 cell (Th1) responses (50, 51, 52, 53). M1 macrophages are characterized by their high antigen presentation capacity, high production of inflammatory cytokines like tumor necrosis factor-alpha (TNF- α) and IL-1, IL-6, IL-12, and IL-23, high production of chemokine C-X-C motif ligand 9 (CXCL9), low IL-10, and increased phagocytic

capabilities which produces nitric oxide (NO) or reactive oxygen intermediates (ROI) (50, 51, 53, 54). One drawback of sustained M1 macrophage activation is that it can be harmful for the host's health and cause disease due to unresolved inflammation which inhibits cell proliferation and causes tissue damage.

M2 macrophages on the other hand are responsible for resolving inflammation, wound healing, and play a major role in immune suppression, angiogenesis, and remodelling of the extracellular matrix (50, 51). They can be further divided into M2a, M2b, M2c and M2d subtypes based on stimuli and changes in transcriptional profile (51, 54). M2 macrophages are involved in T helper type 2 cell (Th2) responses and can be induced/activated by IL-4, IL-10, and IL-13, as well as immune complexes and glucocorticoids (51, 54). Their common characteristics include: poor antigen presentation abilities, decreased phagocytic capabilities, low production of IL-12, high production of immunosuppressive cytokines like TGFβ, IL-10, IL-4, and IL-13, secretion of CCL17 and CCL22, high expression of mannose and scavenger type receptors, and generation of arginase-1 (Arg-1) which can impair proliferation of T lymphocytes and reduce NO production (50, 51, 54, 55). It is important to note that although they have decreased phagocytic capabilities, M2 macrophages are still capable of phagocytosis. While macrophage polarization is often defined in black and white terms, it is important to note that different macrophage subtypes often present these markers at varying expression levels.

1.2.2 Glioblastoma Associated Macrophages in the Tumor Microenvironment

Increasing evidence shows that the TME plays a role in the initiation of cancer and the promotion of tumor proliferation and metastasis (56). It is well known that tumor cells in various cancers will recruit GAMs into the TME by the secretion of chemokines (eg: CCL2). As

previously mentioned, GAMs can make up about 30-50% of the cells in the tumor with the mesenchymal subtype having the highest infiltration. The two major sources of immune cells in GAMs are microglia and macrophages, in which resident microglia accounted for 15% while monocytes/macrophages accounted for 85% (57). Microglia are brain resident macrophages that originate from the embryonic yolk sac while monocytes are immature immune cells circulating in the blood that originate from the bone marrow and differentiate into macrophages when infiltrating the brain (58, 59). It is important to note that resident microglia and peripheral macrophages infiltrating the TME are morphologically indistinguishable and share common surface markers (57, 58, 59). Moreover, they have different preferential localizations in the tumor. Resident microglia are typically localized in the peritumoral regions while peripheral macrophages are localized in the central and perivascular regions (57, 58, 59). This of course differs based on GBM subtype (57).

GAMs are considered to have high phenotypic plasticity and a wide range of functional effects which can be regulated by various microenvironmental factors (60, 61). This high phenotypic plasticity means that the population of GAMs is in a state of constant transition between the M2 and M1 polarization states (60, 61). Therefore, the previous thought that active GAMs are only M2 in phenotype is inaccurate. Various studies have shown that GAMs play a major role in initiating cancer, proliferation, development, and metastasis by maintaining an immunosuppressive environment (56, 60, 61, 62). It does this by secreting various cytokines (eg: IL-10, IL-13, TGFβ), chemokines (eg: CCL2, CCL17, CCL22), growth factors (eg: epidermal growth factor, colony stimulating factor 1, vascular endothelial growth factor), inhibitory immune checkpoint proteins, and enzymes (56, 60, 61, 62). Through this immunosuppressive environment, GAMs are able to promote Treg induction which supports the TME by allowing

tumor escape from immune surveillance, as well as metabolic starvation and inactivation of T-cells (56, 60). Studies also show that the migration of GAMs into necrotic areas to clear apoptotic cells from the prothrombotic milieu is linked to tumor malignancy and aggressiveness (57). Analysis of marker genes from different experimental models and clinical samples by Walentynowicz et al. (63) showed that only a small set of common genes were considered reliable markers of glioma-induced polarization in microglia/macrophages, TGM2 being one of them. GAMs also have a family of receptor tyrosine kinases consisting of Tyro3, Axl, and MerTK, which play a role in tumor cell interactions, macrophage polarization, efferocytosis, and autoimmunity (61). Overall, high infiltration of GAMs is associated with poor prognosis, survival, and response to therapy, making them an attractive target for immunotherapy like innate immune checkpoint blockade (56, 64).

1.2.3 Efferocytosis

Efficient resolution of inflammation is critical in homeostasis and restoring tissue integrity. Typically, the process of resolving inflammation involves the clearing of apoptotic or necrotic cells by phagocytosis before the release of any intracellular components (65, 66). Subsequently, this prevents the activation of an inflammatory response that would have been triggered by the release of immunogenic material (65). If, however secondary necrosis occurs, causing the cytoplasmic and nuclear contents of an apoptotic cell to be released, DAMP signalling would be activated, which induces the expression of inflammatory cytokines and chemokines, and activates the innate and adaptive immune responses (67). This process of macrophages/microglia clearing apoptotic cells without the induction of an immune response by secondary necrosis is called efferocytosis (65, 66, 67). As inflammation is being resolved,

efferocytic macrophages change their phenotype and start producing anti-inflammatory cytokines like IL-10 and TGFβ, while dampening pro-inflammatory cytokines like IL-12 and TNF-α (67). In turn, this helps dampen inflammation and promote tissue repair. This type of immunomodulatory cytokine regulation is coupled with the process of efferocytosis and occurs in both professional and non-professional phagocytic cells (67). It has also been shown that this coupling allows efferocytosis to be actively involved in several pathways that play a crucial role in tissue repair and homeostasis, differentiation, and the resolution of inflammation (65). Due to its pleiotropic nature, any defect in efferocytosis may result in a variety of pathological consequences such as atherosclerosis, inflammatory diseases, and autoimmune diseases (65, 66). In recent studies, efferocytosis has been shown to play a role in the TME, metastasis, and progression of cancers (68, 69). The cancer cells essentially capitalize from the tolerogenic and immunosuppressive nature of efferocytosis to help support cancer progression and provide an escape from immunological surveillance (65, 67).

The initiation of efferocytosis occurs when apoptotic cells release a “find me” signal, which recruits phagocytic cells like monocytes/macrophages (67, 70). There are 4 “find me” signals that have been identified so far: lysophosphotidylcholine (LPC), sphingosine 1-phosphate (S1P), chemokine C-X_3-C motif ligand 1 (CX_3CL1), and nucleotides (e.g.: adenosine triphosphate [ATP]) (67, 70). Following the “find me” signal, apoptotic cells start to release “eat me” signals as surface markers that enable phagocytes to accurately identify apoptotic cells (67, 70). Phosphatidyl serine (PS), the most heavily studied “eat me” signal, is a phospholipid that is typically localized to the inner leaflet of the plasma membrane (67, 70). However, in apoptotic cells, PS rapidly translocates to the outer leaflet of the plasma membrane (67, 70). PS then interacts with the soluble bridging ligands growth arrest specific-6 (Gas6) and protein S1

(ProS1), which indirectly binds to Tyro3, Axl, and MerTK receptors on the surface of phagocytes (65, 67, 71). These three receptors each perform different functions, however, Axl and MerTK receptor kinase activation is essential to the PS dependent phagocytosis of apoptotic cells (65, 67). There have been studies on Axl and MerTK mediated efferocytosis that show a decreased innate immune response in macrophages as well as poor survival in human cancers (67, 72). This decreased response creates a TME that facilitates tumor progression and metastasis. Tyro3, Axl, and MerTK receptors can also activate downstream signalling pathways and regulate cytoskeletal rearrangement when phosphorylated, which allows for engulfment of apoptotic cells after phagocytic recognition and binding (67, 73).

After engulfment, various cytokines cause macrophages to lean towards the M2 alternatively activated immune suppressive phenotype (65, 67). Following Tyro3, Axl, and MerTK receptor phosphorylation, the downstream signalling of the phosphatidylinositol 3 kinase/protein serine threonine kinase (PI3K/Akt) pathway plays a role in the polarization of macrophages (65, 67). Essentially Tyro3, Axl, and MerTK receptors directly bind to PI3K, which then phosphorylates Akt (65, 67). This polarizes macrophages to the M2 alternatively activated immune suppressive phenotype while dampening the M1 classically activated inflammatory phenotype (67). The M2 macrophage will secrete immunosuppressive cytokines in the TME which will recruit FOXP3+ T regulatory cells and suppress the $CD4^+$ and $CD8^+$ effector T cells (65. 67). Conversely, this will downregulate pro-inflammatory cytokine expression, which will ultimately promote a tolerogenic, and immunosuppressed TME causing tumor progression, invasion, and poor survival in cancer patients (67).

1.3 Transglutaminase 2

Tissue transglutaminases are a family of nine proteins. Of particular interest in this study, tissue transglutaminase 2 (TGM2) is a multifunctional calcium dependent enzyme that has been found to play an important role in GBM proliferation and survival, particularly in the mesenchymal subtype (74). However, it is important to note that TGM2 is present in some but not all GBM patients (74). It can either function intracellularly or extracellularly and plays 2 major roles: 1) catalyzing calcium dependent transamidation reactions & binding (extracellular), 2) functioning as a G protein which can control β-adrenergic-receptor-mediated signaling transduction pathways (intracellular) (74, 75, 76, 77, 78). When bound with GTP/GDP, TGM2 is in a closed state conformation functioning as a GTP-binding GTPase which is necessary for cancer stem cell (CSC) survival (74, 75, 76, 77, 78). When bound with Ca2+ however, it is in an open state conformation involved in crosslinking proteins (74, 75, 76, 77, 78).

Recent studies show that only the GTP binding activities of TGM2 are required for the expression of epithelial mesenchymal transition (EMT) markers, as well as epidermal cancer stem cells (ECS) and CSC invasion, migration, and tumour formation (74, 75, 77, 78, 79, 80). Due to the slow proliferation of CSCs and cells undergoing EMT, there is a resistance to most anti-cancer drugs that target rapidly dividing cells (74, 75, 77, 78, 79, 80). There have been other studies looking at TGM2 expression in knockout cancer cell lines which show that TGM2 expression was associated with signalling, migration, metastasis, and EMT (74, 76, 77, 78, 79, 80). TGM2 was also found to be highly expressed in CD44 high (stem cell marker) cells in glioma tissue which plays a role in tumorigenesis (63). Finally, the expression of TGM2 was found to be induced by TGFβ, which plays a role in immune responses as well as monocyte/macrophage functions (eg: migration, differentiation) (63)

1.3.1 TGM2 in efferocytosis

There have been previous studies showing TGM2 knockout mice having defects in in-vivo clearance of apoptotic cells, which resulted in age-dependent autoimmunity (81, 82). This led us to further investigate what role TGM2 plays in efferocytosis, and how it may relate to the progression of tumors. Observations in mice that had TGM2 knocked out showed that they were viable but prone to developing autoimmunity, thymus involution, and other inflammatory diseases (eg: glomerulonephritis) in the long run (81). This was due to impaired efferocytosis in TGM2 null macrophages, as they were able to bind apoptotic cells but had defective engulfment which requires a functional GTP binding site of TGM2 (82). Ultimately, the induction of apoptosis in the knockout mice promoted an inflammatory response due to the lack of immunosuppressive cytokine expression in macrophages in comparison to the wild-type mice (81). It has been shown that the induction of apoptosis causes macrophages to release TGFβ1 in response to the PS on apoptotic cells which requires TGM2 expression in order to be activated (81). Essentially, successful efferocytosis requires TGM2 expression in macrophages. This line of evidence has pushed us to further investigate the role of TGM2 in glioblastoma associated immune cells.

1.4 Study Rationale

Glioblastoma is an incurable disease in need of effective therapeutic treatments. Studies have shown that cancer cells will recruit macrophages into the TME which will then be reprogrammed into the immunosuppressive phenotype that will promote tumour growth. There is evidence that the protein TGM2 has a role in glioblastoma growth and drugs that inhibit its function were tested. Since these studies only focused on the use of TGM2 inhibitors in

glioblastoma, we were interested in looking at TGM2 expression in microglia/macrophages along with the interactions and the role it plays in glioblastoma.

For this study primary glioblastoma cells that are mesenchymal in subtype (termed PriGO17As) were isolated from patient tumours from the Ottawa hospital, as it is the most accurate model of glioblastoma since it replicates the genetic and histopathological characteristics of the donor's tumour. THP-1 cells were used as our initial macrophage model in order to create a clinically relevant model. Since TGM2 has been previously shown to have a role in efferocytosis and the suppression of autoimmunity in mice, we think that targeting TGM2 may prove to be a novel and effective immunotherapy to improve survival in glioblastoma.

1.5 Hypothesis

Based on current knowledge of glioblastoma, the following hypotheses were made:

1. In glioblastoma, TGM2 is predominantly expressed by GAMs.
2. Glioblastoma induces TGM2 expression in macrophage/microglia.
3. TGM2 in GAMs contributes to an immunosuppressive phenotype.

1.6 Objectives

1. Characterize the expression of TGM2 in glioblastoma.
2. Determine the function of TGM2 in glioblastoma with the goal of determining the effect of TGM2 inhibitors.

2 - Materials and methods

2.1 Bioinformatics

Data from the Cancer Genome Atlas (TCGA) database was analyzed using cBioportal (83, 84)using the Cell 2013 RNAseq dataset (85). Data from the Chinese Glioma Genome Atlas (CGGA; 249) database (86) was analyzed using GlioVis (87). As a second way to assess data from the TCGA dataset, Enrichr was used (88, 89). Single cell RNA-seq data from Neftel et al. (90) was analyzed through the Broad Institute Single Cell Portal.

2.2 Antibodies

TGM2 (D11A6) rabbit monoclonal antibody (cat. no. 3557), Iba1 (E4O4W) rabbit monoclonal antibody (cat. # 17198, used for immunohistochemistry), rabbit DA1E monoclonal antibody IgG isotype control (cat. # 3900), and cleaved caspase-3 rabbit monoclonal antibody (cat. # 9664) were all from Cell Signaling Technology. For immunofluorescence in xenografts, mouse monoclonal Anti-Iba1/AIF1 antibody was from Millipore/Sigma (cat. no. MABN92). For immunofluorescence in human samples, anti-CD68 mouse monoclonal antibody was from Millipore/Sigma (AMAB90873). GAPDH mouse monoclonal antibody (ab8245) was from Abcam.

2.3 Cell Culture

PriGO cultures: PriGO17A cells were isolated following a protocol approved by the Ottawa Hospital Research Ethics Board as described previously (47, 91). Glioblastoma cells were grown on plates coated with laminin (Sigma-Aldrich, Oakville, ON, Canada) in Neurobasal A medium supplemented with B27, N2 (all from Life Technologies, Burlington, ON, Canada),

EGF and FGF (Peprotech, Rocky Hill, NJ, USA) and heparin (Stemcell Technologies, Cambridge, MA, USA) at 37°C in 5% O2/CO2. THP-1 cultures: THP-1 monocytes and macrophages were grown in RPMI-1640 medium (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum at 37°C and 5% CO2. To differentiate THP-1 monocytes into macrophages, they are treated with 100ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 hours. PMA media is then removed and regular media with treatment of interest is added. THP-1 cells transduced with a NF-kB luciferase reporter (ATCC, TIB-202-NFkB-LUC2) were also used in place of wild type THP-1 cells.

2.4 Treatments

The following treatments were used separately on THP-1s or in co-culture to study the effects on TGM2 activation: 50 ng/mL of IL-4 for 48 hours, 20 ng/mL of IFN- γ for 48 hours, and 20 ng/mL of TGFβ for 6 hours, 24 hours, and 48 hours (all were obtained from R&D systems, Minneapolis, MN, USA).

2.5 Mouse Xenograft Model

CD1-nude female mice were intracerebrally injected with human PriGO17A cells using a stereotaxic apparatus as described previously (91). Mice were euthanized at the first signs of morbidity. Brains were isolated, fixed with formalin and paraffin embedded in preparation for immunohistochemistry (IHC).

2.6 Western Blot

Western blot analysis was done as described previously (92). Cell lysates were harvested, and protein samples were run on Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 4–12% bis-tris polyacrylamide gel from Invitrogen (Carlsbad, CA, USA). The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences, Chicago, IL, USA). After transfer, membranes were stained with amido black for loading control, then probed with primary TGM2 antibodies at room temperature for 1 hr, followed by rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) for 1 hr. For other loading control, membranes were stripped and probed with anti-GAPDH (Abcam). Secondary HRP conjugated antibodies were detected with Luminata Forte Western HRP Substrate (Thermo Scientific Pierce). Western blots were visualized by chemiluminescence using the ChemiDoc imaging system from Bio-Rad (Hercules, CA, USA).

2.7 Immunohistochemistry

IHC was performed on formalin-fixed, paraffin-embedded tissue sections using the Leica Bond system. For rabbit antibodies, a modification of protocol F (from Leica Bond system) that eliminates the post primary step when using rabbit antibodies on mouse tissue was used. Sections were first treated using an ethylenediaminetetraacetic acid (EDTA) buffer (pH 9.0, epitope retrieval solution 2) for 20 min. Sections were then incubated with a 1:100 dilution of primary antibody for 30 min and detected using an HRP-conjugated compact polymer system. For the rabbit IgG control, a concentration matching the protein concentration of the specific antibody was used. Slides were then stained using Diaminobenzidine (DAB) as the chromogen,

counterstained with hematoxylin, mounted and cover slipped. Whole section digital images were generated using a Zeiss Axioscan Z1 slide scanner.

2.8 Tissue Microarray

Construction of the tissue microarray was described previously (93). Duplicate 1 mm cores were used for each patient. While the original tissue microarray included lower grade glioma patients, only tissue from the eighty-three IDH wild-type glioblastoma patients was analyzed here. Immunohistochemistry for TGM2 was performed as described above.

2.9 Immunofluorescence

Paraffin sections were deparaffinized and treated using an EDTA buffer pH 9.0 for antigen retrieval. Sections were blocked for 30 minutes with Rodent Block M (Biocare RBM961H). Sections were then incubated overnight at 4oC with either no primary antibody, 1:75 dilution of Rabbit TGM2/1:500 Mouse Iba1 and 1:75 dilution of Rabbit TGM2/Mouse Iba1 1:1000. The following day, sections were washed with 1 X TBST and incubated with Goat anti-Rabbit IgG-488 (Invitrogen #A-11008) and Donkey anti-mouse IgG -568 (#A10037) using a 1:500 dilution for 1hour at room temperature. This was followed by incubation for 5 minutes with a quencher (Vector TrueView Autofluorescence Quenching Kit #SP-8400, Vector Labs) to decrease autofluorescence. Sections were then washed, incubated with 5 ug/ml of DAPI (ThermoScientific #62248) and cover slipped.

3 - Results

3.1 Bioinformatic analysis of TGM2 suggests a role in glioblastoma progression

Analysis of the TCGA 2013 database (152 patients) on cBioportal (Figure 1A) and the CGGA database (249 patients) using GlioVis (Figure 1B) showed significantly higher TGM2 mRNA expression in the mesenchymal subtype. Since the mesenchymal subtype, relative to the other molecular subtypes, is known for higher infiltration of GAMs, correlations between TGM2 mRNA and the standard microglia/macrophage marker mRNA were assessed. A positive correlation can be seen between TGM2 mRNA and CD68 and AIF1 (Iba1) mRNA (Figure 2A and B). Another way to assess TGM2 gene expression associations with microglia/macrophages was by the use of Enrichr. The 50 genes that have the highest positive correlation with TGM2 mRNA levels (as determined on cBioportal) were analyzed, and under "cell types" it showed a very strong match with macrophages (Figure 3; 34/50 match between TGM2 signature and macrophages; adjusted $P = 2.8x10^{-15}$, odds ratio 5.35). Finally, TGM2 mRNA expression was assessed from the single cell RNA sequencing (scRNA seq) data (available through the Broad Institute Single Cell Portal) of TGM2 mRNA expression in a subset of GAMs obtained from a paper by Neftel *et al* (90). This study had 28 glioblastoma patients with data from a total of 24,131 cells. Figure 4A shows t-distributed stochastic neighbour embedding (tSNE) analysis with cell type listed on the upper left, while 4B shows TGM2 expression in a subset of macrophages (upper left quadrant) and mesenchymal glioblastoma cells (lower right quadrant). Since this study did not separate microglia from macrophages, marker expression was used for further assessment. The chemokine receptor CCR2 is known to be preferentially expressed in macrophages, while the CX3CR1 chemokine receptor is preferentially expressed in microglia (31). TGM2 is shown to cluster with CCR2 expression, rather than CX3CR1 (Figure 4B & C).

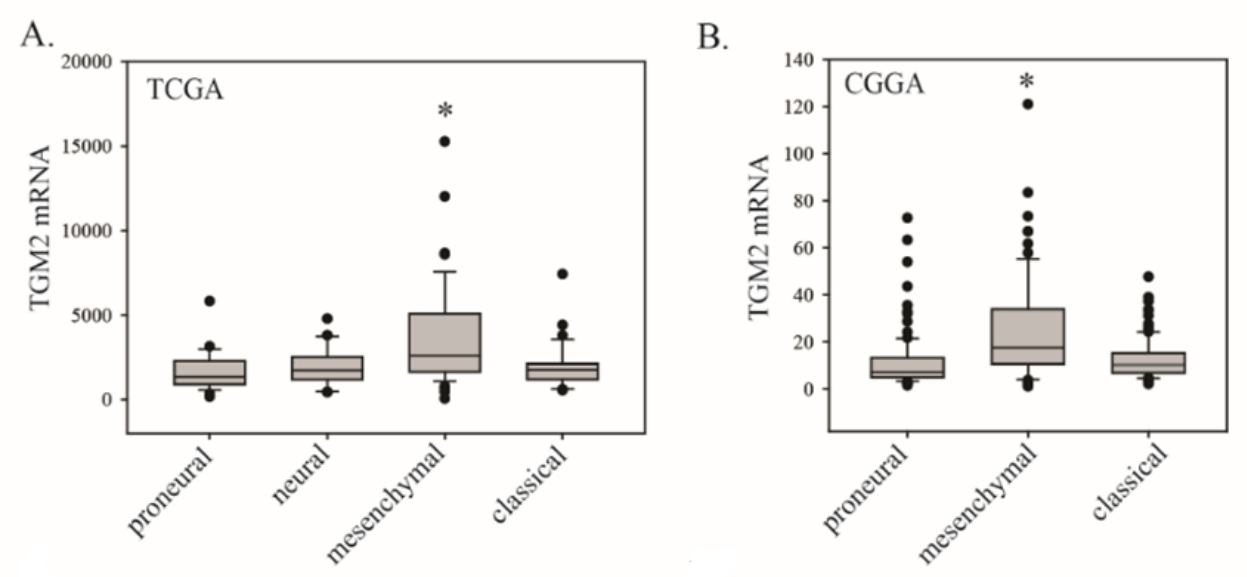

A.
TCGA
TGM2 mRNA
20000
15000
10000
5000
0
*
proneural
neural
mesenchymal
classical
B.
CGGA
TGM2 mRNA
140
120
100
80
60
40
20
0
*
proneural
mesenchymal
classical

Figure 1. ***TGM2 mRNA expression in glioblastoma.*** Analysis of (A) TCGA and (B) CGGA datasets using a Kruskal-Wallis One Way Analysis of Variance on Ranks analysis to look at TGM2 expression in the different subtypes of glioblastoma as data were not normally distributed. To isolate the group or groups that differ from the others, a multiple comparison procedure (Dunn's Method) was performed.

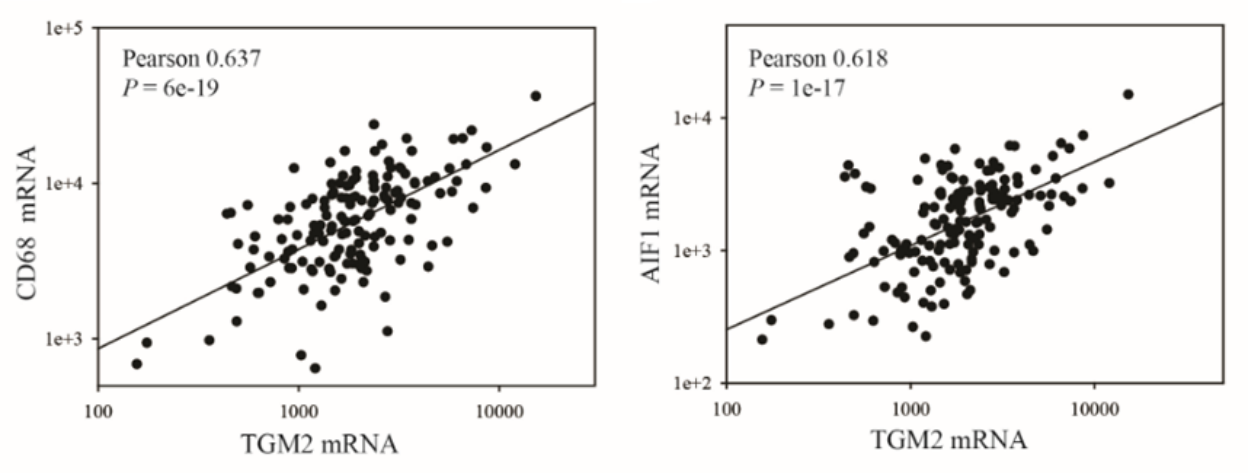

Pearson 0.637
P = 6e-19
CD68 mRNA
1e+5
1e+4
1e+3
100
1000
10000
TGM2 mRNA
Pearson 0.618
P = 1e-17
AIF1 mRNA
1e+4
1e+3
1e+2
100
1000
10000
TGM2 mRNA

Figure 2. ***TGM2 mRNA correlation with pan macrophage/microglia markers.*** A correlation of TGM2 mRNA levels with CD68 and AIF1 (Iba1) in the TCGA and CGGA datasets

	Gene	corr.
1	IL4R	0.705
2	TGFBR2	0.699
3	TNFRSF1B	0.677
4	FMNL1	0.669
5	MLKL	0.667
6	TMEM106A	0.667
7	ST3GAL1	0.666
8	ENG	0.662
9	DAB2	0.662
10	B4GALT1	0.660
11	LTBP2	0.656
12	TRPM2	0.656
13	ARHGAP4	0.656
14	CYP1B1	0.655
15	ARHGAP30	0.654
16	JAK3	0.652
17	HAS1	0.652
18	DEF6	0.651
19	SLCO2B1	0.650
20	STING1	0.650
21	GPR132	0.650
22	IL1R1	0.649
23	PLCG2	0.648
24	MFSD1	0.648
25	CSF2RB	0.648

	Gene	corr.
26	SUSD6	0.647
27	ITGAM	0.646
28	LILRB1	0.643
29	ARPC1B	0.642
30	RASAL3	0.642
31	PRKCD	0.642
32	SLC7A8	0.639
33	CTSZ	0.638
34	GIMAP1	0.637
35	RASSF5	0.635
36	RAB11FIP1	0.635
37	RHBDF2	0.633
38	IL15RA	0.633
39	GIMAP8	0.632
40	CD4	0.632
41	SH3TC1	0.632
42	DOCK2	0.632
43	ALOX5	0.631
44	LILRB2	0.631
45	ANXA11	0.631
46	SERPINB9	0.630
47	PLCB2	0.630
48	ADGRE2	0.630
49	SLC16A5	0.630
50	LAIR1	0.630

Enrichr: Cell Types: ARCHS4 Tissues: macrophage; adjusted *P* value 8e-19; odds ratio 16.45; combined score 760. 34/50 match

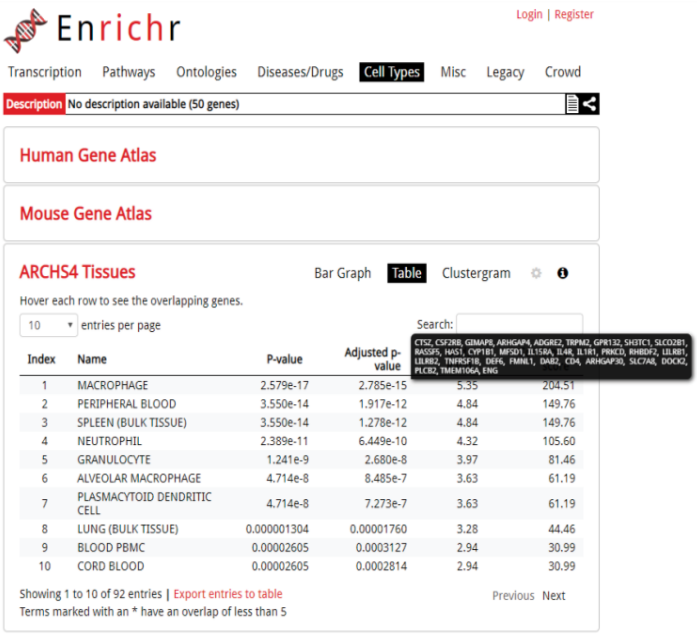
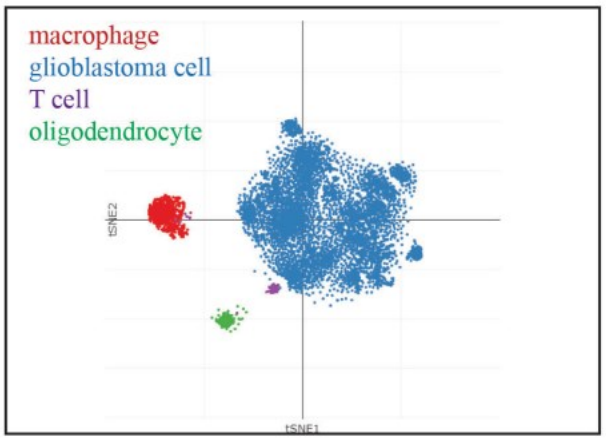
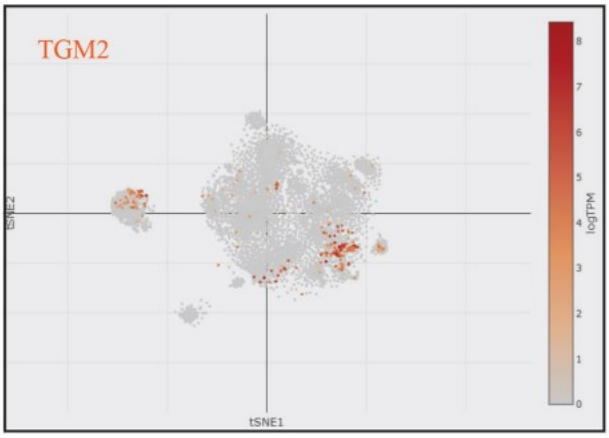


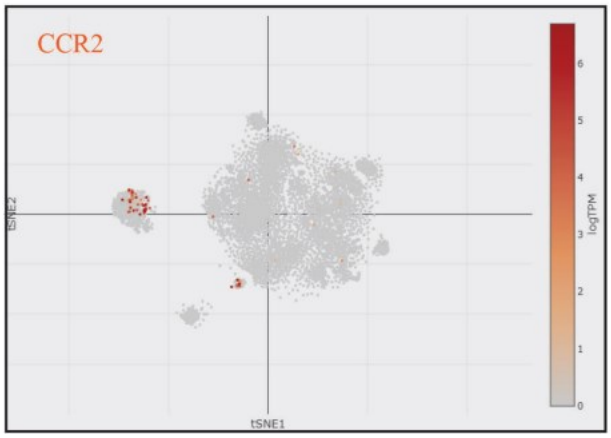
Figure 3. ***TGM2 mRNA expression in glioblastoma analyzed in Enrichr.*** The fifty genes showing the strongest positive correlation with TGM2 mRNA expression by Spearman correlation. Yellow highlights show match to macrophage cell type identified using Enrichr under Cell Types/ARCHS4 Tissues (adjusted $P = 2.8 \times 10^{-15}$, odds ratio 5.35).

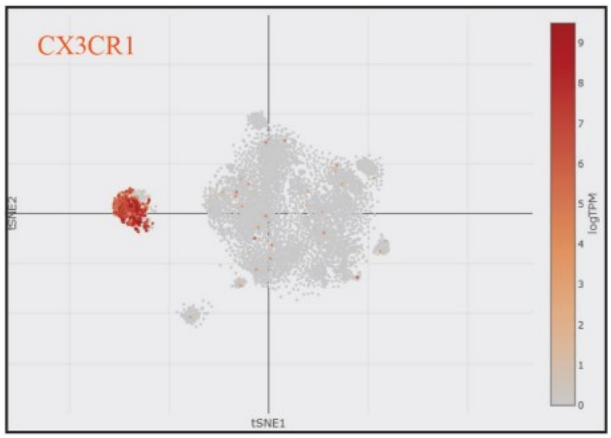
A

B


macrophage
glioblastoma cell
T cell
oligodendrocyte
tSNE2
tSNE1


TGM2
tSNE2
tSNE1
logTPM


CCR2
tSNE2
tSNE1
logTPM


CX3CR1
tSNE2
tSNE1
logTPM

C

D

Figure 4. ***scRNA seq data of TGM2 mRNA expression in a subset of GAMs by Neftel et al.***

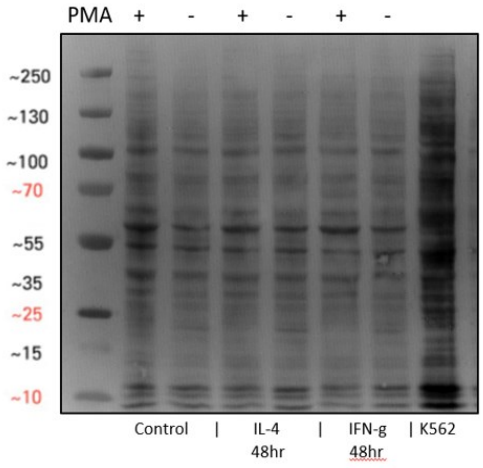
Data is pooled from 28 patients and CD45 immune depletion step was done before sequencing. tSNE analysis with cell type designations (A). TGM2 selection shows expression in a subset of macrophages and mesenchymal glioblastoma (B). CCR2 macrophage marker selection (C). CX3CR1 microglia marker selection.

3.2 Cell Culture experiments identify TGM2 expression in macrophages

To study TGM2 expression in cell culture, THP-1s were either differentiated with PMA for 48 hours or left undifferentiated followed by treatments with 50ng/mL IL-4, 20ng/mL INFγ, or 20ng/mL TGFβ1 for 6, 24, and 48 hours. Differentiated and Undifferentiated macrophages were treated with IL-4 and TGFβ since they are known to induce TGM2 expression in THP-1s. Treatment with IFN- γ, which polarizes macrophages into the M1 phenotype, was done to observe whether or not pro-inflammatory macrophages express TGM2. K562 cells (first human myelogenous leukemia cell line) were used as a control for TGM2 expression (band is around 62-77 kDa). Differentiated THP-1 cells in Figure 5B show stronger expression of TGM2 in comparison to undifferentiated THP-1 cells which have weak/undetectable TGM2 expression. Expression of TGM2 is also seen to increase after 48 hr treatment with either IL-4 or INFγ (Figure 5B). A time course treatment for differentiated THP-1 cells with TGFβ in Figure 6B shows higher TGM2 expression at the 48 hr time point. For this blot, IL-4 treated THP-1s and K562 cells were used as controls. In PriGO17As, TGM2 expression was not detected with or without TGFβ1 treatment (Figure 7B).

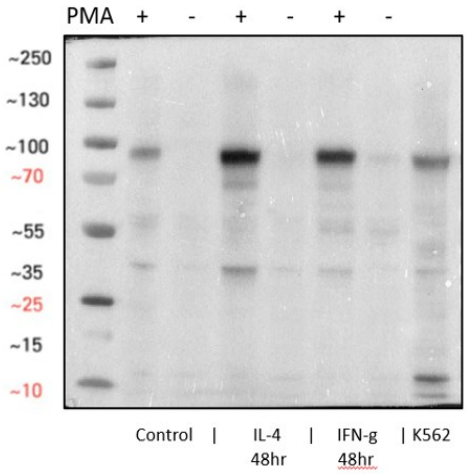
A

Amido Black



B

TGM2



C

GAPDH

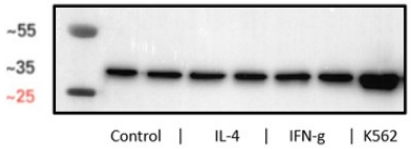


Figure 5. PMA differentiated THP-1s have higher expression of TGM2 in all treatments.

Amido black staining for loading control (A). TGM2 probing (B). GAPDH probing for loading control (C).

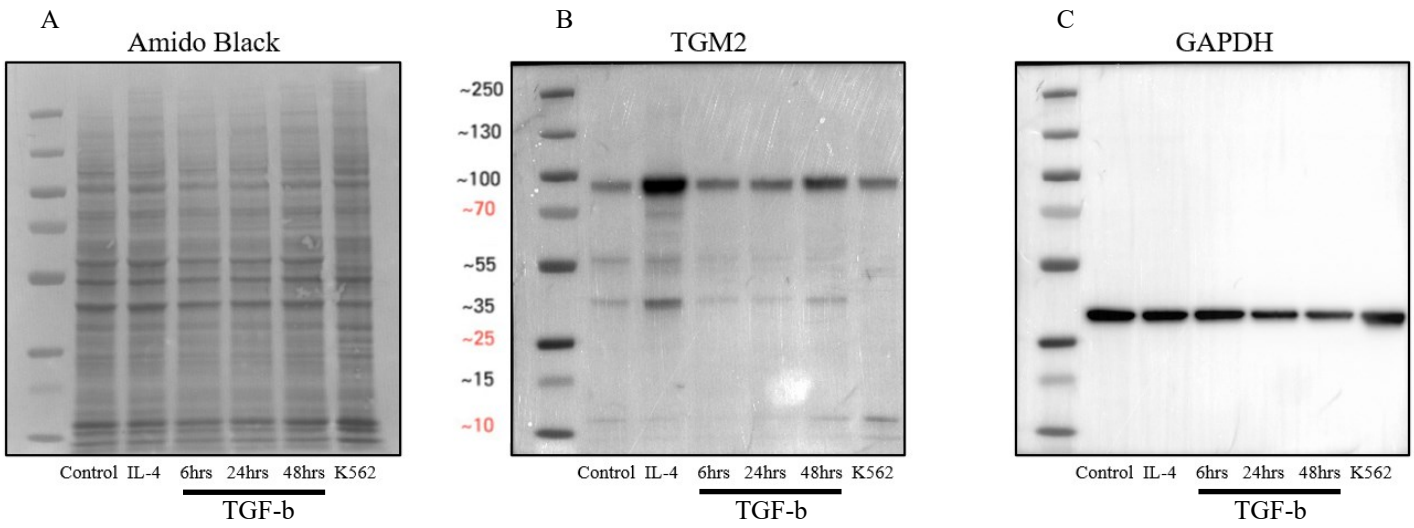

A
Amido Black
Control IL-4 6hrs 24hrs 48hrs K562
TGF-b
B
TGM2
~250
~130
~100
~70
~55
~35
~25
~15
~10
Control IL-4 6hrs 24hrs 48hrs K562
TGF-b
C
GAPDH
Control IL-4 6hrs 24hrs 48hrs K562
TGF-b

Figure 6. THP-1 cells treated with TGFβ for 48 hours had the highest TGM2 expression in comparison to other time points however, treatment with IL-4 had the highest TGM2 expression. Amido black staining for loading control (A). TGM2 probing (B). GAPDH probing for loading control (C).

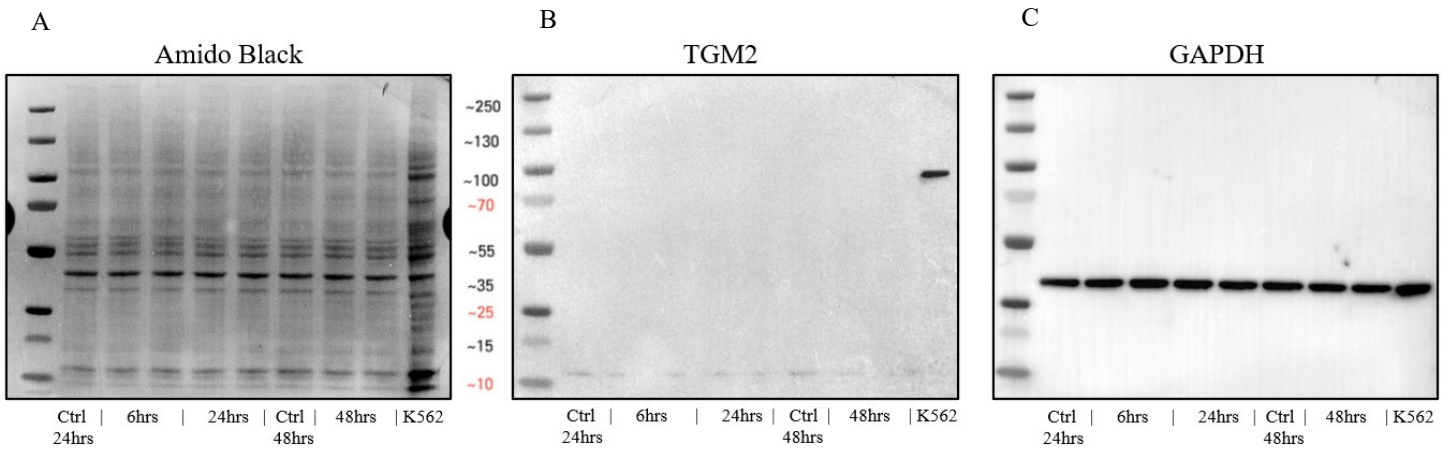

A
Amido Black
B
TGM2
C
GAPDH
~250
~130
~100
~70
~55
~35
~25
~15
~10
Ctrl 24hrs
6hrs
24hrs
Ctrl 48hrs
48hrs
K562

Figure 7. PriGO17A cells treated with or without TGFβ had no TGM2 expression. Amido black staining for loading control (A). TGM2 probing (B). GAPDH probing for loading control (C).

3.3 Immunohistochemistry analysis reveals TGM2 expression in GAMs that is consistent in both mouse and human models

To model mesenchymal-subtype glioblastoma in a xenograft model, human PriGO17A glioblastoma cells were intracerebrally injected into nude immunocompromised mice. IHC in Figure 8 showed marked engagement and activation of mouse macrophage/microglia within the tumour microenvironment. IHC was done again for TGM2 staining in normal mouse brain (Figure 9A). Note that positive staining was absent in no primary and non-specific antibody controls (Figure S1 in appendix). Microglia had weak positive staining (Figure 9B). Endothelial cells in normal brain vasculature were also positively stained (Figure 9C). Strong positive TGM2 staining is seen in the pia mater of the outermost meninges and the ependymal cells lining the lateral ventricle (Figure 9D and E). On the other hand, Figure 10A shows dark staining of tumor tissue in the brain of an immunocompromised mouse injected intracerebrally with PriGO17A cells. Within the large tumor, there are diffused borders (Figure 10B), positive staining of neovasculature (Figure 10C), dark TGM2 staining of the microglia (Figure 10D), and strong staining of macrophages around regions of necrosis (Figure 10E) that are characterized by pseudopalisading cells and microglia/macrophages clearing away apoptotic cells. To confirm the presence of TGM2 in GAMs, double immunofluorescence was done on sections for Iba1 (red, Figure 11A) and TGM2 (green, Figure 11B). When merged, there is a clear overlap in staining which confirms that TGM2 is indeed coming from the microglia/macrophages (Figure 11C).

To assess the expression of TGM2 in multiple glioblastoma patients, IHC was performed on a tissue microarray (TMA) containing duplicate 1 mm cores from 83 patients on 4 slides. Scoring data for TMA can be seen in Figure S2 (appendix). The patterns seen were similar to those observed in our xenograft models. Dark TGM2 staining of microglia/macrophages (Figure

12A), positive staining of endothelial cells surrounding neovasculature (Figure 12B), and areas of necrosis (Figure 12C). Lastly, in certain rare cases, tumor tissue had no expression of TGM2 (Figure 12D). Since pathologists typically do not take tissue cores with areas with necrosis from donor blocks, IHC was done for whole sections of patient samples stained with TGM2 in Figure 13 in order to identify and observe areas of necrosis in our human model. Areas of necrosis in Figure 13A & B are marked with N where macrophages surrounding those areas of necrosis have darker TGM2 staining than macrophages that are further away. The area of necrosis in Figure 13B closely resembles what is seen in our mouse model. To confirm the presence of TGM2 in human GAMs, double immunofluorescence was done on whole patient sections for CD68 (Figure 14, 1st column from the left) and TGM2 (Figure 14 2nd column from the left). The section in row A is a region containing macrophages and endothelial cells, row B is a higher magnification of a region with neovasculature that has some presence of macrophages, row C is a Z stack of row B emphasizing the co-localization of TGM2 and CD68 in macrophages, and row D is a necrotic region. The merged image (Figure 14 3rd column from the left) confirms that macrophages in our human model also express TGM2 and is consistent with our mouse model.

In Figure 15, the different regions of necrosis caused by thrombotic events in neovasculature can be seen in both the xenograft (Figure 15A) and human (Figure 15C & E) models after staining with TGM2. Close up images of the necrotic regions in both models show that the microglia/macrophages are infiltrating those regions and engaged in efferocytosis (Figure 15B [mouse], and 15D & F [human]). It is important to note that morphologically apoptotic cells appear rounded and shrunken due to nuclear and cytoplasmic condensation in comparison to necrotic cells which swell up, bleb, and become leaky before finally erupting and releasing its contents (94). In order to confirm that the dying/dead cells in areas of necrosis are

apoptotic cells that are potentially being efferocytosed, IHC was done on our xenograft model and probed with cleaved caspase 3 (as seen in Figure 16A &B). Figure 13C shows a close-up image of a macrophage engulfing multiple apoptotic cells.

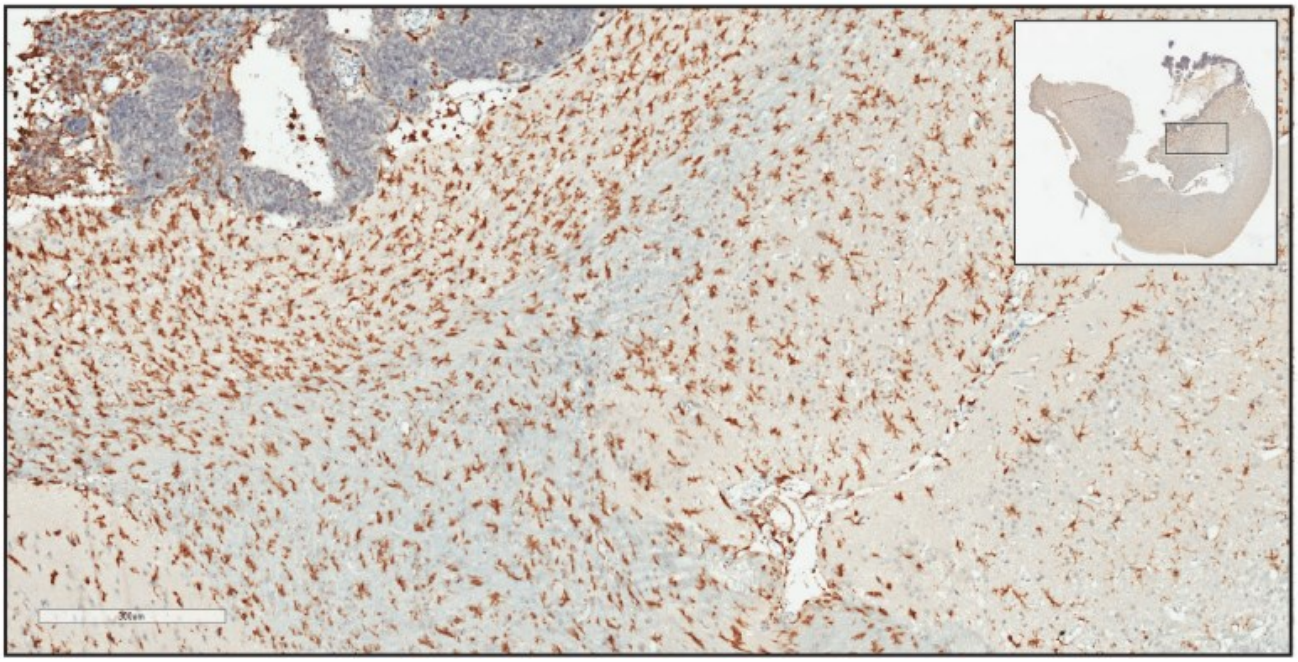
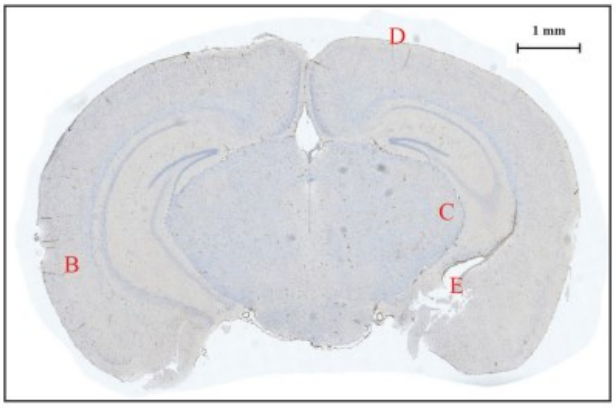
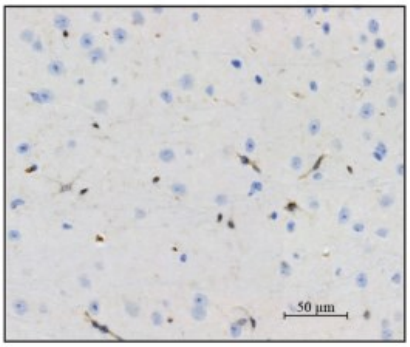


Figure 8. Nude mouse xenograft shows active engagement of mouse microglia/macrophages with human PriGO17A cells. The grey area is the site of intracerebral injection, and the brown dots are microglia/macrophages stained with Iba1.

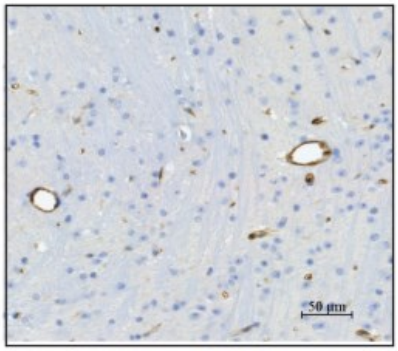
A.



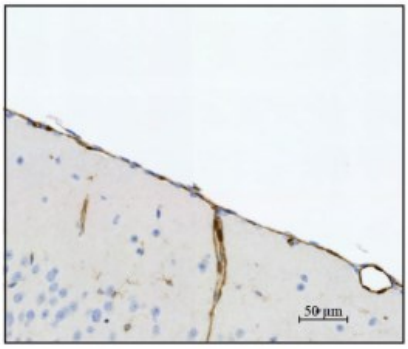
B.



C.



D.



E.

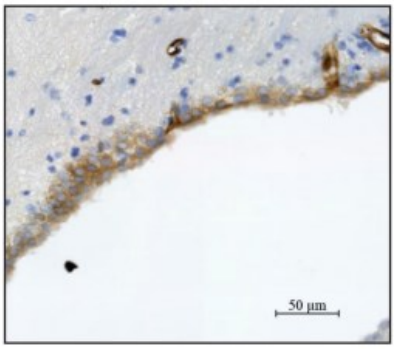


Figure 9. IHC of healthy mouse brain probed with TGM2. (A) overview of brain section analyzed. (B) faint staining of healthy microglia. (C) positive TGM2 staining of endothelial cells surrounding the blood vessels. (D) positive TGM2 staining of pia mater (innermost layer of meninges) (E) positive TGM2 staining of the ependymal cells lining the lateral ventricles.

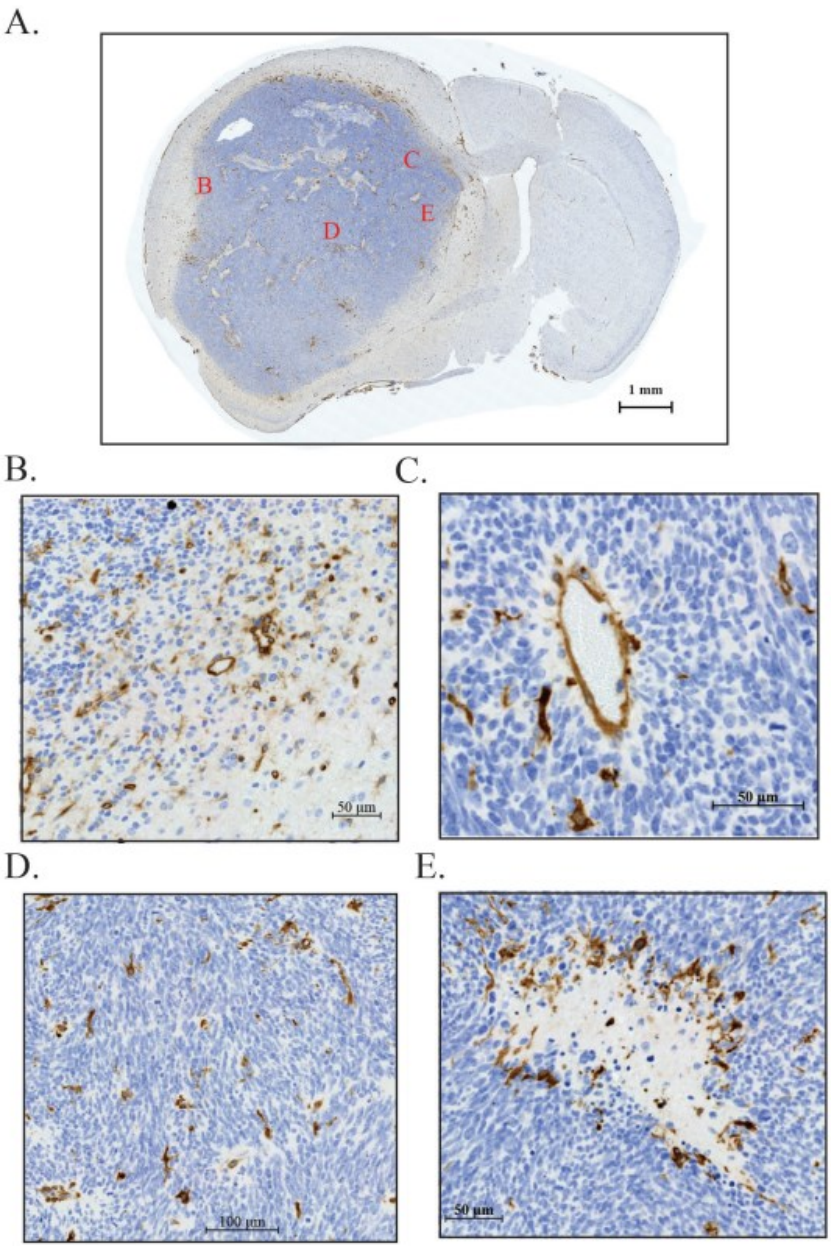
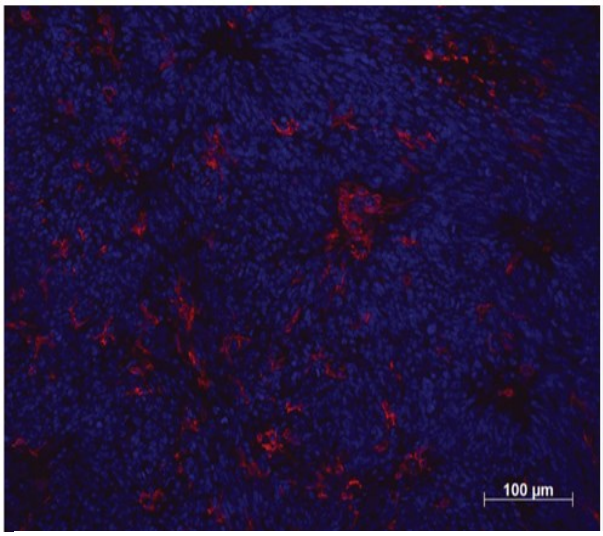

A.
B
C
D
E
1 mm
B.
50 µm
C.
50 µm
D.
100 µm
E.
50 µm

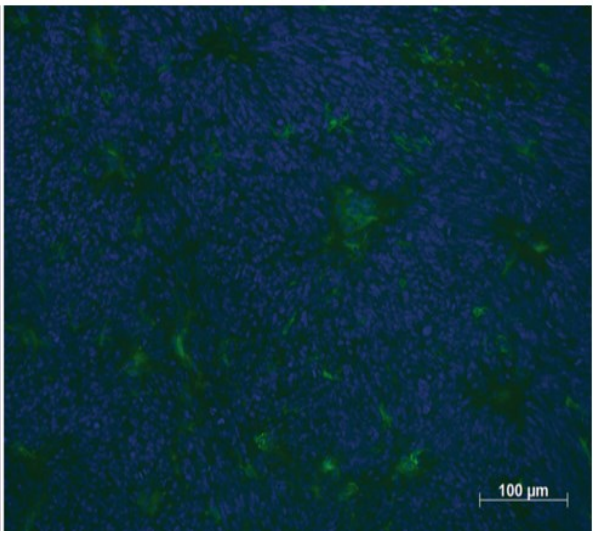
Figure 10. IHC of mouse brain with glioblastoma probed with TGM2. (A) overview of brain section analyzed. (B) diffused border betw. tumor tissue and healthy tissue. (C) positive TGM2 staining of endothelial cells in neovasculature. (D) darker staining of microglia. (E) region of necrosis surrounded by TGM2 stained microglia/macrophages clearing up apoptotic cells.

A



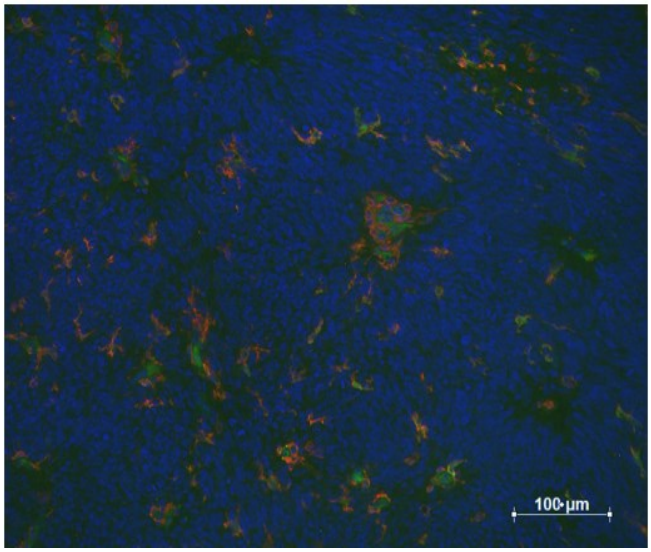
Iba1 (macrophages)

B



TGM2

C



Merged

Figure 11. Double immunofluorescence staining of mouse brain for conformation of TGM2 in GAMs. DAPI (in blue) is staining the nuclei, Alexa Fluor 488 (in green) is staining for TGM2 (in B), and Alexa Fluor 568 (in red) is staining for Iba1 (in A). (C) merged image.

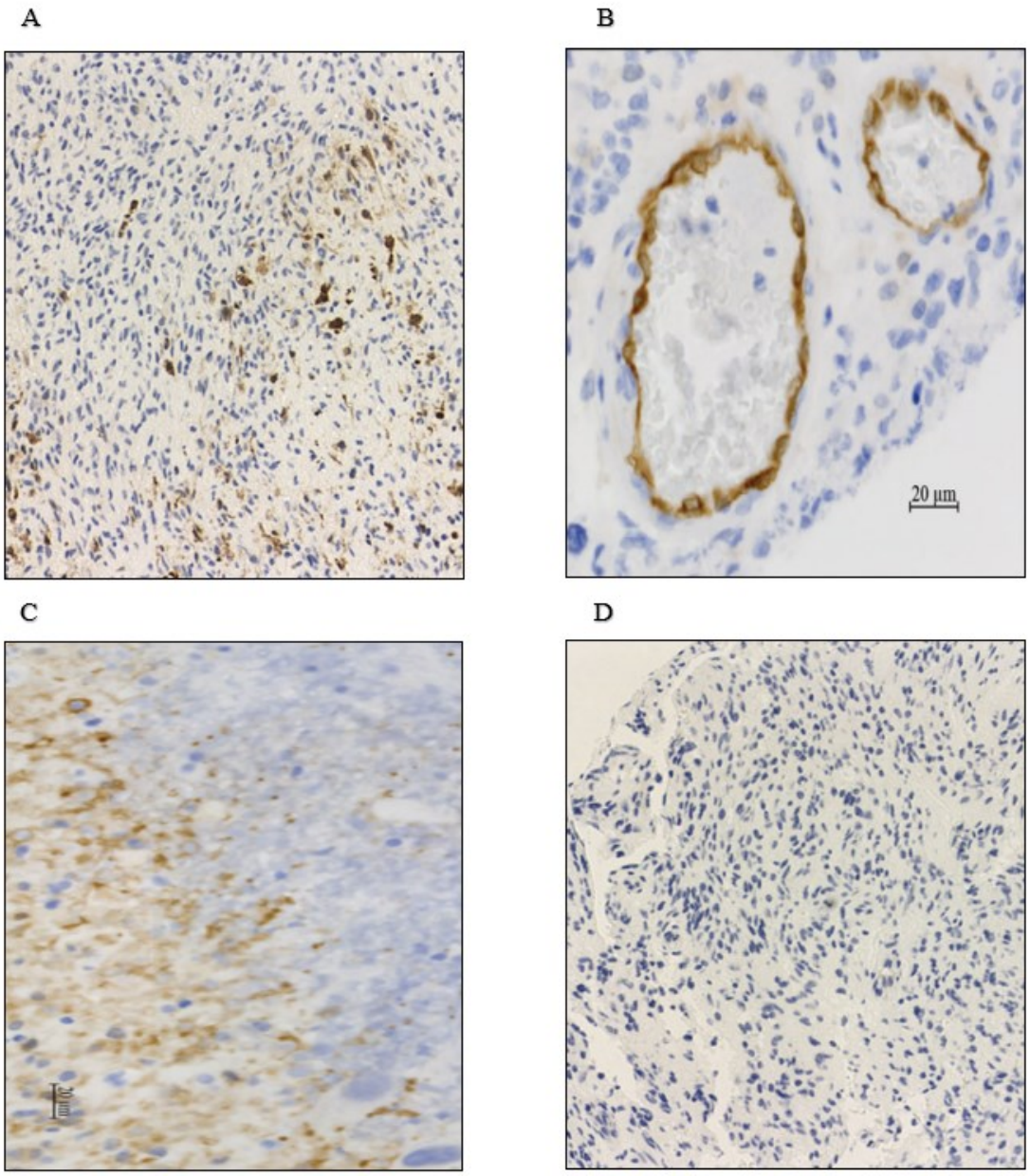
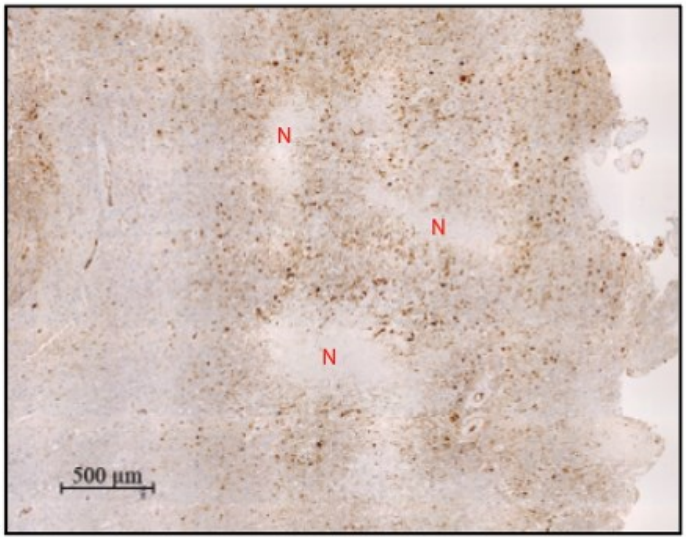

A
B
20 µm
C
D

Figure 12. Human glioblastoma tissue microarray taken from 83 patients. (A) dark TGM2 staining of microglia/macrophages. (B) positive TGM2 staining of endothelial cells surrounding neovasculature. (C) areas of necrosis. (D) glioblastoma tissue with no TGM2 expression.

A



B

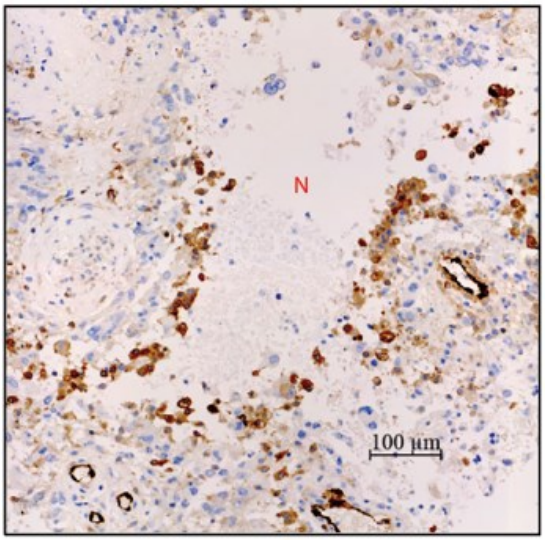


Figure 13. IHC of TGM2 stained whole sections from patient samples identifying areas of necrosis. Areas of necrosis in A and B marked with N in whole patient sections where macrophages surrounding the area of necrosis are seen to have dark TGM2 staining.

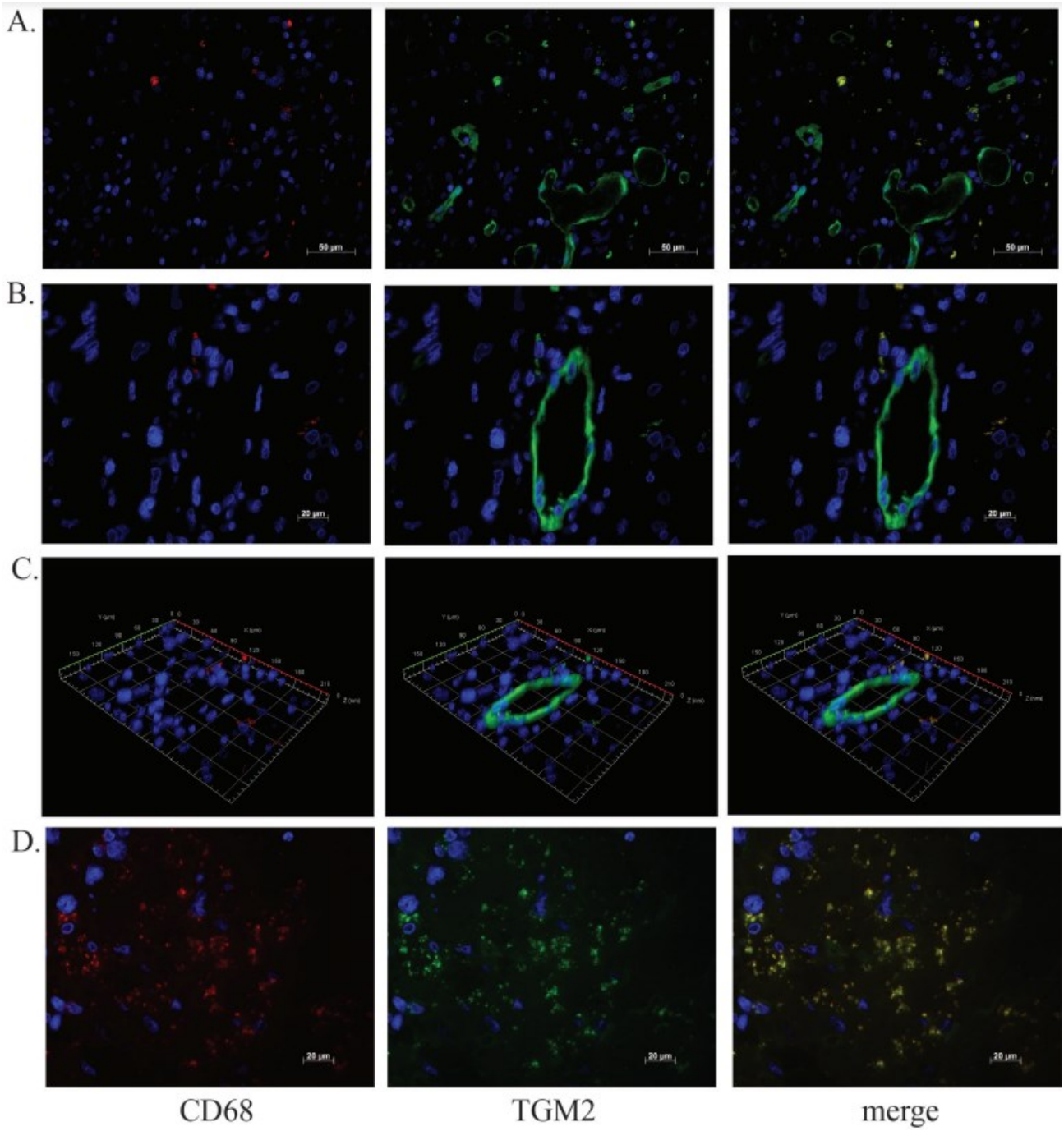
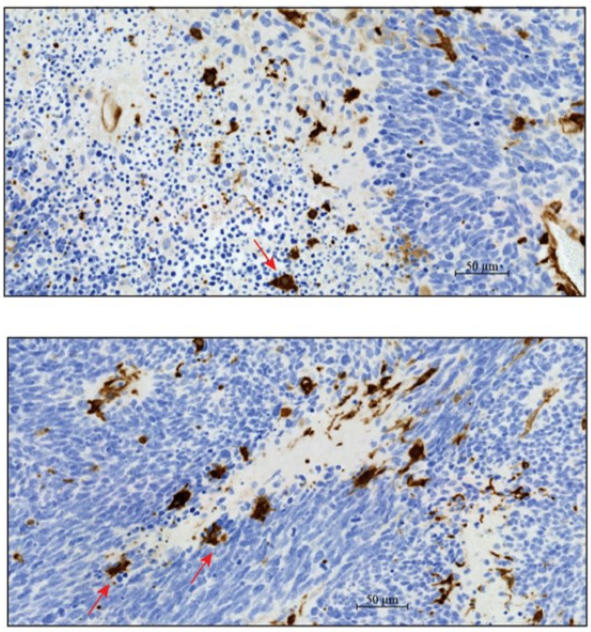
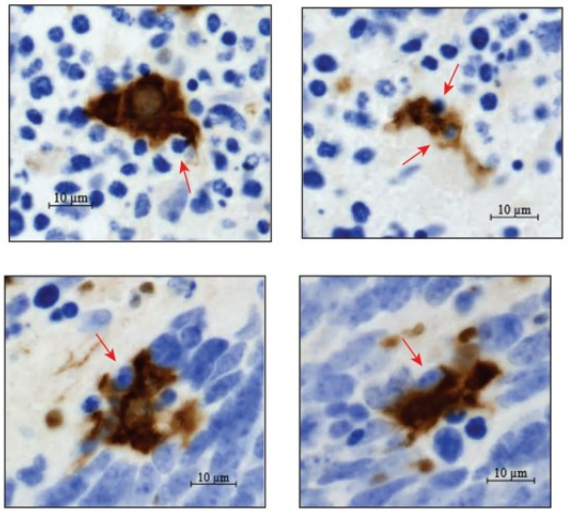

A.
B.
C.
D.
CD68
TGM2
merge

Figure 14. Double immunofluorescence staining with CD68 and TGM2 for whole sections from patient samples. Each column from left to right is as follows: CD68 staining (red Alexa Fluor 568), TGM2 staining (green Alexa Fluor 488) and the merged image. Row A shows a region containing macrophages and endothelial cells. Row B shows a higher magnification of neovasculature with the presence of some macrophages next to them. Row C shows a Z stack of row B, which emphasizes TGM2 and CD68 co-localization in macrophages. Row D shows an area of the tumor that has a necrotic region.

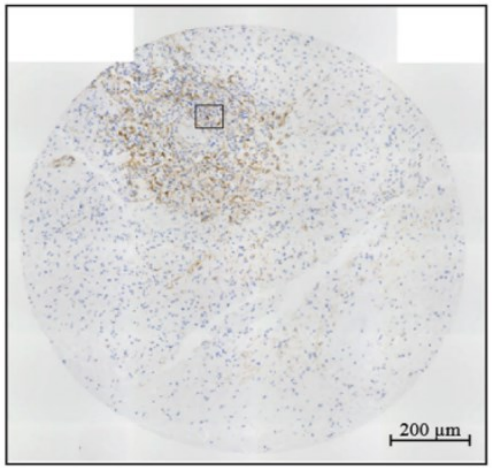
A



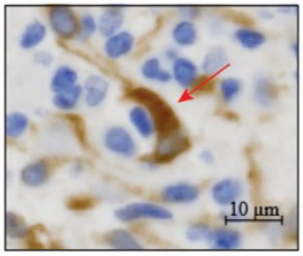
B



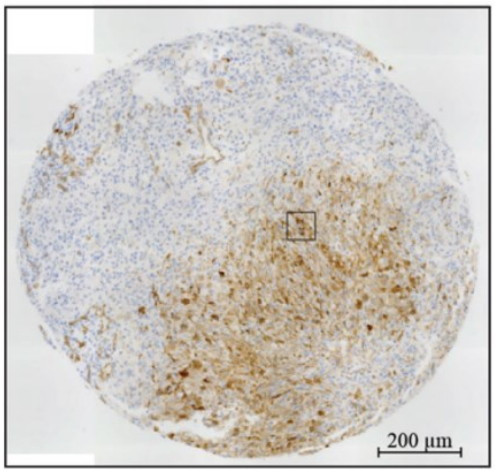
C



D



E



F

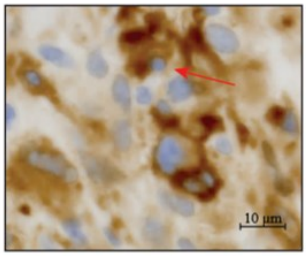
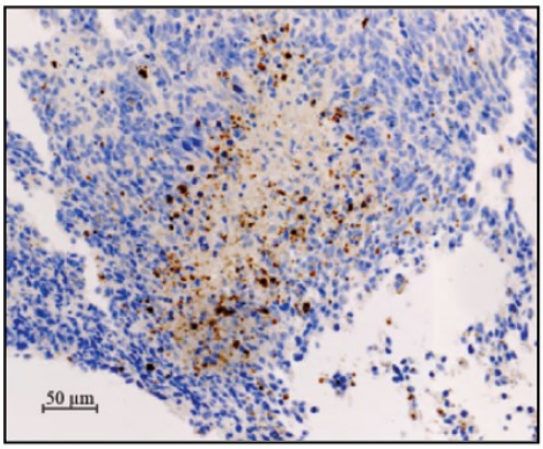
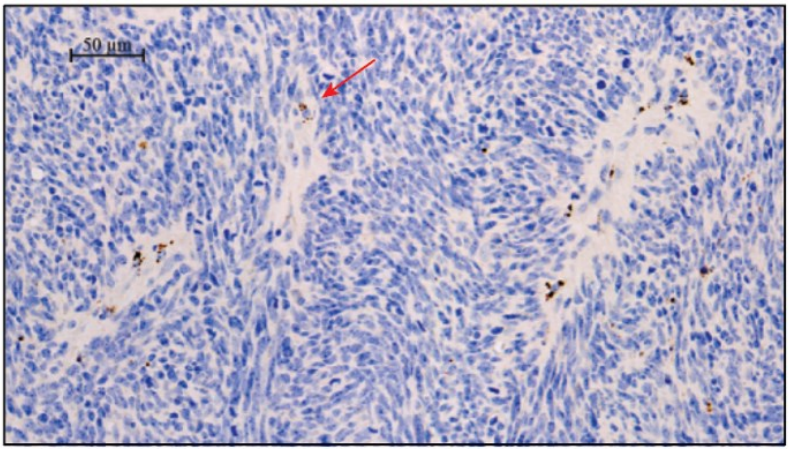


Figure 15. IHC of efferocytosis occurring in areas of necrosis in both mouse and human models. Areas of necrosis in the xenograft model (A) and human model (C & E). Close up images show that both models are engaged in efferocytosis (B, D, & F).

A



B



C

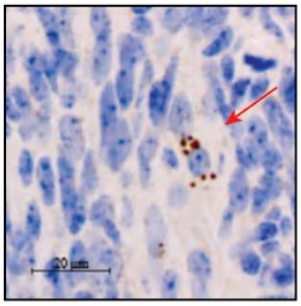


Figure 16. IHC of cleaved caspase 3 probed xenografts identifying apoptotic cells. Areas of necrosis in the xenograft model with cleaved caspase 3 staining of apoptotic cells in brown (A & B). Close up image of a macrophage engulfing apoptotic cells (C).

4 - Discussion

Previous studies on the role of TGM2 in glioblastoma focused on expression in glioblastoma cells rather than in other cells in the TME. A subset of mesenchymal subtype glioblastoma cells were found to express TGM2 (37, 38). Glioblastomas recruit microglia and macrophages and these can sometimes comprise up to 50% of the cells in the tumor (37, 38). This is especially evident in mesenchymal subtype glioblastoma. In our study, we are focused on showing that microglia/macrophages are the major source of TGM2 in glioblastoma and determining the role of microglia/macrophage-expressed TGM2 in glioblastoma pathogenesis.

Analysis of the TCGA and CGGA datasets in Fig 1A & B showed higher TGM2 mRNA expression in the mesenchymal subtype in comparison to the other molecular subtypes which is consistent with literature (74). Correlations between TGM2 mRNA and the standard microglia/macrophage markers CD68 and Iba1 mRNA were assessed in Figure 2A & B. This showed high positive correlation which agrees with higher infiltration of GAMs in the mesenchymal subtype (37, 38). To further assess the association in gene expression of TGM2 with microglia/macrophages from the TCGA dataset, Enrichr was used. There was a very strong match with macrophages which suggests that macrophages are a major source of TGM2 in glioblastoma. TGM2 mRNA expression assessed from the scRNA seq data by Neftel *et al* (90) in Figure 4B showed that TGM2 is expressed in a subset of macrophages and mesenchymal glioblastoma. In the scRNAseq data, TGM2 clustered with CCR2 expression rather than CX3CR1 (Figure 4B). These markers distinguish macrophages from microglia, with CCR2 expression being restricted to the former. The clustering of TGM2 expression with CCR2 suggests that macrophages, rather than microglia, have the highest TGM2 expression. Since the

study by Neftel *et al*. performed a CD45 immune depletion step prior to scRNA sequencing, the total level of TGM2 mRNA expression between glioblastoma cells and macrophages cannot be compared. Analysis of TCGA bulk RNA-seq data does however indicate that macrophages are probably the largest source of TGM2 mRNA in glioblastoma tumours.

In order to study the expression of TGM2 in our cell culture model, we used PMA differentiated THP-1s and treated them with IL-4, INFγ, or TGFβ1. These cytokines were chosen based on their well-established roles in polarizing macrophages and previous literature indicating that they could regulate TGM2 expression (95). Consistent with previous literature (95), differentiated THP-1 cells showed stronger TGM2 expression in comparison to undifferentiated THP-1 cells which had nearly undetectable TGM2 expression (Figure 5B). Also consistent with previous literature (95), expression of TGM2 increased after 48 hr treatment with either IL-4 or INFγ (Figure 5B). Given that IL-4 has immune suppressive effects and INFγ has inflammatory effects, this suggests that TGM2 expression is associated with general macrophage activation, rather than with a specific polarization state. Treatment of differentiated THP-1 cells with TGFβ1, was also found to increase the expression of TGM2, consistent with previous literature (81). Finally, PriGO17As treated with or without TGFβ1 in Figure 7B showed no TGM2 expression. This is consistent with literature where the presence of TGM2 was observed in some but not all mesenchymal subtype glioblastoma cells (74). These Western blots also validate the TGM2 antibody to be used for the IHC studies described here.

Initial IHC of mesenchymal glioblastoma in a nude mouse xenograft model in Fig 8 showed us that mouse macrophages/microglia actively engaged the tumor, similar to what is observed in the human disease (37, 38). Based on this, IHC was performed on this xenograft model and on normal mouse brain for comparison (Figures 9A-E & 10A-E). TGM2 was present

at low levels in normal brain, but much darker TGM2 staining was evident in the xenograft. The darker staining was especially evident in areas of necrosis within the tumour. In normal brain, positive TGM2 staining was seen in endothelial cells, in pia mater mesothelial cells, and in ependymal cells lining the lateral ventricles. The presence of TGM2 in normal brain tissues raises the question of potential toxicities with TGM2 inhibition. However, studies from our collaborator Dr Keillor have shown negligible toxicity of TGM2 inhibitors in mice (74, 96, 97). Also, in a paper by Szondy et al (81), TGM2 null mice were observed to have defective clearance of apoptotic cells due to deficient activation of TGFβ1, and long-term effects such as autoimmunity, thymus involution, and immune complex glomerulonephritis. There were however no significant brain abnormalities reported for TGM2 knockout mice. We think that TGM2 is possibly playing a role in cross linking the extracellular matrix in the above-mentioned positively stained sites by transamidation. Since other transglutaminases were found to also have transamidation activities (75), we speculate that since the TGM2 knockout mouse did not have brain abnormalities, other transglutaminases are compensating for the absence of TGM2. Double immunofluorescence with Iba1 and TGM2 in Figure 11 further confirmed that TGM2 is being produced by GAMS. It is important to note that these cells are most likely macrophages since bioinformatic analysis of TGM2 mRNA expression suggested high positive correlation in macrophages.

IHC performed on human samples in Figure 12 showed similar staining patterns observed in our xenograft models. It is however important to note that areas of necrosis in the human model showed TGM2 expression to be more diffusely distributed in comparison to the nude mouse xenograft model. Further assessment of necrotic regions in whole sections of patient samples in Figure 13 (especially Figure 13B) resembled the dark TGM2 staining surrounding

areas of necrosis seen in our mouse model. Double immunofluorescence of whole sections from patient samples stained with CD68 and TGM2 in Figure 14 further confirmed that GAMs in our human model also express TGM2. The merged Z stack image in Figure 14 row C emphasized the intracellular co-localization of CD68 and TGM2 in macrophages. CD68 is known to be a protein that primarily localizes to endosomes/lysosomes in macrophages (98). Since TGM2 co-localizes with CD68, this could indicate that it is also present in endosomes/lysosomes, consistent with a role in efferocytosis. To confirm the localization of TGM2, additional fluorescence microscopy-based techniques or fractionation procedures can be done.

It is thought that thrombotic events in neovasculature cause regions of necrosis where macrophages will infiltrate in order to engulf dying/dead cells. Figure 15 shows these regions of necrosis in both the xenograft (Figure 15A) and human (Figure 15C & E) models probed with TGM2. Close up images of these regions of necrosis in both models (Figure 15B [mouse] and 15D & F [human]) showed microglia/macrophages infiltrating those regions and efferocytosing apoptotic cells. We assumed that these dying/dead cells are apoptotic and that the macrophages are efferocytosing them since morphologically apoptotic cells appear rounded and shrunken due to nuclear and cytoplasmic condensation (94). To confirm that the dying/dead cells in regions of necrosis are apoptotic, sections from the xenograft model in Figure 16 were probed with cleaved caspase 3. Figures 16A & B confirmed that the cells are indeed apoptotic, and the close-up image in Figure 16C showed a macrophage engulfing those apoptotic cells. It is important to note that the number of apoptotic bodies present in areas of necrosis in our nude xenograft model may appear to be more abundant than what is observed in the human model since nude mice lack Tregs which are known to have a role in enhancing macrophage efferocytosis (99).

Regarding efferocytosis in glioblastoma, as mentioned in the introduction, efferocytosis has been shown to play a role in the TME, metastasis, and progression of cancers (65, 67). We think that the generation of necrotic regions recruits macrophages to efferocytose apoptotic bodies which promotes an immunosuppressive phenotype in macrophages. This could help cancer cells evade immune surveillance and capitalize from the tolerogenic and immunosuppressive nature of efferocytosis to help support tumor progression. A paper by Tawil *et al* (100) showed that although glioblastomas actively generate vasculature, they also promote thrombotic events by overexpressing tissue factor (among multiple other mechanisms) which leads to the generation of necrotic regions and promotes cancer aggressiveness. From this, we speculate that it might be possible that glioblastomas are evading immune surveillance and maintaining their aggressive growth by balancing angiogenesis and apoptosis activated immunosuppression.

Based on the data presented, we think that TGM2 expressed by GAMs may be a good target for immunotherapy against glioblastoma. Studies done in animal models have shown that blocking MerTK driven efferocytosis alone or in combination with other immune checkpoint inhibitors (eg: anti-PD-L1) increased tumor immunogenicity and improved survival (67). In the previously mentioned mouse model by Szondy et al (81), inducing apoptosis in normal mice increased the levels of TGM2 which increases the activation of TGFβ1, and in turn increases the clearance of apoptotic cells by efferocytosis (positive feedback loop). As previously mentioned, since TGM2 is present in the brain under normal conditions, TGM2 knockout mice were devoid of brain abnormalities, and other transglutaminases also have transamidation activities, this would suggest that the function of TGM2 in these normal tissues is non-essential, meaning that the selective inhibition of TGM2 would be well tolerated. It is important to note that TGM2 in

efferocytosis is dependent on G-protein activities rather than transamidation activities (82) so using TGM2 inhibitors that only block GTP binding sites (*e.g*. NC9 developed by Dr. Keillor) rather than both sites may be preferable.

In order to confirm these speculations, cell-based screening assays could be done to observe the effects of TGM2 inhibitors on efferocytosis. These assays include: efferocytosis assay to visualize phagocytic and apoptotic cells and quantify efferocytosis, ELISA to measure immune-suppressive cytokines released by macrophages during efferocytosis (eg: TGF β1, IL-10, IL-13), RT-qPCR to measure gene expression of efferocytosis related genes like MerTK, PD-L1, and Arg-1, or flow cytometry for the objective measurement of efferocytosed apoptotic cells.

When it comes to glioblastoma patients, the standard treatment is a combination of radiation and temozolomide, which induces substantial cancer cell death. Given the role of TGM2 in the tolerogenic clearance of apoptotic cells (65, 67), we think that using TGM2 inhibitors in combination with radiation and temozolomide as a treatment may be able to promote tumour immunogenicity and help overcome the difficulties in providing effective immunotherapy for glioblastoma.

In conclusion, based on bioinformatics, cell culture experiments, IHC on mouse and human samples, and double immunofluorescence, I have been able to determine that TGM2 is predominantly expressed by glioblastoma-associated macrophages and that glioblastoma promotes TGM2 expression in these. Based on previous literature and the data presented here, TGM2 likely has an essential role in the efferocytosis of dying glioblastoma cells and the maintenance of an immunosuppressive tumour microenvironment. Future work will be done in cell culture to fully characterize macrophage efferocytosis and its effects on immune suppressive cytokine production in the absence and presence of TGM2 inhibitors. In addition, the effects of

TGM2 inhibitors on glioblastoma growth in immunocompetent mouse models of glioblastoma will be assessed. This research will help design further studies that will evaluate the use of TGM2 inhibitors as therapeutic agents for glioblastoma.

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Contribution of Collaborators

The contents of this manuscript were written by Mara Elgafarawi with editorial suggestions from Dr. Ian Lorimer. All of the experiments presented in this manuscript are the work of Mara Elgafarawi with the exception of IHC which was done by pathology core and Margarita Lui, and the tissue microarray courtesy of Dr. John Woulfe and Dr. David Munoz.

Appendix

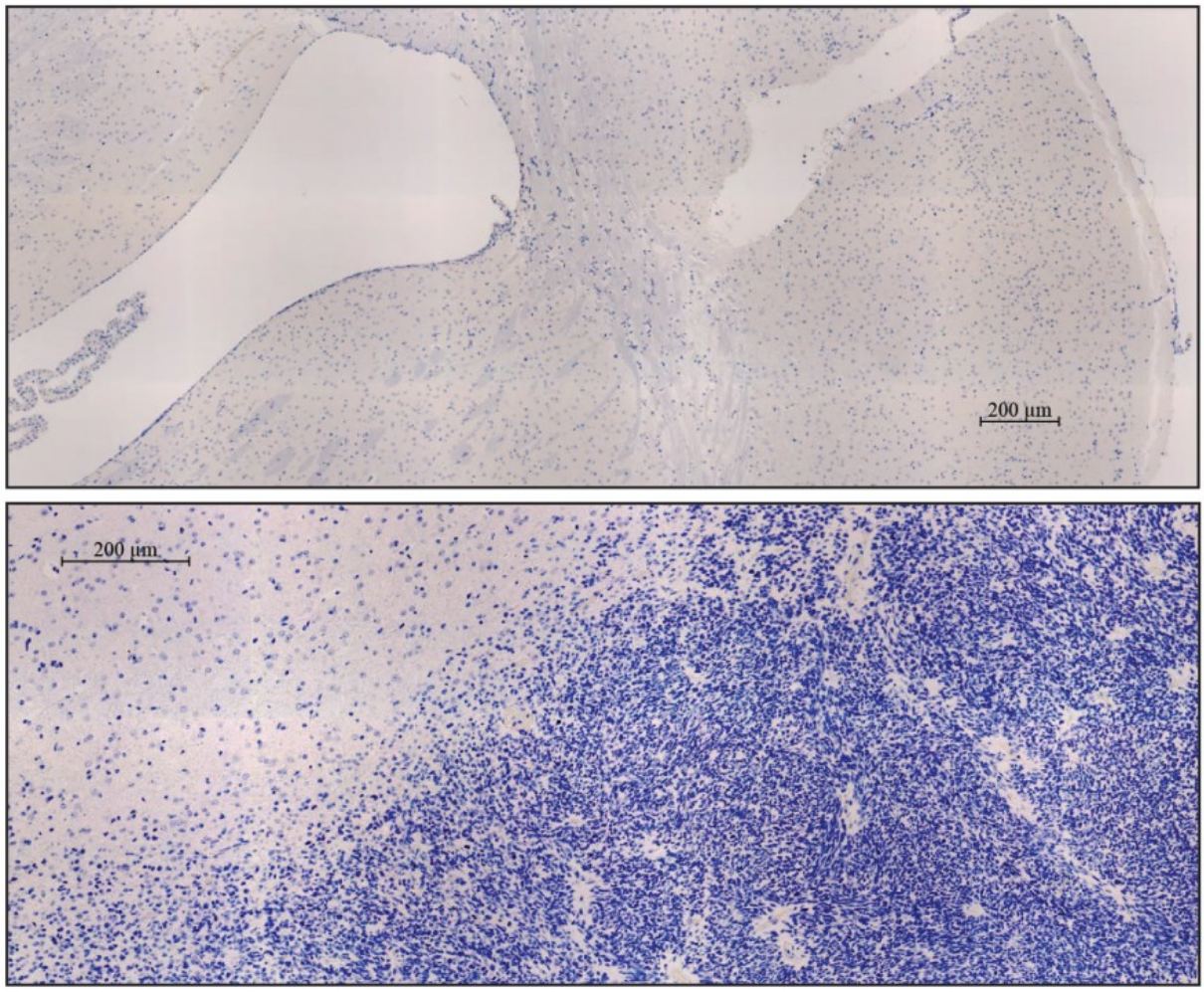

Figure S1 no primary and non-specific antibody controls in mouse xenograft model

TMA#	Core # (from scan)	SAMPLE ID	all negative	uniform positive cancer cell	endothelial	sporadic positive cells	focal positive	necrosis present?	comments
8	1	S19-9405 4A		n	y	y	n	n	
	2	S19-688 2B		y?	y	y	n	n	uniform positive, with sporadic very dark positive
	3	S19-688 2B		y?		y	y	n	weak uniform positive, with sporadic very dark positive
	4	S19-6232 1B		n	y	n	n	n	
	5	S19-6232 1B		n	y	n	n	n	
	6	S19-6017 C		n	y	n	n	n	
	7	S18-11377 8B		n	y	n	n	n	
	8	S18-11377 8B		n	y	n	n	n	
	9	S18-11075 2A		n	n	y	n	n	
	10	S18-1821 2A		n	y	y	y	n	
	11	S18-1821 2A		n	y	y	n	n	very few cells positive
	12	S18-2862 D		n	y	y	y	y	few positive
	13	S18-19332 2A		n	y	y	n	n	many endothelial, few dark sporadic
	14	S18-19332 2A		n	y	n	n	n	
	15	S18-16928 2A		n	y	y	y	n	large +ve focal patch containing sporadic very dark cells
	16	S19-3207 C		n	y	y	n	n	
	17	S19-4196 2A		n	y	y	n	n	
	18	S19-3207 C		n	y	y	n	n	
	19	S19-4196 2A		n	y	n	n	n	
	20	S19-6017 C		n	y	y	n	n	
	21	S19-901 B		y	y	y	n	n	weak positive cancer cells
	22	S18-11075 2A		n	y	y	n	n	
	23	S19-7928 3C		n	y	y	n	n	
	24	S18-2862 D		n	y	y	n	n	many sporadic postive w mphage morph
	25	S18-12174 A		n	y	y	y	y	weak focal positive staining
	26	S19-10153 A	y						
	27	S19-10153 A	y						
	28	S18-13439 2C		n	y	y	n	n	
	29	S19-9289 2A		n	y	y	n	n	
	30	S19-9289 2A		n	y	y	n	n	
	31	S18-13439 2C		n	y	y	n	n	
	32	S19-901 B	y						
	33	S18-11039 B		n	y	y	n	n	
	34	S19-7928 3C		n	y	y	n	n	
	35	S18-11039 B		n	n	y	n	?	
	36	S18-5272 A		n	y	y	n	n	
	37	S18-3314 2A		n	y	y	n	n	mostly endothelial
	38	S18-12174 A		n	y	y	n	n	very few sporadic
	39	S18-3314 2A		n	y	y	y	n	strong focal positive in vicinty of damaged blood vessel
	40			n	y	y	n	n	MARKER 1
	41		y						MARKER 2
	42	S19-9405 4A	y						
	43	S18-5272 A		n	y	y	y	?	low cell density

TMA#	Core # (from scan)		all negative	uniform positive cancer cell	endothelial	sporadic positive cells	focal positive	necrosis present?	comments
9	1	S18-19882 A		n	y	y	n	n	
	2	S18-21383 2A		n	y	y	n	n	
	3	S18-10905 A		n	y	y	n	n	
	4	S18-22710 4B		n	y	y	n	n	
	5	S18-22710 4B		n	y	y	n	n	
	6	S18-21731 5A		n	y	y	y	n	
	7	S18-10905 A		n	y	y	n	n	
	8	S18-22460 2A		n	n	y	n	n	a few faint positive cells
	9	S18-13710 2B		n	n	y	n	n	a few faint positive cells
	10	S18-13710 2B		n	n	y	n	n	sporadic very dark cells and extensive weaker staining
	11	S18-22238 2B		n	y	y	n	n	
	12	S18-12115 A		n	n	y	n	n	a very few faint positive cells
	13	S18-7198 3A	y						
	14	S18-7198 3A	y						
	15	S18-20382 2B		n	y	y	n	n	sporadic very dark cells
	16	S18-20382 2B		n	n	y	n	n	
	17	S18-22238 2B		n	y	y	n	n	
	18		y						MARKER 2
	19	S18-13681 2A		n	y	y	n	n	
	20	S18-13375 2B		n	y	y	n	n	all faint
	21	S18-13375 2B		n	y	y	n	n	all faint
	22	S18-1910 2A		n	y	y	n	n	all faint
	23	S18-1910 2A		n	y	y	n	n	
	24		y						MARKER 1
	25	S18-12553 2A		n	y	y	n	n	sparse, weak
	26	S18-12553 2A		n	n	y	y	?	
	27	S18-14101 A		n	n	y	n	n	sparse
	28	S18-22460 2A		n	y	y	n	n	sparse
	29	S18-13681 2A		n	y	y	n	n	sparse
	30	S18-8889 6A		n	y	y	n	n	
	31	S18-1004 2A		n	y	y	y	n	
	32	S18-8889 6A		n	y	y	n	n	sparse
	33	S18-12339 2A		n	y	y	n	n	
	34	S18-12339 2A		n	n	y	n	n	
	35	S18-9448 A		n	n	y	y	n	very focal
	36	S18-17530 2A		y?	n	y	y	n	sporadic very dark with mphage morphology
	37	S18-21731 5A		n	n	y	n	n	
	38	S18-17530 2A		n	n	y	y	n	small piece tissue
	39	S18-19882 A		n	y	y	n	n	

TMA#	Core # (from scan)		all negative	uniform positive cancer cell	endothelial	sporadic positive cells	focal positive	necrosis present?	comments
10	1			n	y	y	n	n	MARKER 1
	2			n	y	y	n	n	MARKER 2
	3	S18-12610 2A		n	y	n	n	n	
	4	S18-15965 2A		n	n	y	n	y?	
	5	S18-17755 2A		n	y	n	n	n	very little tissue
	6	S17-94 2B		y?	y	y	n	n	weak cancer cell, dark sporadic
	7	S17-2343 2A		n	y	y	n	n	dark sporadic
	8	S17-7262 3C		n	y	y	y	n	weak positive
	9	S17-7311 A		n	n	y	y	n	
	10	S17-4439 A		n	y	n	n	n	weak staining
	11	S17-14933 1A		n	y	y	n	n	sparse, faint sporadic
	12	S18-12610 2A		n	y	n	n	n	areas of faint diffuse staining
	13	S18-15965 2A		n	n	y	n	n	very weak, sparse sporadic
	14	S17-94 2B		n	n	y	n	n	dark sporadic, background of faint positive
	15	S17-2343 2A		n	n	y	n	n	faint sporadic
	16	S17-7262 3C		n	y	y	n	n	faint
	17								no tissue
	18	S17-14933 1A		n	y	y	n	n	mostly endothelial
	19	S18-12176 B		n	y	y	n	n	faint, sparse
	20	S17-4294 B		n	y	y	y	n	one strong focal area around bv
	21	S17-5356 2A	y						
	22	S17-3905 2A		n	n	y	y	n	
	23	S18-12176 B		n	y	y	n	n	mostly endothelial
	24	S17-4294 B		n	y	y	n	n	faint
	25	S17-5356 2A		n	n	y	n	n	dark sporadic, very little tissue
	26	S17-1371 13B		n	y	y	n	n	mostly medium sporadic
	27	S17-1371 13B		n	y	y	n	n	
	28	S17-8524 B		n	n	n	n	n	faint diffuse background staining
	29	S17-12038 2B		n	y	y	n	n	almost all endothelial
	30	S17-12038 2B		n	y	y	n	n	
	31	S17-13731 B		n	y	y	y	y	
	32	S17-13731 B		n	y	y	y	y	

TMA#	Core # (scan to 25)		all negative	uniform positive cancer cell	endothelial	sporadic positive cells	focal positive	necrosis present?	comments
11	1	S17-10305 2B		n	y	y	n	n	
	2	S17-18330 B		n	y	y	y	n	
	3	S17-18756 3A		n	y	y	n	n	mostly endothelial
	4	S17-10103 C		n	y	y	n	n	
	5	S17-12255 9A		n	y	y	n	n	
	6	S17-11023 2A		n	y	y	n	n	very faint
	7	S17-22218 1B		n	y	y	n	n	
	8								no tissue
	9	S17-10305 2B		n	y	n	n	n	
	10								no tissue
	11	S17-18756 3A		n	n	y	y	n	
	12	S17-10103 C		n	y	y	n	n	mostly endothelial
	13	S17-12255 9A		n	y	y	y	n	
	14	S17-9675 2A		n	n	y	n	n	
	15								very little tissue
	16	S17-22098 A		n	n	y	n	n	sporadic dark
	17	S17-17437 13B		n	y	y	n	n	mostly endothelial
	18	S17-9675 2A		n	y	y	n	n	
	19	S17-22098 A		n	y	y	n	n	
	20	S17-14567 3A		n	n	y	y	n	
	21	S17-19039 A		n	y	n	n	n	some very faint diffuse staining
	22	S17-11235 2A		n	y	y	n	n	mostly endothelial
	23	S17-17332 2C		n	y	y	n	n	faint
	24	S17-11235 2A		n	y	y	n	n	
	25	S17-17332 2C		n	y	y	n	n	mostly endothelial, sporadic faint
	26	S17-17837 2B		n	n	y	y	n	
	27	S17-17837 2B		n	n	y	y	n	no endothelial cells
	28	S17-18245 B		n	n	y	y	n	
	29	S17-18245 B		n	y	y	n	n	
	30	S17-19056 2A		n	y	y	n	n	
	31	S17-19056 2A		n	y	y	n	n	
	32	S17-11016 C		n	y	y	n	n	
	33								skip, counted twice
	34	S17-21863 2A		n	y	y	y	n	
	35	S17-11009 B		n	n	y	n	n	
	36	S17-11005 A		n	n	y	n	n	
	37	S17-10838 2B		n	n	y	n	n	very few positive
	38	S17-14567 3A		n	y	y	n	n	
	39	S17-21863 2A		n	y	n	n	n	
	40	S17-11009 B		n	y	y	n	n	
	41	S17-11005 A		n	y	y	n	n	
	42	S17-10838 2B		n	y	n	n	n	
	43	S17-17437 13B		n	y	n	n	n	
	44	S17-17457 19A		n	y	y	n	n	
	45	S17-20984 2C		n	y	y	n	n	
	46	S17-20127 A		n	y	y	n	n	
	47	S17-12835 2B		n	y	n	n	n	
	48	S17-20127 A		n	n	y	n	n	
	49	S17-12835 2B		n	y	y	n	n	

Figure S2 Scoring done for human glioblastoma tissue microarray taken from 83 patients