Stimulation of glioblastoma proliferation by macrophages through CD47/SIRPα

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Abstract

Glioblastoma is the most common and aggressive type of adult brain tumour with a need for new treatments. CD47 has been shown to be overexpressed on some human cancers and to interact with macrophages but its role in glioblastoma has not yet been fully explored. Here, we identify a novel role for the CD47/SIRPα interaction between glioblastoma and macrophages in that it can stimulate glioblastoma proliferation in co-cultures. Blocking either CD47 or SIRPα resulted in decreased glioblastoma proliferation. Furthermore, we show that macrophage stimulated glioblastoma proliferation is not occurring through downstream signalling of SIRPα but likely through CD47 to the PI3Kβ pathway. Initial results of co-cultures using glioblastoma cells which express CD47 that is GPI linked to the membrane, and therefore cannot signal downstream, support these findings. The implication of this research is the possibility to develop new therapies targeted at CD47 to decrease glioblastoma proliferation.

Acknowledgements

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List of Abbreviations

CSF-1	Colony stimulating factor – 1
GPI	Glycophosphatidylinositol
HLH	Hemophagocytic lymphohistiocytosis
HSC	Hemotopoeitic stem cells
Ig	Immunoglobulin
ITIM	Immunoreceptor tyrosine-based inhibitor motif
LPS	Lipopolysaccharide
MDSC	Myeloid-derived suppressor cells
MRI	Magnetic resonance imaging
NK	Natural killer
PI3K	Phosphoinositide 3-kinase
PriGO	Primary glioblastoma
RTK	Receptor tyrosine kinase
SH2	Src homology region 2
SIRPα	Signal regulatory protein alpha
TAM	Tumour associated macrophage
TSP	Thrombospondin
WHO	World Health Organization

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1 – Introduction

1.1 - Glioblastoma

1.1.1 - Glioblastoma – disease, prognosis and treatment

Glioblastoma is the most common and aggressive type of adult brain tumour. According to the World Health Organization (WHO) it is classified as a grade IV astrocytoma based on morphology and pathology (1). Grade IV is the most severe of the astrocytomas and is distinguished pathologically by its irregular nuclei, scant cytoplasm, increased cellularity, brisk mitotic activity, necrosis and microvascular proliferation (2).

Glioblastoma commonly presents with headaches, seizures and/or focal neurological deficits as a result of the tumour mass affecting normal brain function. It is diagnosed using magnetic resonance imaging (MRI) or computed tomography followed by pathological analysis of a biopsy (3). Standard initial treatment involves surgical resection and a combination of radiation with an oral chemotherapy called temozolomide (3). Following treatment, MRI is used for response assessment. Treatment is difficult due to the heterogeneity of the tumours, their invasive nature and the challenges associated with the blood brain barrier. A phase II trial of Temsirolimus, a small-molecule inhibitor of mTOR, in recurrent glioblastoma patients found only a minor improvement in time to progression (4). Some success has been found in a clinical trial testing a medical device that delivers low-intensity intermediate-frequency alternating electric fields to the tumour. Use of the device resulted in a benefit in progression free survival but not in overall survival (5). Despite advancements in

treatments such as surgical techniques, radiation and chemotherapy, glioblastoma is associated with a median overall survival of only one year and thus there is a clear need for new therapeutics.

Multiple cells of origin have been proposed for glioblastoma. It has been suggested that glioblastoma may arise from adult neural stem cells or multipotent neural progenitor cells that arise from the subventrical zone and the subgranular zone in the brain (6). Glioblastoma may also arise from more differentiated lineages such as oligodendrocyte precursor cells, astrocytes or mature neurons (7-8). Glioblastoma has the potential to originate from a variety of neural cells which may contribute to the heterogeneity of the disease.

Despite differences in cells of origin and genetics, a common feature shared by glioblastoma is its high invasive potential. Even radical neurosurgery where the whole hemisphere of the brain afflicted with glioblastoma was removed eventually lead to relapse in the opposite hemisphere (9). This suggests that some glioblastoma cells were able to invade into the other hemisphere. Even with modern microsurgery techniques, the diffuse border of glioblastoma makes complete resection impossible and recurrence frequently occurs.

Single glioblastoma cells can migrate throughout the brain travelling along Scherer's secondary structures; brain parenchyma, existing blood vessels, white matter tracts and the subarachnoid space (10). Neural precursor cells have been shown to migrate along white matter tracts and blood vessels suggesting a similar mechanism for

migration. This distinctive pattern of glioblastoma invasion distinguishes it from systemic tumours that have metastasized to the brain.

1.1.2 - Genetics

Traditionally it was thought that glioblastoma can arise through two different pathways leading to tumour types with distinct molecular genetic abnormalities. Secondary or type 1 glioblastoma are tumours that progress from lower grade astrocytomas, they often contain TP53 mutations and make up only 5% of glioblastoma cases (11). Primary or type 2 glioblastoma are the most common and arise rapidly *de novo* without evidence of a clinical precursor. Primary glioblastoma are characterized by high frequencies of EGFR amplification, PTEN loss and INK4A loss (11). Secondary glioblastoma have frequent PDGF/FGF amplification, p53 mutation, Rb loss, PTEN loss and IDH1 mutations (2).

Glioblastoma has been reclassified by molecular subtype based on RNA expression analysis of clinical samples led by The Cancer Genome Atlas (12). Initially four subtypes were identified: classical, mesenchymal, neural and proneural, however, further analysis revealed a fifth subtype, glioma-CpG island methylator phenotype (G-CIMP) (12-13). CIMP tumours have hypermethylation of CpG islands and tend to be secondary tumours with *IDH1* mutations (13). The G-CIMP subtype is associated with younger age and TP53 mutations. In the Proneural subtype alterations of *PDGFRA* are common (12). The neural subtype is classified based on the expression of neuron markers such as *NEFL, GABRA1, SYT1* and *SLC12A5* and most closely resembles

normal brain tissue (13). Chromosome 7 amplification paired with chromosome 10 loss is common in glioblastoma and was seen in 100% of the Classical subtype tumours (13). The classical subtype is further characterized by high level of EGFR amplification. Finally, the mesenchymal subtype is characterized by hemizygous deletions of 17q11.2, a region containing *NF1* and expression of mesenchymal markers such as *CHI3L1* and *MET* (12, 14). Although these are the typical characteristics of each subtype, these events are in no way mutually exclusive and there tends to be a lot of overlap and variation within a tumour. It was also found that the different subtypes respond to therapy differently, with CIMP having the longest survival time. This suggests that methods to classify tumours according to components that most strongly promote their growth may be beneficial in creating targeted therapeutic approaches to the disease.

1.1.3 - Heterogeneity

Tumour heterogeneity is a major challenge in the treatment and diagnosis of cancer. Similar to many cancer types, intertumoural heterogeneity can be observed in glioblastoma. Intertumoural heterogeneity is where tumours from different patients exhibit distinct genetic lesions and expression programs that lead to different outcomes and therapeutic responses. Furthermore, glioblastoma have also been shown to display intratumoural heterogeneity where cells from the same tumour contain different mutations or exhibit distinct phenotypic or epigenetic states (15). Through single cell analyses, it was found that cells within glioblastoma also display a spectrum of stemness and differentiation states (15). Receptor tyrosine kinases (RTK) have been

targeted in many therapeutics however, single cell analyses reveal a mosaic of RTK expression within a single tumour (15). This likely contributes to targeted therapy resistance in glioblastoma. For example, a therapeutic approach targeting the EGF receptor was a promising candidate given that EGFR overexpression is common in glioblastoma. However, EGFR inhibitors showed no significant benefit in glioblastoma clinical trials (16). Further signalling redundancy is also possible in that glioblastoma can use other signalling pathways to bypass RTKs altogether. Increased heterogeneity is associated with decreased survival, emphasizing the need to consider both intertumoural and intratumoural heterogeneity when developing new treatments.

1.1.4 - Primary cultures

This study uses glioblastoma cell cultures that were isolated from tumour samples of patients that underwent surgery at the Ottawa Hospital and are therefore referred to as primary glioblastoma cells or PriGO cells. The cells were grown on laminin coated culture flasks in serum-free neural stem cell media that was supplemented with growth factors and vitamins at 37°C and 5% O_2. Cells isolated and cultured under similar conditions have also been referred to as glioblastoma tumour-initiating cells and glioblastoma stem cells. These conditions preserve the genetic profile of the original tumour in culture as well as its invasiveness in intracranial xenografts (17-19). Another important characteristic of these primary cells is their potential to differentiate, mimicking the heterogeneity of glioblastoma. Primary glioblastoma cell cultures have been shown to express markers of neural stem cells including nestin,

SOX2 and vimentin (17-19). These cells are cultured in the absence of serum as serum has been shown to induce aberrant differentiation leading to the expression of multiple lineage markers as well as senescence (17, 20). Furthermore, the culture of primary glioblastoma cells in the presence of serum results in a loss of invasive potential, both *in vitro* and *in vivo,* leading to the formation of well circumscribed tumours (17). Culturing primary glioblastoma cells in this way preserves the heterogeneity and phenotypic characteristics of the cells and provides a powerful model to study their behaviour.

1.2 - Macrophages and Cancer

Tumour immune surveillance has been of interest in recent years and much has been done to study the role of the adaptive immune system. More recently, the innate immune system and the role of macrophages has been of interest in regulating tumour immunity. The observation of macrophages in tumours has been known for quite some time but until recently the experimental focus has been on malignant cells. The following subsections provide the relevant information on macrophages.

1.2.1 - Macrophage Polarization

Macrophages are polarized on a spectrum from M1 to M2. M1 or classically activated macrophages participate in the coordinated response to immunogenic antigens primarily through production of proinflammatory cytokines (such as TNF-α, IL-1 and IL-12), upregulation of cell surface molecules necessary for antigen presentation and an enhanced ability to phagocytose pathogenic material (21). M1 macrophages

originate upon encounter with IFN-γ and/or microbial stimuli such as lipopolysaccharide (LPS). They are characterized by high IL-12 production and activation of a T-cell response, cytotoxic activity against phagocytozed microorganisms and neoplastic cells, high expression of reactive oxygen intermediates and good capability as an antigen presenting cell (22). In general, M1 macrophages can be considered soldiers; they defend the host from viral and microbial infections, fight against tumours, produce high amounts of inflammatory cytokines and activate the immune response (23).

M2 or alternatively activated macrophages release immunosuppressive cytokines (such as IL-10, IL-6 and TFG-β), downregulate cell surface molecules necessary for antigen presentation and have decreased phagocytic capacity (24). M2 macrophages can be stimulated with IL-4 and IL-13, immune complexes or with IL-10 and glucocorticoids (25). The hallmarks of M2 macrophages are IL-10 high, IL-12 low, secretion of CCL17 and CCL22, high expression of mannose, scavenger and galactose-type receptors, poor antigen-presenting capability and promotion of wound-healing (22). M2 macrophages can be considered workers of the host; they promote scavenging of debris, angiogenesis and remodelling and repair of damaged tissues. The loss of equilibrium of M1 and M2 cell number may lead to pathological events. For instance, an excess of M1 macrophages could induce chronic inflammation whereas an increase in M2 macrophages could promote severe immune suppression (22). While the M1 and M2 phenotypes provide a useful way to characterize macrophages, it is important to emphasize that these cells are plastic and can exist anywhere on the M1 to M2 spectrum.

1.2.2 - Tumour Associated Macrophages

The tumour microenvironment consists of not only cancer cells but of many non-malignant resident cell types such as macrophages (26). It is becoming increasingly clear that the tumour microenvironment plays a role in promoting the tumour to its full neoplastic phenotype. Tumour associated macrophages (TAMs) have pivotal roles in the progression and metastasis of tumours.

In normal tissues, pathogens or wounds result in the local expression of growth factors that recruit circulating monocytes and stimulate them to differentiate into macrophages. The macrophages then mediate the immune response. Tumours release similar growth factors and chemokines to recruit macrophages to the tumour microenvironment (27). A correlation has been shown that increased TAM density leads to poorer prognosis for many cancer types (27).

TAMs have a pro-neoplastic role by influencing aspects of tumour biology such as; production of molecules that affect cell growth, enhancement of angiogenesis, modulations of the inflammatory response and catalysis of structural changes of the extracellular matrix (28). The pro-angiogenic role of TAMs is highlighted by the correlation between increased TAM numbers and high vascular grades in many tumours including, glioma, squamous cell carcinoma of the esophagus, breast, bladder and prostate carcinoma (22). TAMs also aid in tumour dissemination by promoting invasion characteristics of malignant cells and by making their movement easier by a direct action on the tumour microenvironment (29). TAMs exert strong immune suppressive activity, not only by producing IL-10 but also through the secretion of

chemokines, which preferentially attract a subset of T-cells that are devoid of cytotoxic functions (such as Tregs) (30). Moreover, these chemokines are inducible by IL-4, IL-10 and IL-13, creating a M2-mediated immunosuppressive loop (22).

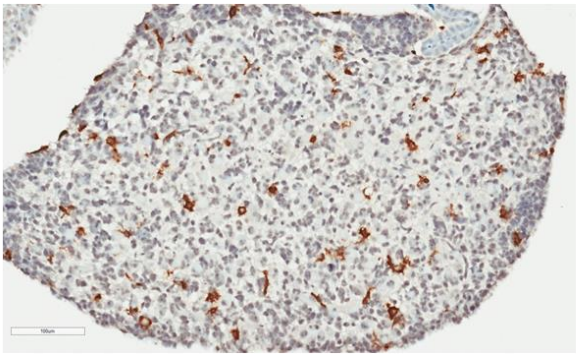
There is increasing evidence that TAMs play an important role in tumour development and thus these cells have been considered an attractive target for anti-cancer therapies. There are currently a few anti-macrophage approaches that are under evaluation pre-clinically. In a breast cancer murine model, it was found that malignant cells recruit macrophages through CCL5 (31). Treatment of murine breast cancers with Met-CCL5 (a receptor antagonist) led to a decreased number of infiltrating macrophages and this was associated with a significantly reduced tumour size (31). Furthermore, the anti-tumoural drug Trabectin (Yondelis) has immunomodulatory properties on mononuclear phagocytes. It has been shown that this drug showed a selective cytotoxic effect on monocytes and macrophages while sparing the lymphoid subset (32). Recent studies have shown interest in using macrophages as natural vectors to deliver therapeutic molecules to the tumour, since they are able to infiltrate tumours spontaneously. Intratumoural injection of macrophages transduced with an IL-12 recombinant adenoviral vector in a prostate cancer model led to the reduction of primary tumour growth and decreased lung metastases with a higher number of tumour infiltrating CD4+ and CD8+ T-cells compared to control (33). This evidence supports the role macrophages have in promoting the tumour microenvironment.

1.2.3 - Macrophage/microglia infiltration of glioblastoma

TAMs in glioma are believed to originate from two distinct sources. Microglia are the resident macrophages of the brain and are believed to monitor their local neural tissue through extensive ramifications (34). Microglia are a distinct lineage that arise from the embryonic yolk sac (35). The second source of macrophages comes from the peripheral bone marrow-derived mononuclear cells. The monocytes exit the blood stream and differentiate into macrophages once they enter the tissue. It is still not known how to differentiate the lineage of origin of the two types of macrophages so phenotypic and functional differences are unknown (21).

Multiple studies have found that microglia and macrophages are able to infiltrate gliomas (36, 37). In xenotransplantion of PriGO cells microglia/macrophages stained with Iba1, a microglia/macrophage specific calcium-binding protein, are found within the tumour (figure 1.1A). Furthermore, macrophages/microglia can be seen migrating towards to the tumour (figure 1.1B). The microglia/macrophage population is often the dominant glioma-infiltrating immune cell making up anywhere from 30-50% of the cell population in gliomas (38). There are a multitude of factors that result in microglia/macrophage chemoattraction such as the release of chemokines and other factors. One such chemokine is monocyte chemoattractant protein-1 (MCP-1) or CCL2 which has been shown to increase microglial infiltration and is associated with more aggressive gliomas (39). Some other chemoattractive factors that are released by glioma cells are hepatocyte growth factor and scatter factor (40).

A.



B.

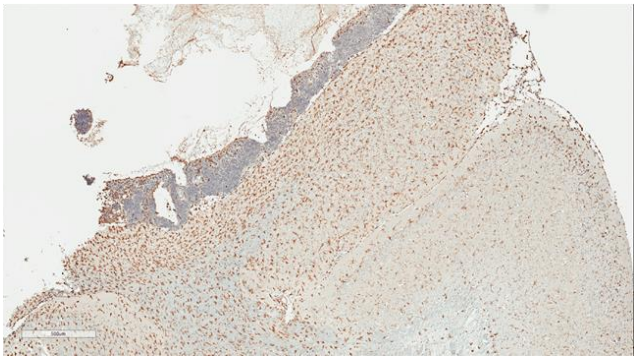


Figure 1.1. Macrophages/microglia migrate towards and infiltrate PriGO xenotransplantations. (A) Iba1 stained macrophages/microglia are found in PriGO8A cell growths. (B) Iba1 stained macrophages/microglia are seen at a higher density surrounding PriGO17A tumour. Immunohistochemistry experiments were completed by Manijeh Daneshmand.

Research suggests that TAMs within malignant gliomas tend to be the immunosuppressive M2 macrophages (41, 42). Reduced TNF-α expression by TAMs was observed during late stages of tumour growth, consistent with an M2 phenotype (42). Human monocytes co-cultured with glioblastoma cells have reduced CD80 and HLA-DR expression which are necessary for T-cell activation (41). Furthermore, glioma TAMs have been shown to be deficient in phagocytosis. Monocytes conditioned with glioma cells isolated from patients undergoing craniotomy had reduced ability to phagocytose bacterial cell wall particles compared to astrocyte-conditioned and unconditioned monocytes (43). These findings suggest the glioma tumour microenvironment is able to re-educate macrophages into an immunosuppressive phenotype which supports tumour progression.

There has been increasing evidence that microglia/macrophages are able to promote glioma growth and migration. One study found that mouse microglia/macrophages increased the motility of murine glioma cells (GL261) by 3 fold whereas endothelial cells and oligodendrocytes only weakly promote glioma motility (44). Microglia release factors such as stress-inducible protein 1 (STI1), epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) which act to increase glioma proliferation and migration (45-47). It is becoming increasingly evident that TAMs are recruited to the evolving tumour via secretion of chemokines and then interact with the tumour environment to promote the glioma invasion and growth. However, it is still not clear exactly which molecules are responsible for the interactions between glioma and microglia/macrophages and further characterization is needed.

1.3- CD47/SIRPα – protein and interactions

1.3.1 - Integrin associated protein (CD47)

CD47, also known as integrin associated protein was originally discovered as a plasma membrane molecule that co-purified with the integrin αvβ3 from leukocytes and placenta (48). Since then, it has been shown to be associated with many different families of integrins and it can modulate their activity. Furthermore, CD47 has been shown to interact with signal regulatory protein alpha (SIRPα) and thrombospondin (TSP).

CD47 is a cell surface marker that is expressed on many normal cells in most tissues at low levels. It is a member of the immunoglobulin superfamily of membrane proteins. CD47 contains five transmembrane regions, a single IgV-like domain at its N-terminus and a short cytoplasmic tail (49). The Ig domain is required for CD47 interaction with its associated integrins (αvβ3 and α2β1) and its ligands TSP and SIRPα. The expression of CD47 varies according to immune status or disease. For example memory T cell progenitors are associated with high CD47 levels in order to prevent them from being phagocytized (50). Similar things can be observed with hematopoietic stem cells (HSC) and progenitors which increase their expression of CD47 once mobilized by cytokine induction in order to prevent phagocytosis.

CD47 can interact with two families of proteins, TSP and SIRP. The major secreted ligand for CD47 is TSP1 however, there are a few other members of the thrombospondin family that are able to bind to CD47 but with a much lower affinity (51). Two members of the signal regulatory protein family are receptors for CD47. SIRPα has

defined signalling functions whereas SIRPγ lacks a cytoplasmic domain and is thought to signal indirectly through lateral associations with other membrane proteins (52). Lateral interactions between CD47 and SIRPα have also been suggested and proteolytic shedding of the extracellular domain of SIRPα may provide a second soluble ligand for CD47 (53). Direct binding studies have demonstrated that TSP1 inhibits SIRPα binding to cells expressing CD47 (51). This suggests with competitive binding of TSP1 and SIRPα to a single site on CD47, steric inhibition of binding to distinct but proximal sites or allosteric inhibition (54). The CD47/SIRPα binding structure has been crystallized but the location of the TSP binding site remains unclear (55).

It has been suggested that CD47 may be able to interact with G proteins. It was found that antibodies to the G protein alpha subunit could immunoprecipitate affinity-labeled CD47 and monoclonal antibodies against CD47 could immunoprecipitate the alpha and beta G protein subunits (56). The treatment of cells with pertussis toxin eliminated the co-immunoprecipitation of CD47 and Gi (56). The five transmembrane segments of CD47 along with the two transmembrane segments of its associated integrin could form a seven transmembrane spanning complex that may function in G protein activation (figure 1.1). Different integrin partners and/or CD47 isoforms may be able to influence the ability of the complex to activate specific heterotrimeric G proteins (48).

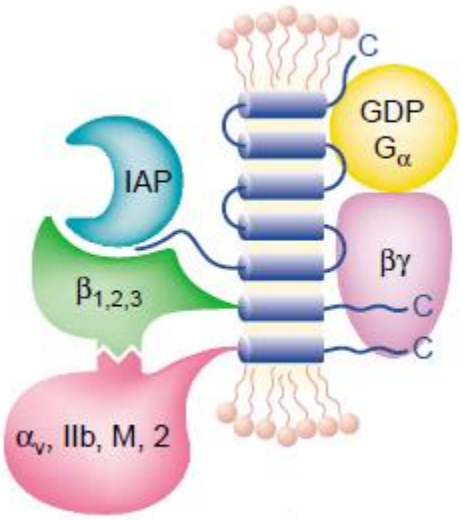

C
GDP
G_α
IAP
βγ
$\beta_{1,2,3}$
C
C
α_v, IIb, M, 2

Figure 1.2. CD47 interaction with G-proteins. It is speculated that CD47 can associate with one of its integrins, forming a typical seven transmembrane segment capable of activating a heterotrimeric G protein. Image modified from (48).

1.3.2 - Signal regulatory protein alpha (SIRPα)

SIRPα was originally identified as a highly expressed glycoprotein in the brain (57). Further studies revealed that it is a membrane protein found mainly on myeloid and neural cells such as: monocytes, most populations of tissue macrophages, granulocytes, some populations of dendritic cells and some bone marrow progenitor cells (58). The levels of SIRPα on macrophages appear quite stable and do not seem to be affected by inflammatory conditions (59). SIRPα contains three Ig-like domains in the extracellular region, a single transmembrane region and a cytoplasmic region that contains four tyrosine residues with immunoreceptor tyrosine-based inhibitory motifs (ITIM) (60). Upon phosphorylation of the tyrosine residues, the ITIM mediates the recruitment and activation of Src homology region 2 (SH2) domain containing tyrosine phosphatases SHP-1 and SHP-2 (58). Tyrosine phosphorylation of the cytoplasmic region of SIRPα is mediated by various growth factors and cytokines as well as by integrin mediated cell adhesion to extracellular matrix proteins (58). SHP-1 is predominantly expressed in hematopoietic cells and acts to dephosphorylate the phosphoprotein substrates and thereby affects intracellular signalling pathways, most often in an inhibitory fashion (61). SHP-2 is expressed in most cell types and regulates the small guanosine triphosphate (GTP)-binding proteins Ras and Rho, contributing to the positive control of cell growth and migration (62, 63). SIRPα acts as a docking protein to recruit SHP-1 or SHP-2 to the cell membrane in response to external stimuli, these phosphatases are important for downstream signalling.

SIRPα is part of a paired receptor family containing: inhibitor receptor (SIRPα), activating receptor SIRPβ1, non-signalling receptor SIRPγ as well as some other proteins that have not yet been characterized (64). There exists variability of SIRP genes across different species however, SIRPα and SIRPβ1 are present in both mammals and chickens (64). One of the main setbacks in determining the expression of paired receptors is that many reagents including monoclonal antibodies may cross react with other closely related members of the family (65).

1.3.3 - CD47/SIRPα interaction

The Ig-like domain of CD47 interacts with the NH_2–terminal domain of SIRPα. The interaction of human SIRPα and CD47 is of low affinity which is common for interactions between leukocyte surface proteins (66). Binding of SIRPα by CD47 promotes tyrosine phosphorylation of SIRPα in macrophages which recruits SHP-1 and SHP-2 and promotes the activation of Rho (figure 1.2) (67). Less is known about the downstream signalling pathway of CD47 suggesting possible unknown roles for CD47/SIRPα in tumour/macrophage interactions (figure 1.2). The mechanism in which the CD47/SIRPα complex is removed from the cell surface is still unclear. A probable mechanism is endocytosis of the CD47/SIRPα complex by either cell. A study showed that CD47 expressed on the surface of cultured mouse hippocampal neurons undergoes *trans*-endocytosis by neighbouring astrocytes expressing endogenous SIRPα in order to terminate the cellular responses triggered by the interaction (68).

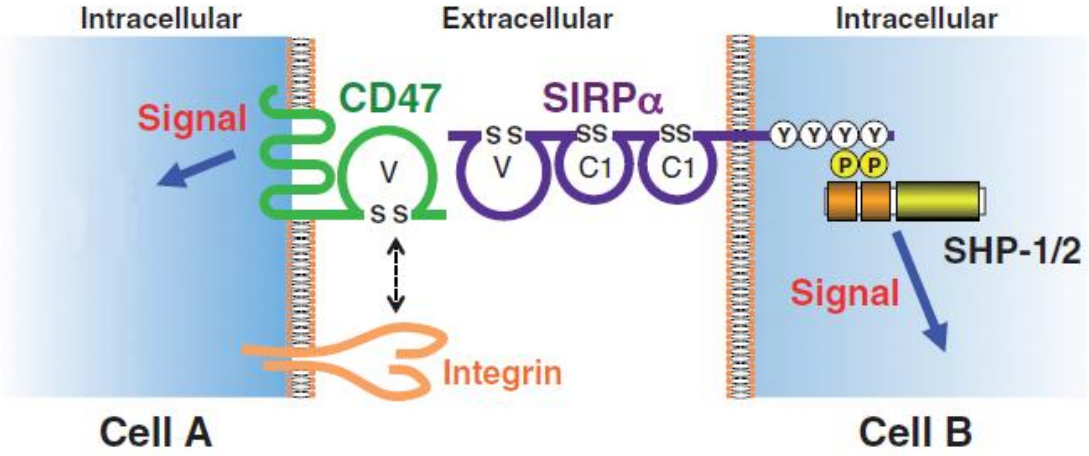

Intracellular
Extracellular
Intracellular
Signal
CD47
V
S S
SIRPα
S S
V
SS
C1
SS
C1
Y Y Y Y
P P
SHP-1/2
Signal
Integrin
Cell A
Cell B

Figure 1.3. Model of the CD47/SIRPα interaction. CD47 binds SIRPα which leads tyrosine phosphorylation and activation of SHP-1/SHP-2. Downstream signalling of CD47 is still unclear. Image modified from (69).

SIRPα is highly polymorphic whereas the ligand binding domain of CD47 is nonpolymorphic (70). However, it has been shown that several different SIRPα alleles all bind CD47 with similar affinity (71). This is expected as most of the polymorphisms in SIRPα result in changes in surface residues that are distantly adjacent from the binding site and are therefore unlikely to affect ligand binding.

One of the better characterized roles of the CD47/SIRPα interaction is host cell phagocytosis. The binding of CD47 to SIRPα on macrophages leads to inhibition of phagocytosis through inhibition of myosin accumulation at the submembrane assembly site of the phagocytic synapse which is required for particle internalization (72). When CD47 binds SIRPα, Src family kinases promote ITIM phosphorylation which enhances phosphatase recruitment to a phosphotyrosine site on non-muscle myosin II and inhibits its activity thereby limiting phagocytosis (72). For this reason, CD47 is often termed a “don’t eat me signal” and a marker of self. Early evidence for this role came from the infusion of CD47-deficient erythrocytes into wild-type mice which were cleared much faster than their wild-type counterparts (73). It was also shown that the phagocytosis of CD47-deficient red blood cells by isolated splenic macrophages from wild-type mice was enhanced *in vitro* (73). Based on the involvement of CD47-SIRPα interactions in phagocytosis, deregulation of CD47 and/or SIRPα expression in disease could disturb homeostasis and cause altered host blood cell clearance and thereby contribute to pathogenesis of blood disorders. This is observed during exacerbations of hemophagocytic lymphohistiocytosis (HLH) where CD47 is selectively downregulated on HSCs which renders the cells prone to phagocytosis by macrophages (74). The

decrease in CD47 expression on HSCs contributes to the dramatic reduction in the number of HSCs on the bone marrow of HLH patients. It is clear that CD47 binds to SIRPα, leading to inhibitory signalling but the signalling pathways downstream of CD47 are still unclear.

1.3.4 - Cancer

Various cancers from both hematologic and non-hematologic origin have been shown to express relatively high levels of CD47 compared to their normal counterparts, with the highest levels found on cancer stem cells (75). It has also been demonstrated that CD47 is expressed on many solid tumour types (76). Higher CD47 expression levels correlated with poor prognosis and higher resistance to chemotherapy (75). It is hypothesized that CD47 over-expression on tumours allows them to escape immune surveillance through evasion of phagocytosis. This occurs through the binding of CD47 on tumour cells to SIRPα on macrophages which inhibits phagocytosis and promotes tumour survival. This was observed when forced expression of CD47 in a CD47-deficient myeloid leukemia cell line lead to aggressive dissemination and death in xenografted mice compared to the minimal engraftment in the CD47 deficient cells (77). Because downstream signalling of CD47 is not perfectly understood, there may be other potential mechanisms by which CD47 can promote tumour growth.

The use of blocking antibodies to inhibit the CD47-SIRPα pathway is beginning to be explored as a cancer therapeutic. One study found that incubating tumour cells and macrophages with either an anti-CD47 antibody or an anti-SIRPα antibody leads to

phagocytosis of the tumour cells where a non-blocking antibody had no effect (75). Furthermore, they were able to show *in vivo* that the elimination of the tumour cell via the blocking CD47 antibody was due to phagocytosis as treatment with anti-CD47 antibody in mice that were depleted of phagocytes did not show any antitumour effects (75). A tumour-eliminating effect has been observed in non-hematopoietic solid tumours treated with anti-mouse CD47 (76). However, if blocking inhibitory signalling through SIRPα on macrophages was sufficient to activate innate tumour immunity, deletion of the cytoplasmic tail of SIRPα in host mice should decrease tumour growth but this was not observed (65). This suggests that the mechanism of action of these antibodies is not fully understood and further investigation is needed.

Anti-CD47 antibodies may also play a role in eliminating tumour cells through Fc-dependent mechanisms such as; antibody-dependent cellular cytotoxicity and complement dependent cytotoxicity. A study showed that a particular anti-CD47 antibody was able to induce NK cell mediated cytotoxicity against head and neck cancer cell lines (78). Human and mouse NK cells express very little to no SIRPα so it can be concluded that the effects observed was not due to the blockage of the CD47-SIRPα interaction. Thus far there have not been many reports on antibody-dependent cellular cytotoxicity and complement dependent cytotoxicity. These mechanisms are likely dependent on the specific anti-CD47 antibody IgG isotype as each isotype has different abilities to stimulate cytotoxicity. Although the CD47/SIRPα remains a promising target for cancer therapeutics, more work needs to be done in determining the mechanistic data involved in this interaction.

1.4 - Study Rationale

Macrophages are able to infiltrate the glioblastoma tumour microenvironment and can be re-educated to promote tumour growth. This makes the tumour/macrophage interaction highly attractive to study as a potential therapeutic target. For this study we isolated glioblastoma cells from patient tumours (primary glioblastoma cell cultures) which are the most accurate model of glioblastoma as they replicate the genetic and histopathological characteristics of the original patient's tumour. Additionally, primary monocytes were used to generate macrophages in order to create a clinically relevant model. CD47 expressed on glioblastoma can interact with SIRPα expressed on macrophages. Some roles for the CD47/SIRPα interaction have been explored, such as phagocytosis, but because of the unknown downstream signalling pathways of CD47, more research must be done to better understand the interaction. The CD47/SIRPα tumour/macrophage interaction may prove to be a novel and effective target to improve survival in glioblastoma.

1.5 - Hypothesis

I hypothesize that macrophages stimulate glioblastoma proliferation through the CD47/SIRPα, specifically through downstream signalling of CD47.

1.6 - Objectives

1. Establish experimental conditions for the co-culture of primary glioblastoma cells and primary macrophages.
2. Examine the effects of macrophage and glioblastoma cell co-culture on glioblastoma cell proliferation.
3. Investigate the role of CD47/SIRPα on macrophage stimulated glioblastoma proliferation.

2 - Materials and Methods

2.1 Antibodies and chemicals:

CD47 mouse monoclonal antibody was from Invitrogen (Carlsbad, CA, USA). CD200 mouse monoclonal antibody was from R&D Systems (Minneapolis, MN, USA). SIRPα/β (CD172) mouse monoclonal antibody was from BioLegend (San Diego, CA, USA). Cetuximab was from Bristol-Myers Squibb (New York, NY, USA). GAPDH mouse monoclonal antibody was from Abcam (Cambridge, MA, USA). STAT6 and pSTAT6 were from Cell Signalling Technologies (Danvers, MA, USA).

The following inhibitors were used in this study: NSC87877 (Tocris Bioscience, Minneapolis, MN, USA), AZD8186 and BYL719 (Cayman Chemical, Ann Arbor, MI, USA).

2.2 Cell culture:

PriGO cultures: PriGO cultures were isolated following a protocol approved by the Ottawa Hospital Research Ethics Board as described previously (79). Cells were grown on laminin-coated plates in Neurobasal A medium supplemented with B27, N2 (all from Life Technologies, Burlington, ON, Canada), EGF and FGF2 (Peprotech, Rocky Hill, NJ, USA) at 37°C in 5% O_2/CO_2.

Macrophage cultures: Whole blood was collected from healthy volunteers and separated into its components using Lymphoprep (STEMCELL Technologies, Vancouver, BC, CA). Monocytes were isolated from blood using CD14 MicroBeads (Miltenyi Biotec, Auburn, CA, USA). Monocytes were differentiated into macrophages by

culturing them in RPMI-1640 medium (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 10% Human AB serum (Thermo Fisher Scientific, Waltham, MA, USA) and 10ng/mL CSF-1 (R&D Systems, Minneapolis, MN, USA) for 7 days.

2.3 Cell counts:

Cell counts were analyzed by the Invitrogen Countess instrument (Carlsbad, CA, USA) or by haemocytometer using trypan blue exclusion for viable cells.

2.4 Co-cultures:

PriGOs were plated on laminin-coated 96 well plates at a density of 5000 cells per well and allowed to adhere overnight. Macrophages were harvested by cell scraping and stained with 10µM Cell Tracker Red (Invitrogen, Carlsbad, CA, USA) for 20-30 minutes. Following staining, macrophages were added to the 96 well plate at a density of 5000 cells per well. Co-cultures took place in PriGO media supplemented with 10ng/mL CSF-1 to support macrophages. The plate was scanned and cells were counted using Cellomics ArrayScan (Thermo Fisher Scientific, Waltham, MA, USA).

2.5 Western blotting:

Western blotting was performed as described previously (80). After electrophoretic transfer from the gel, membranes were stained with amido black to confirm that equal sample loading and transfer was achieved. Chemiluminescence from

HRP conjugated secondary antibodies was detected with the Alpha Innotech Fluorchem system (Santa Clara, CA, USA). Cerebral cortex protein medley was purchased from Clontech (Mountain View, CA, USA).

2.6 Immunofluorescence:

Immunofluorescence microscopy was performed as described previously (81). Cells were grown in 6-well dishes containing glass coverslips pre-coated with laminin. Cells were washed in cold PBS and fixed in cold 4% paraformaldehyde for 30 minutes. Cells were washed in PBS 3 times for 5 minutes and then blocked in 5% normal goat serum for 30 minutes at room temperature. Cells were incubated with the primary antibody for 1 hour at room temperature and then washed 3 times for 5 minutes in PBS. Cells were then incubated with secondary antibody for 1 hour at room temperature and washed 3 times for 10 minutes in PBS. Coverslips were mounted on slides with Prolong gold with DAPI (Invitrogen, Carlsbad, CA, USA) and allowed to air dry overnight in the dark. Slides were then sealed with mounting media. Analysis was carried out in ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.7 Plasmid Constructs

Full length cDNA encoding CD47 was amplified from PriGO8A cells. PCR was used to add a DNA sequence for a GPI anchor to a DNA sequence for the extracellular domain of CD47. Refer to appendix 1 for the final sequence. For constitutive lentiviral

vector production, GPI anchored CD47 was subcloned into pLeti-CMV-Puro using BamH1/Sal1 restriction sites.

Transduction with lentiviral vectors: Replication-incompetent lentiviral particles were made using the four-plasmid transfection methods described previously (82). PriGO cells were transduced by incubation with lentivirus-containing supernatant added to regular media for 24h and selected for with 0.5ug/mL puromycin (Sigma-Aldrich, Oakville, ON, Canada).

2.8 Statistics

Comparisons between two groups were performed using two-tailed t-tests with a p value < 0.05 considered significant.

3 – Results

Aim 1: Establish co-culture conditions and a labelling system for PriGO cells and macrophages

In order to study the interactions between macrophages and primary glioblastoma cells, conditions for their co-culture were established. Primary glioblastoma cells were obtained from patients undergoing surgery at the Ottawa Hospital. Monocytes were obtained from blood samples of healthy volunteers and were isolated magnetically using CD14 MicroBeads (MACS). Approximately 2 million monocytes were isolated from 15mL of blood, consistent with the manufacturer's report (83). Cells were cultured in RPMI media with human serum, penicillin and streptomycin. Differentiation of monocytes into macrophages was induced with the addition of 10ng/mL colony-stimulating factor (CSF-1) and replenishing media every 2 days for one week (figure 3.1). This results in M_0 macrophages according to standardized nomenclature and experimental guidelines (84).

To successfully create co-cultures, the macrophages must be able to live in the same conditions as the PriGO cells (Neurobasal A media supplemented with B27, N2, EGF and FGF2 at 37°C in 5% O_2/CO_2). Culturing macrophages in the PriGO media resulted in cell death with very few cells remaining after 5 days (figure 3.2A). Supplementing the media with 10ng/mL CSF-1 resulted in macrophages survival after 5 days although some cell death still occurred (figure 3.2B). The addition of 10ng/mL CSF-1 had no effect on PriGO cell growth.

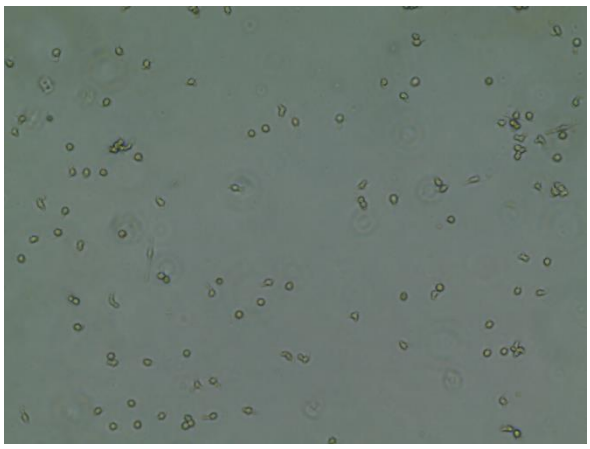


Figure 3.1. Monocytes differentiated into macrophages by addition of CSF-1. Monocytes isolated from whole blood using CD14 MicroBeads were differentiated into macrophages by adding 10ng/mL CSF-1 and replenishing media every 2 days for one week.

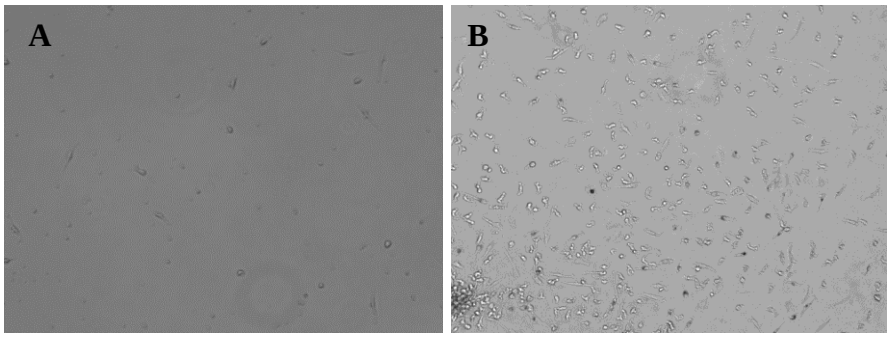

A
B

Figure 3.2. Monocytes grown in Neurobasal A media. (A) Monocytes cultured in Neurobasal A media showed cell death after 5 days. (B) Addition of CSF-1 to the media resulted in favourable culture conditions and cell survival at 5 days.

In order to be able to easily identify a macrophage versus a PriGO cell in co-culture, a dual labelling system was established. Macrophages were chemically labelled with 10μM CellTracker^{TM} Red (Life technologies) for 20-30minutes (figure 3.3A). Cell Tracker^{TM} Red is a stable, non-toxic fluorescent probe that passes through the cell membrane and reacts with thiol groups. Since macrophages are not dividing, the CellTracker^{TM} Red will remain concentrated in the cell throughout its life. Multiple concentrations and incubation times were tested to find the optimal conditions for probing macrophages. PriGO cells were labelled with eGFP through lentiviral transduction (figure 3.3B). These experiments have established the conditions necessary for the co-culture and identification of macrophages and PriGO cells.

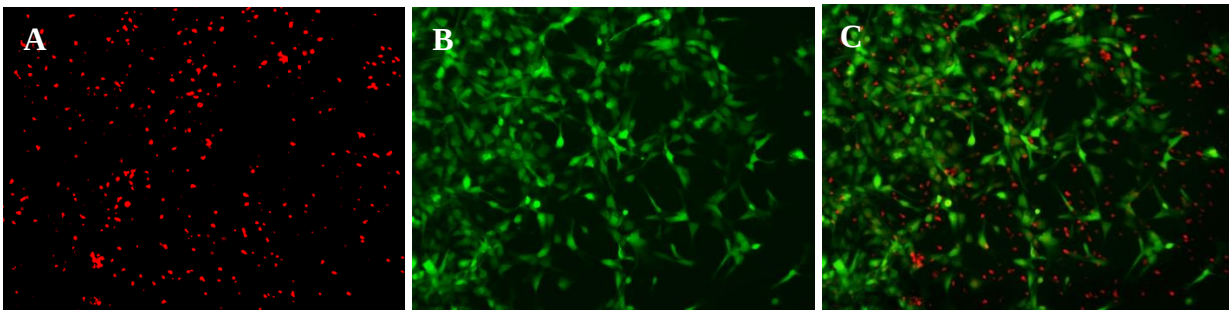

A
B
C

Figure 3.3. Macrophages labelled with Cell Tracker Red. (A) Macrophages were incubated with 10μM CellTracker^{TM} Red for 30 minutes at 37°C. No sign of diminished labelling was apparent after 9 days in culture. (B) PriGO 8A cells labelled with eGFP. (C) Merge.

Aim 2 – Examine the effects of macrophage and glioblastoma cell co-culture on glioblastoma proliferation

To study glioblastoma/macrophage interactions under a range of conditions simultaneously, co-culture experiments were conducted in 96 well plates. Co-cultures of PriGOs and macrophages were followed using a Cellomics ArrayScan instrument (Figure 3.4A and B). The Cellomics ArrayScan scans each plate and provides an image of each well, following the scan, image analysis tools are used to quantify the number of cells per well. The PriGO8A cell line was used for initial experiments. PriGO8As are primary glioblastoma cells acquired from a patient undergoing surgery at the Ottawa Hospital. Images of the raw data clearly show that co-culture of PriGO8A and macrophages resulted in increased PriGO8A proliferation compared to control (figure 3.4C-E). Quantification of cell numbers using the ArrayScanner confirmed that macrophages stimulate PriGO8A proliferation (figure 3.4E).

Co-culture experiments were then repeated with the addition of EGF, a growth factor typically added to the PriGO culture media that had been previously eliminated due to potential reactivity with antibodies. Although PriGO8A proliferation in the absence of macrophages increased, macrophage stimulated PriGO8A proliferation was similar to what was observed in the absence of EGF (figure 3.5).

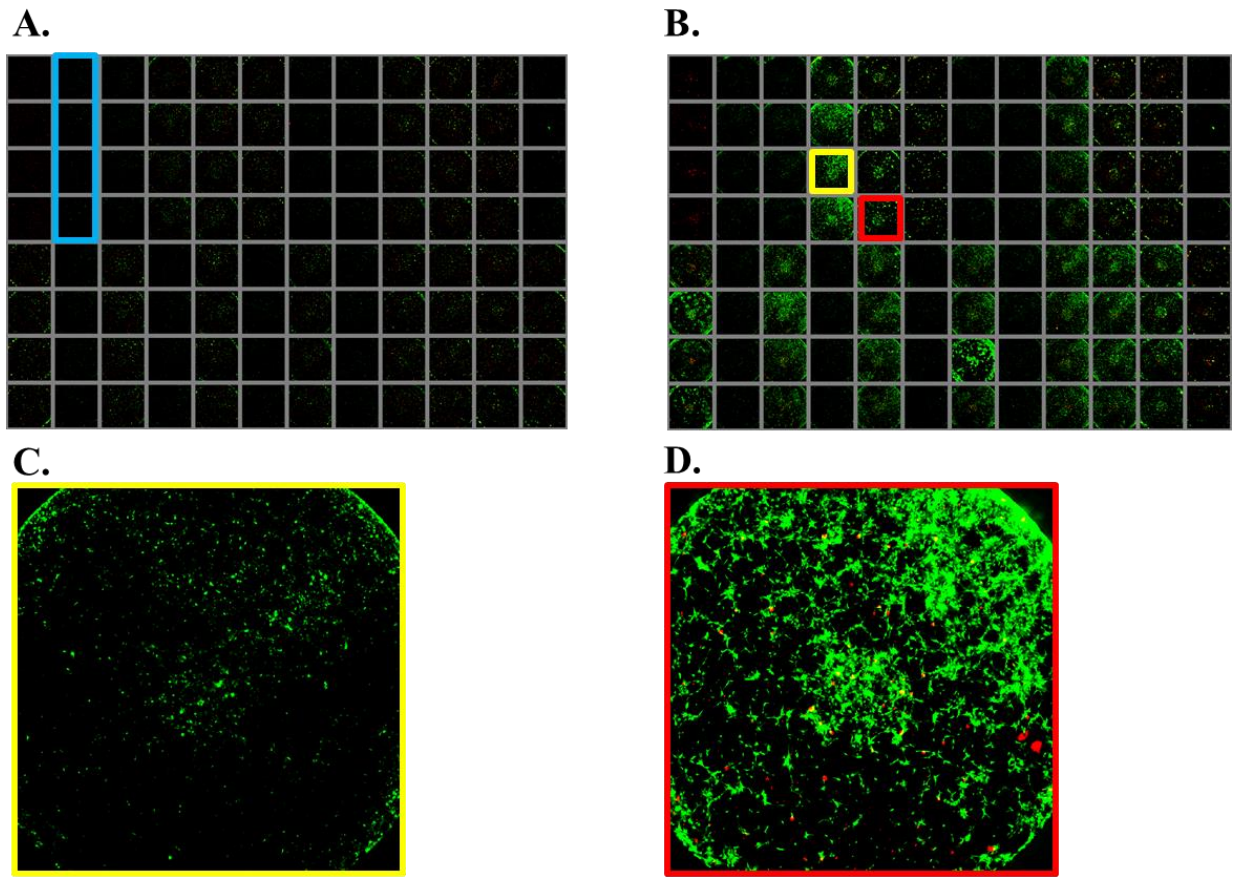

A.
B.
C.
D.
E.
3000
2500
2000
1500
1000
500
0
GFP
8 -EGF +CSF1
8 -EGF +CSF1 +mac
*
0
2
4
24
48
72
96
120
Time (h)

Figure 3.4. Co-culture of PriGO8A and macrophages. (A) Example of Array Scan of 96 well plate at 24h showing GFP-labelled PriGO8A and Cell Tracker Red labelled macrophages. Each condition is repeated in quadruplicate as represented by the blue rectangle. (B) Example of Array Scan at 120h, visible PriGO8A growth is observed in many conditions. Yellow square represents the well in figure C and red square represents the well in figure D. (C) Example of one well of PriGO8A after 120h. (D) Example of one well of PriGO8A co-cultured with macrophages after 120h. (E) Co-culture of macrophages and PriGO8A increases glioblastoma growth compared to control, shown as an example of 5+ similar experiments. Error bars represent standard deviation. Macrophage stimulated PriGO8A growth is significantly different than PriGO8A growth after 24h. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.

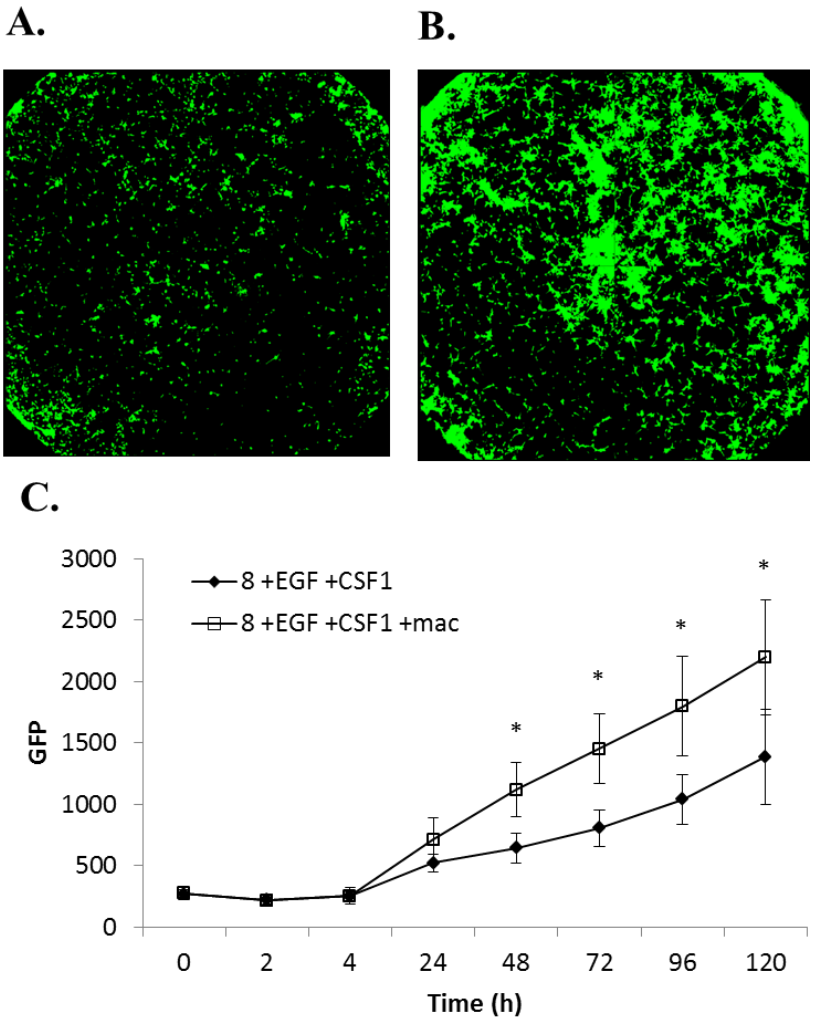

A.
B.
C.
3000
2500
2000
1500
1000
500
0
GFP
8 +EGF +CSF1
8 +EGF +CSF1 +mac
*
*
*
*
0
2
4
24
48
72
96
120
Time (h)

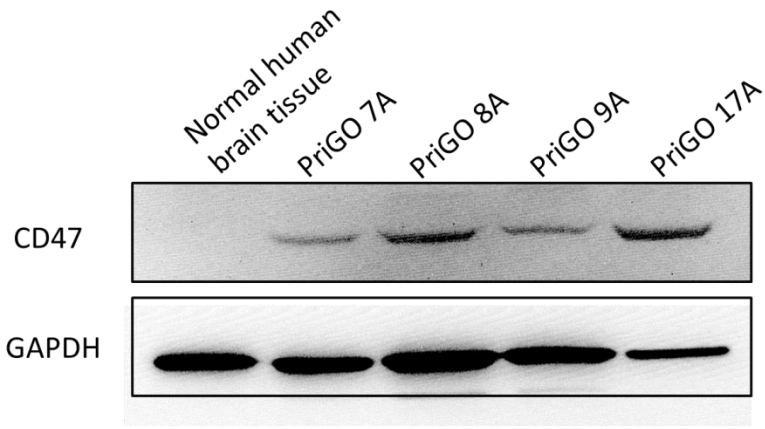
Figure 3.5. Co-culture of PriGO8A and macrophages in the presence of EGF. (A) Example of one well of PriGO8A after 120h. (B) Example of one well of PriGO8A co-cultured with macrophages after 120h. (C) Co-culture of macrophages and PriGO8A increases glioblastoma growth compared to control, shown as an example of 5+ similar experiments. Error bars represent standard deviation. Macrophage stimulated PriGO8A growth is significantly different than PriGO8A growth after 48h. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.

Aim 3 – Investigate the role of CD47/SIRPα in macrophage stimulated glioblastoma proliferation

To determine the role of the CD47/SIRPα interaction in macrophage stimulated glioblastoma cell proliferation CD47 expression on PriGO cells was analyzed. Western blot revealed that CD47 is expressed in all PriGO cell lines at higher levels than normal human brain tissue (figure 3.6A). CD47 expression was further evaluated by immunofluorescence on non-permeabilized cells which confirmed that CD47 is expressed on the cell surface of PriGO cells (figure 3.6B).

To determine whether the increase in glioblastoma growth is occurring through the CD47/SIRPα interaction, a blocking anti-CD47 antibody was added to the co-culture. Addition of the anti-CD47 antibody resulted in prevention of the macrophage stimulated PriGO8A growth (figure 3.7A). To confirm this result a blocking anti-SIRPα antibody was added to the co-culture. Addition of the anti-SIRPα antibody decreased macrophage stimulated glioblastoma growth in a dose dependent manner (figure 3.7B). This indicates that the CD47/SIRPα interaction between glioblastoma and macrophages plays a role in macrophage stimulated glioblastoma proliferation.

A.


Normal human brain tissue
PriGO 7A
PriGO 8A
PriGO 9A
PriGO 17A
CD47
GAPDH

B.

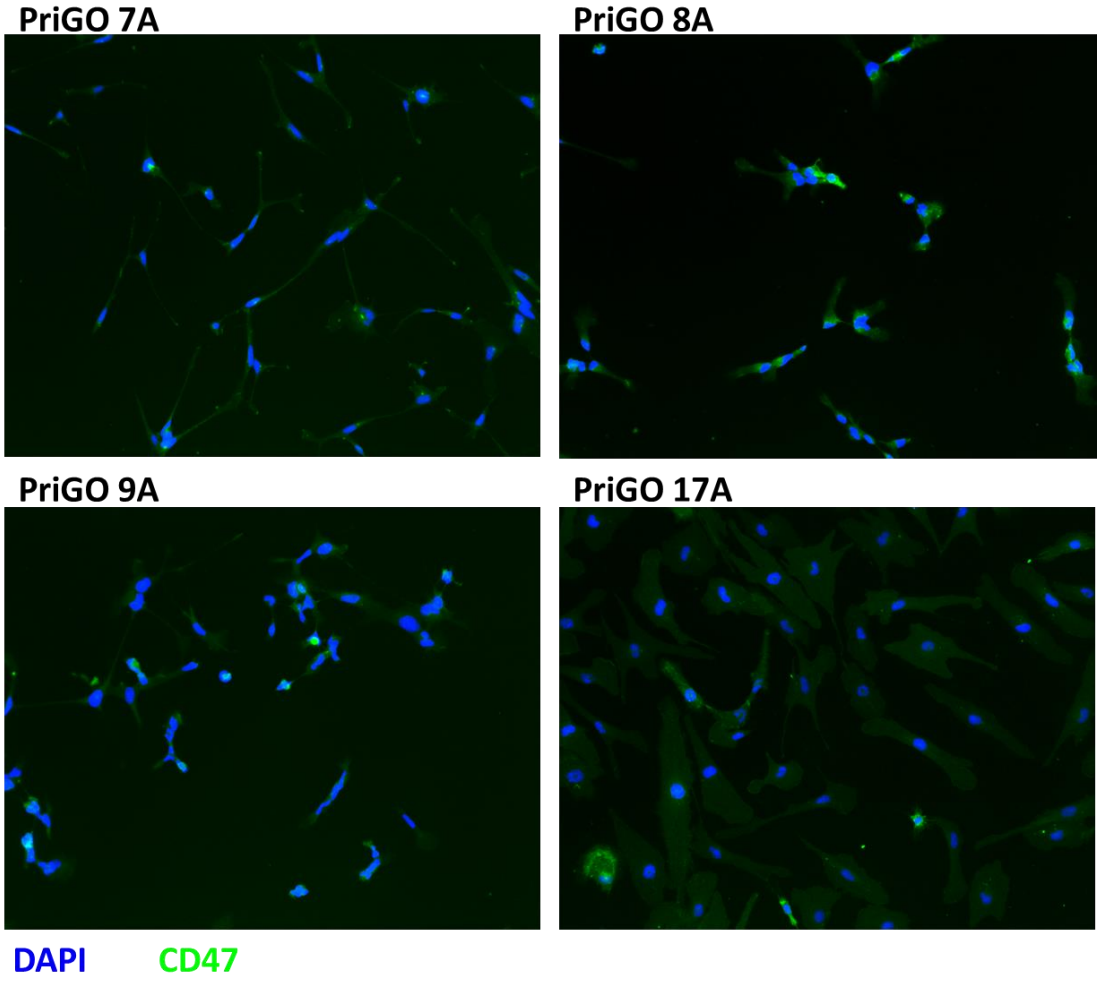
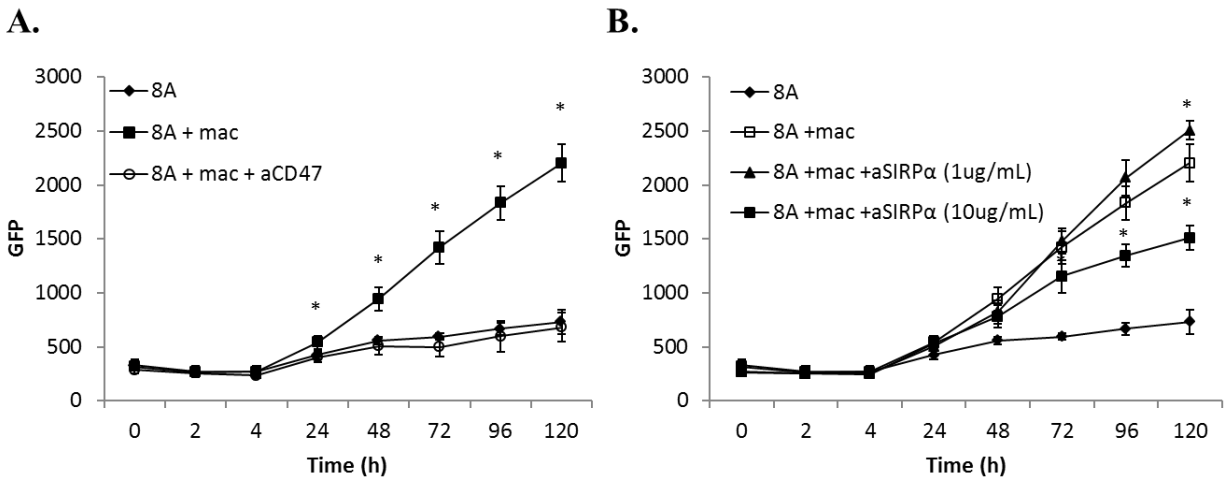

PriGO 7A
PriGO 8A
PriGO 9A
PriGO 17A
DAPI
CD47

Figure 3.6. CD47 expression on PriGO cells. (A) Total cell lysates were analyzed by Western blotting for expression of CD47. (B) Immunofluorescence microscopy was performed for CD47 on PriGO cells.


A.
B.
3000
2500
2000
1500
1000
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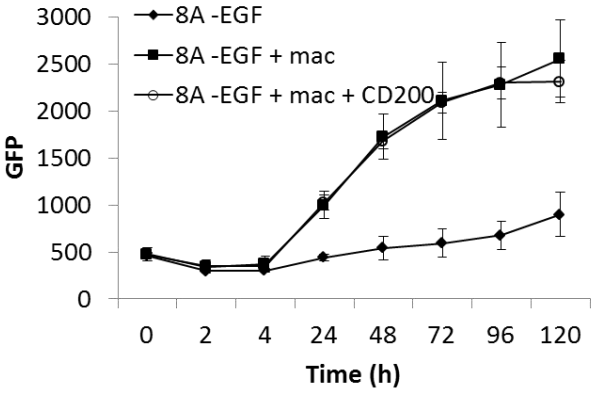
0
GFP
0
2
4
24
48
72
96
120
Time (h)
8A
8A + mac
8A + mac + aCD47
8A +mac
8A +mac +aSIRPα (1ug/mL)
8A +mac +aSIRPα (10ug/mL)
*

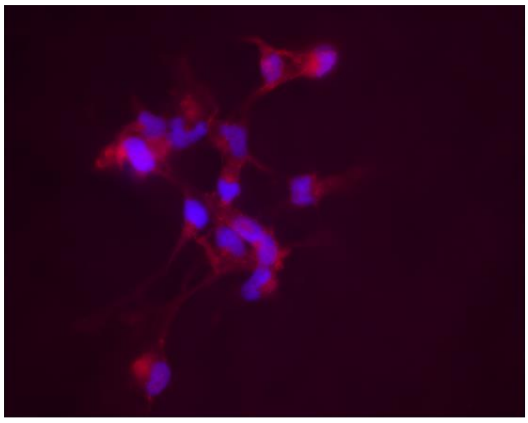
Figure 3.7. Macrophage stimulated glioblastoma cell growth is dependent on the CD47/SIRPα interaction in PriGO8A. (A) Addition of 10ug/mL of anti-CD47 blocks macrophage stimulated cell growth, shown as an example of 3 experiments. Macrophage stimulated PriGO8A proliferation is significantly different than co-cultures treated with anti-CD47 after 24h. (B) Inhibition with anti-SIRPα blocks macrophage stimulated cell growth in a dose dependent manner, shown as an example of 3 experiments. There is a significant difference between macrophage stimulated PriGO8A proliferation and co-cultures with 10ug/mL anti-SIRPα after 48h. Error bars represent standard deviation. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.

Next, control experiments were performed to ensure that the observed reduction in macrophage stimulated PriGO8A proliferation through the addition of anti-CD47 or anti-SIRPα was not a general antibody effect. CD200 is an IgG1 antibody with low binding affinity to PriGO cells. Addition of CD200 to co-cultures resulted in no change in macrophage stimulated PriGO8A proliferation (figure 3.8A). The use of antibodies can result in opsonization of the cell and render it susceptible to FCγR mediated phagocytosis by the macrophages. To ensure that the observed effects of anti-CD47 were not due to FCγR activation, cetuximab, a humanized IgG1 antibody which binds the EGFR receptor on PriGO cells and activates the FCγR on macrophages was added to co-cultures. First, binding of cetuximab to PriGO8A was confirmed with immunofluorescence (figure 3.8B). Addition of cetuximab to co-cultures had very little effect on macrophage stimulated PriGO8A proliferation (figure 3.8C). These experiments illustrate that the observed effects of macrophages on PriGO8A proliferation are CD47/SIRPα specific and not due to a general antibody effect or due to activation of the FCγR.

A.



B.



DAPI Cetuximab

C.

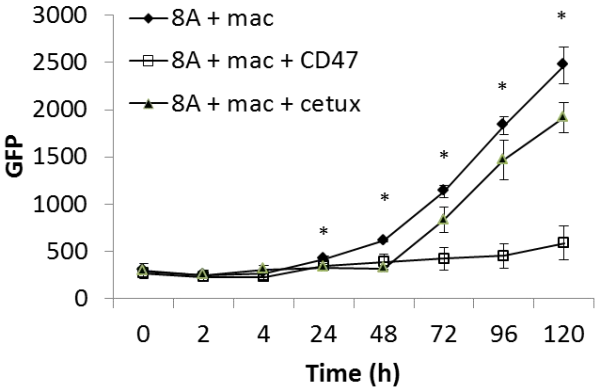
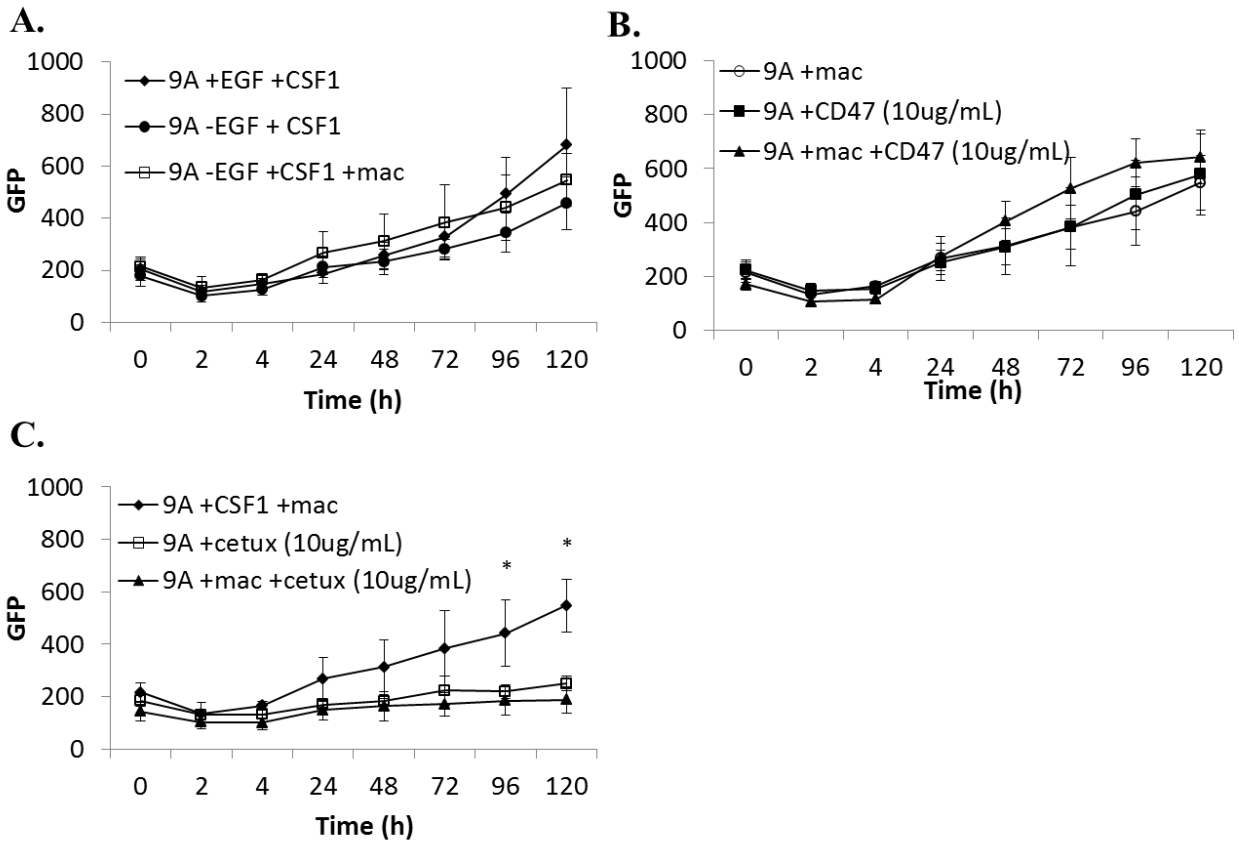


Figure 3.8. Antibody controls. (A) Addition of CD200, an IgG1 antibody, to co-cultures of macrophages and PriGO8As resulted in no significant change in macrophage stimulated PriGO8A proliferation. Graph shown as an example of 3 experiments. (B) Immunofluorescence on PriGO8A cells showing cetuximab binding to the cell. (C) Addition of cetuximab, an EGFR binding antibody which activates the FCγR, to co-cultures had little effect on macrophage stimulated PriGO8A proliferation compared to anti-CD47. Graph shown as an example of 3 experiments. Although adding cetuximab significantly decreased PriGO8A proliferation, the decrease is not comparable to anti-CD47. Error bars represent standard deviation. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.

Co-culture experiments were repeated with PriGO9A, another primary glioblastoma cell line. It was observed that the addition of macrophages to PriGO9As had no effect on cell proliferation (figure 3.9A). Furthermore, the addition of anti-CD47 had no effect on the PriGO9A (figure 3.9B). The addition of cetuximab, which binds to the EGFR, resulted in decreased PriGO9A proliferation both in the presence or absence of macrophages (figure 3.9C). This suggests that PriGO9As are more reliant on the EGFR signalling pathway for proliferation. Previously, it has been shown that amplification of EGFR is higher in PriGO9As compared to PriGO8As consistent with the idea that PriGO9As rely on EGFR signalling for proliferation.

Next, co-culture experiments were repeated in PriGO17A. No difference in proliferation was observed after the addition of macrophages (figure 3.10A). Interestingly, addition of anti-CD47 resulted in decreased PriGO17A proliferation in the presence and absence of macrophages (figure 3.10B). Microarray data has shown that the PriGO17A have higher levels of thrombospondin which is a ligand for CD47. Therefore, it is possible that TSP-1 binds CD47 which promotes cells proliferation and blocking this interaction with anti-CD47 reduces proliferation.


A.
1000
800
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400
200
0
GFP
9A +EGF +CSF1
9A -EGF + CSF1
9A -EGF +CSF1 +mac
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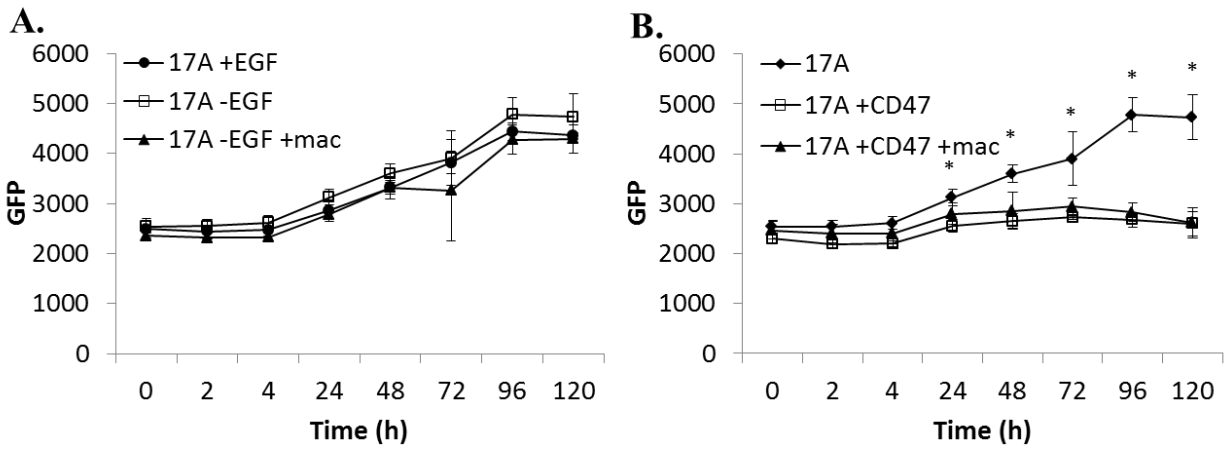
48
72
96
120
Time (h)
B.
1000
800
600
400
200
0
GFP
9A +mac
9A +CD47 (10ug/mL)
9A +mac +CD47 (10ug/mL)
0
2
4
24
48
72
96
120
Time (h)
C.
1000
800
600
400
200
0
GFP
9A +CSF1 +mac
9A +cetux (10ug/mL)
9A +mac +cetux (10ug/mL)
*
*
0
2
4
24
48
72
96
120
Time (h)

Figure 3.9. PriGO9A co-cultures. (A) Co-culture of PriGO9A and macrophages had no significant effect on PriGO9A proliferation. (B) Addition of anti-CD47 to co-cultures had no significant effect on proliferation. (C) Addition of cetuximab (a blocking EGFR antibody) resulted in decreased PriGO9A proliferation. PriGO9A proliferation in co-culture with cetuximab was significantly different from PriGO9A co-culture proliferation after 96h. Figures are an example of 3 similar experiments. Error bars represent standard deviation. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.


A.
6000
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1000
0
GFP
17A +EGF
17A -EGF
17A -EGF +mac
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96
120
Time (h)
B.
6000
5000
4000
3000
2000
1000
0
GFP
17A
17A +CD47
17A +CD47 +mac
*
*
*
*
*
0
2
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24
48
72
96
120
Time (h)

Figure 3.10. PriGO17A co-cultures. (A) Co-culture of PriGO17A and macrophages had no significant effect on PriGO17A proliferation. (B) Addition of anti-CD47 to cultures resulted in reduced proliferation. PriGO17A proliferation was significantly decreased when cultured with anti-CD47 after 24h. Figures are an example of 3 similar experiments. Error bars represent standard deviation. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.

In order to determine whether macrophage stimulated PriGO8A cell growth was occurring through downstream signalling of CD47 or SIRPα, a SHP1/2 inhibitor was added to co-cultures. To verify that the SHP1/2 inhibitor is functioning in macrophages, macrophages were treated with 20ng/mL IL-4 for 48h which is known to reduce pSTAT6 expression through SHP1/2. A second group of macrophages was treated with 500nM of the SHP1/2 inhibitor for 48h before treatment with IL-4 which should block the reduction in pSTAT6 expression. Western blot analysis of cell lysates revealed that treatment with the SHP1/2 inhibitor reduced pSTAT6 reduction after treatment with IL-4. STAT6 was used as a control (figure 3.11A). This illustrates that the SHP1/2 inhibitor is effective in macrophages. Addition of the SHP1/2 inhibitor to co-cultures resulted in no change in glioblastoma cell growth suggesting that the stimulated proliferation occurs through CD47 (figure 3.11B).

It has been suggested that CD47 downstream signalling occurs through G proteins (48). One way that G proteins are able to stimulate proliferation is through activation of phosphinositide 3-kinase beta (PI3Kβ) via the βγ subunit (48). To investigate the role of PI3K in macrophage stimulated glioblastoma cell proliferation, selective inhibitors of PI3Kα and PI3Kβ were added to co-cultures. Addition of either inhibitor decreased PriGO8A growth. Addition of the PI3Kβ resulted in a decrease in macrophage stimulated glioblastoma cell growth (figure 3.12A). Addition of the PI3Kα inhibitor resulted in no change in macrophage stimulated glioblastoma cell growth (figure 3.12B). These data suggest that CD47 may be signalling through PI3Kβ but not PI3Kα. This provides support that CD47 may signal through Gβγ as PI3Kβ and not PI3Kα is a downstream signalling molecule of Gβγ.

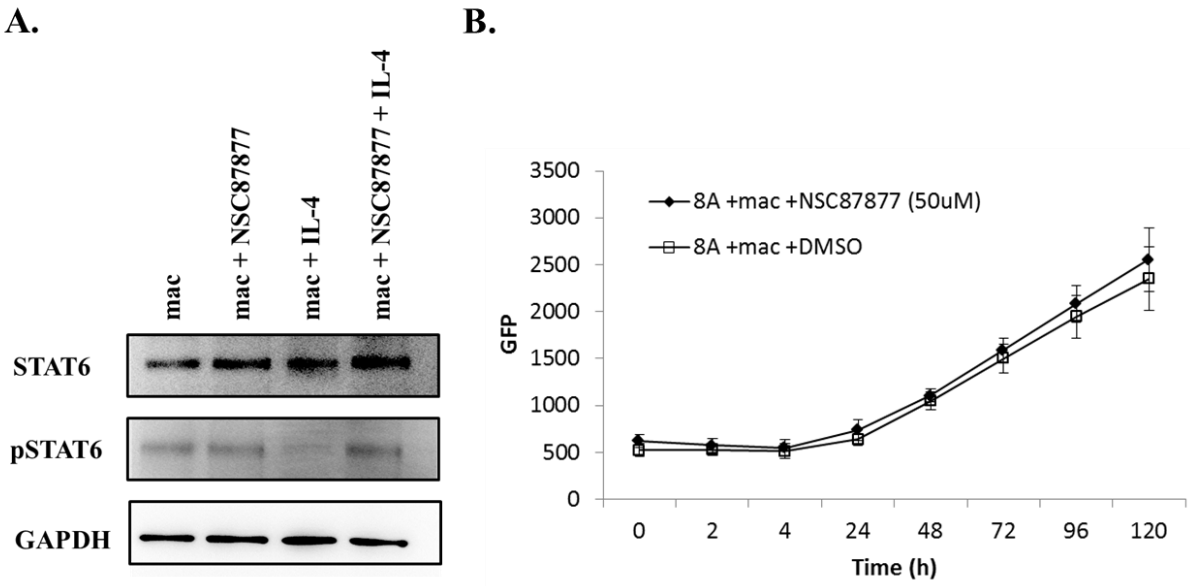
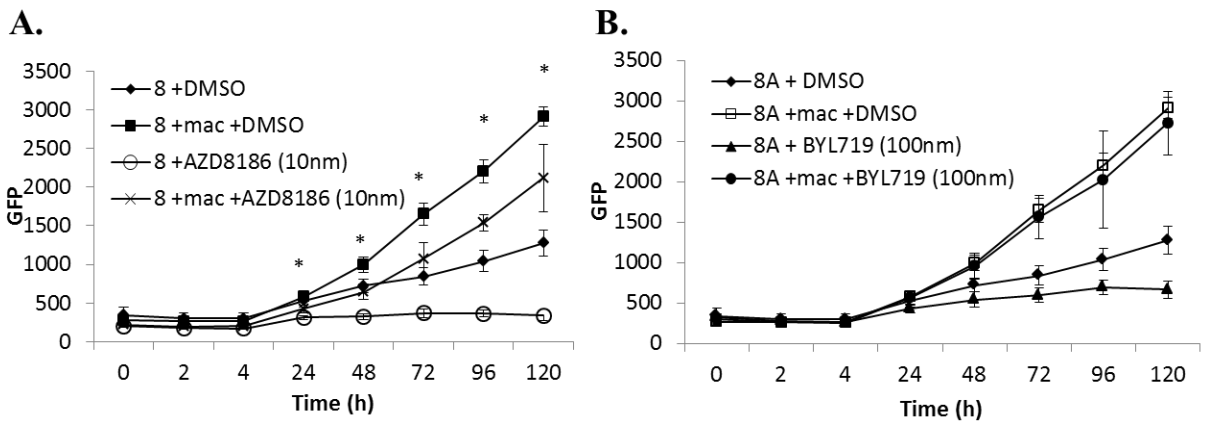

A.
mac
mac + NSC87877
mac + IL-4
mac + NSC87877 + IL-4
STAT6
pSTAT6
GAPDH
B.
8A +mac +NSC87877 (50uM)
8A +mac +DMSO
GFP
3500
3000
2500
2000
1500
1000
500
0
0
2
4
24
48
72
96
120
Time (h)

Figure 3.11. Addition of a SHP1/2 inhibitor has no effect on macrophage induced glioblastoma cell proliferation. (A) Western blot demonstrating that treatment of macrophages with 500nM of the SHP1/2 inhibitor blocks pSTAT6 reduction after treatment with 20ng/mL IL-4. This shows that the SHP1/2 inhibitor is effective. (B) Addition of a SHP1/2 inhibitor did not significantly effect macrophage stimulated PriGO8A proliferation, suggesting that the increased glioblastoma cell growth does not occur through downstream SIRPα signalling. Figure is an example of 3 similar experiments. Error bars represent standard deviation. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.


A.
3500
3000
2500
2000
1500
1000
500
0
GFP
8 +DMSO
8 +mac +DMSO
8 +AZD8186 (10nm)
8 +mac +AZD8186 (10nm)
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2
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120
Time (h)
B.
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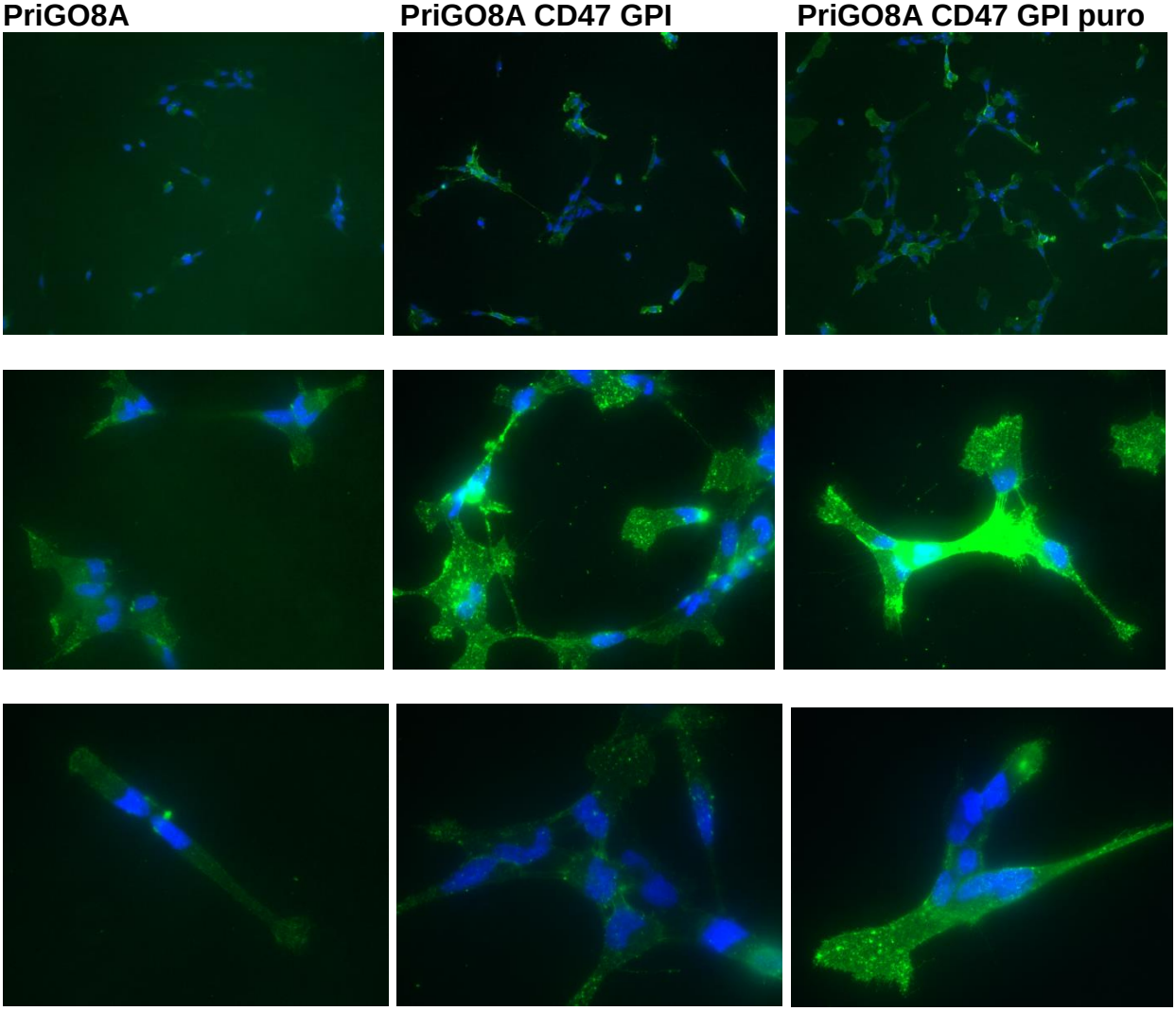
3000
2500
2000
1500
1000
500
0
GFP
8A + DMSO
8A +mac +DMSO
8A + BYL719 (100nm)
8A +mac +BYL719 (100nm)
0
2
4
24
48
72
96
120
Time (h)

Figure 3.12. Addition of PI3K inhibitors. (A) Addition of a PI3Kβ inhibitor resulted in decreased macrophage stimulated glioblastoma cell growth. There is a significant difference in PriGO8A proliferation between PriGO8A and macrophage co-culture and co-culture with the inhibitor after 24h. (B) Addition of a PI3Kα had no significant effect on macrophage stimulated glioblastoma cell growth. Figures are an example of 3 similar experiments. Error bars represent standard deviation. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.

To confirm that intracellular signalling of CD47 activated by macrophages contributes to glioblastoma proliferation a dominant negative approach was used. A protein consisting of the CD47 extracellular domain with a carboxy terminal 26 amino acid glycosylphosphatidylinositol (GPI) anchor from CD59[32-34] was engineered. A lentiviral expression vector was then created that drives expression of the engineered CD47 GPI anchored protein. The protein retains its extracellular domain and therefore its ability to bind with SIRPα but is incapable of downstream signalling and will compete with endogenous CD47. PriGO8As were then transduced with the CD47 GPI lentivirus. Immunofluorescence of transduced PriGO8As shows a higher expression of CD47 than untransduced PriGO8As, additionally, selection with puromycin further increased CD47 expression (figure 3.13A). This result was confirmed with western blot (figure 3.13B).

PriGO8As transduced with the CD47 GPI construct were tested in the co-culture assay. Early results show that PriGO8A CD47 GPI cells proliferate less in the presence of macrophages compared to wild-type PriGO8A (figure 3.14). These data are not conclusive as baseline growth for PriGO8A was lower than normal. These findings will be validated by using Flow Cytometry.

A.



B.

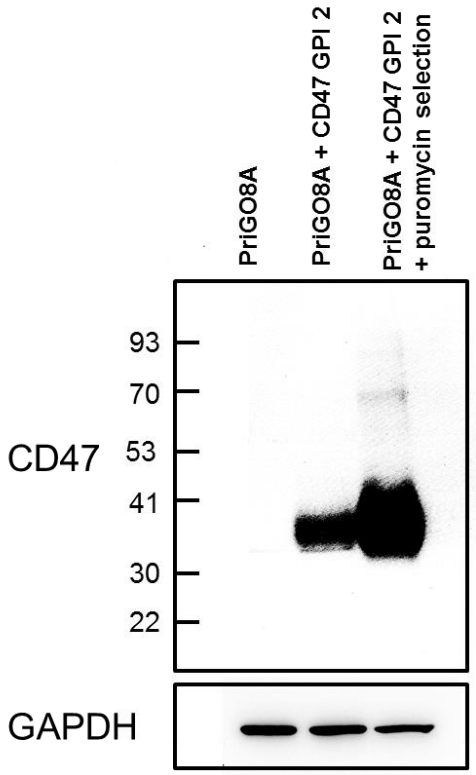

PriGO8A
PriGO8A + CD47 GPI 2
PriGO8A + CD47 GPI 2
+ puromycin selection
93
70
CD47
53
41
30
22
GAPDH

Figure 3.13. CD47 GPI anchor expression on PriGO8A. (A) Immunofluorescence of CD47 in non-permeabilized cells. From left to right: wild-type PriGO8A, PriGO8A transduced with the CD47 GPI anchor lentivirus and PriGO8A transduced with the CD47 GPI anchor lentivirus plus selection with 0.5µg/mL puromycin. Top row is 20X magnification, middle row 40X magnification and bottom row is 63X magnification. (B) CD47 western blot of PriGO8A transduced with CD47 GPI lentivirus with and without puromycin selection.

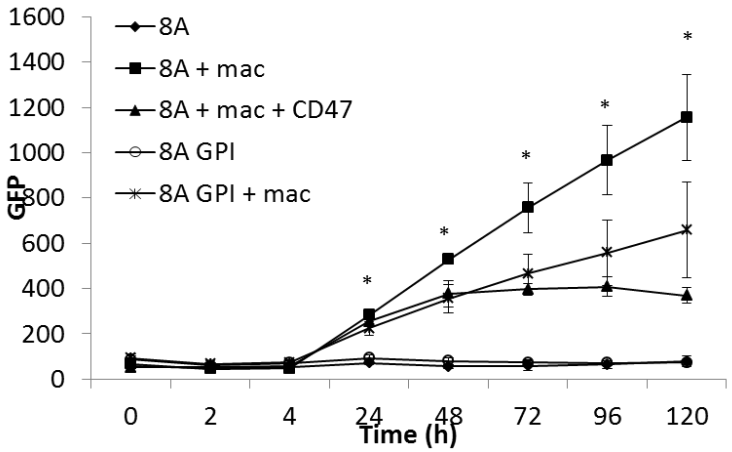
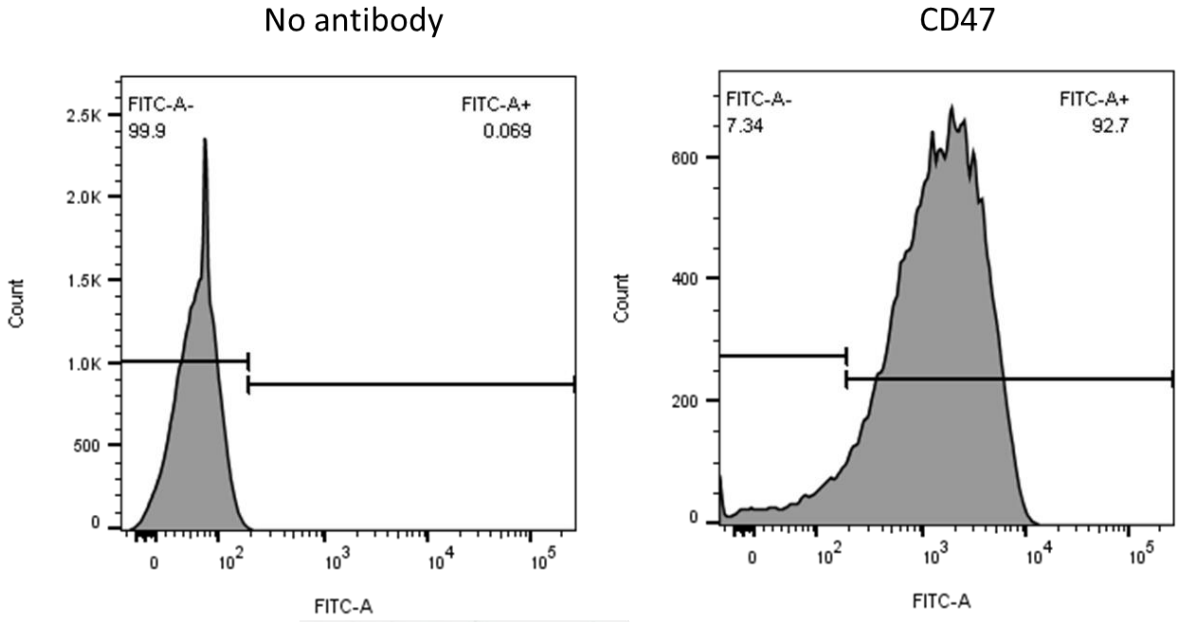

1600
1400
1200
1000
800
600
400
200
0
GFP
8A
8A + mac
8A + mac + CD47
8A GPI
8A GPI + mac
*
*
*
*
*
0
2
4
24
48
72
96
120
Time (h)

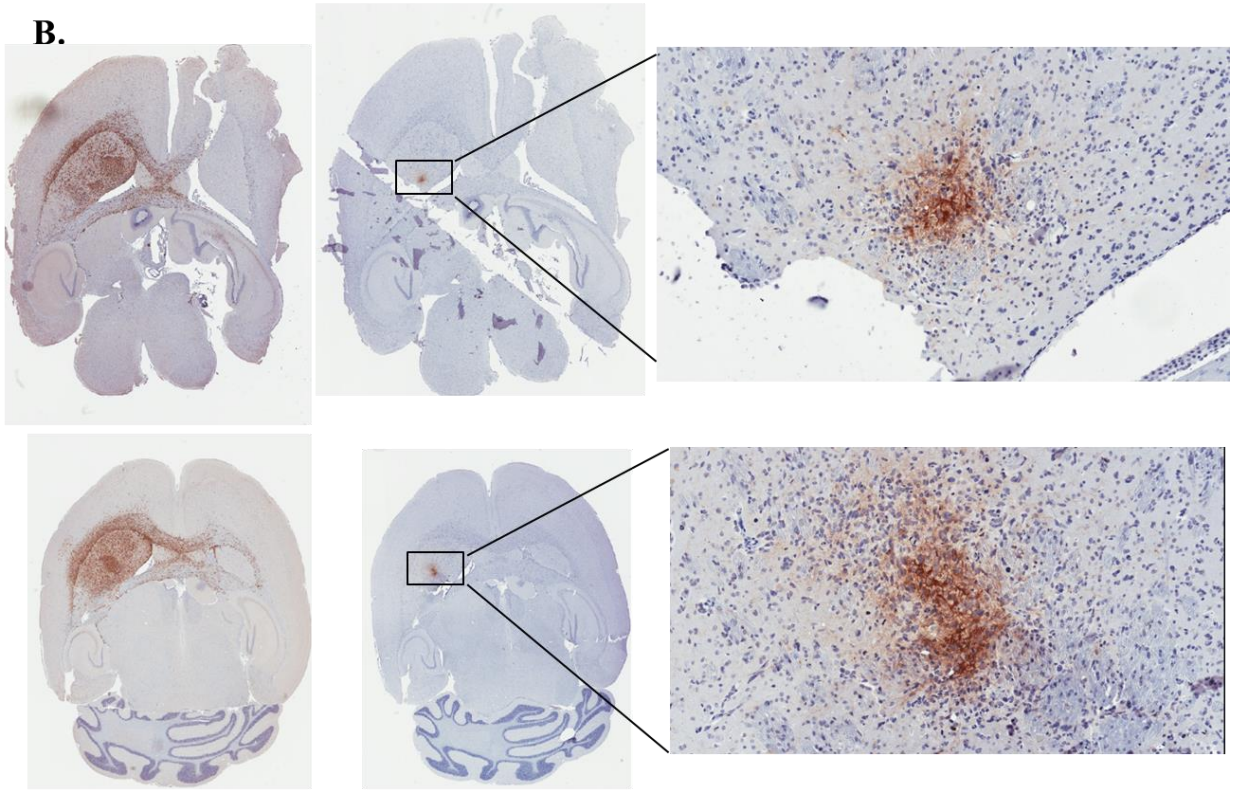
Figure 3.14. Co-culture of PriGO 8A CD47 GPI and macrophages. Preliminary data shows that PriGO8As transduced with the CD47 GPI construct have significantly reduced proliferation in the presence of macrophages than PriGO8A after 24h. Error bars represent standard deviation. Statistics were done using a two-tailed t-test and results considered significant if $p<0.05$.

We then wanted to test the CD47 GPI construct *in vivo*. The expression levels of the CD47 GPI construct were verified using flow cytometry. Results demonstrate that there is a wide range of CD47 GPI expression in PriGO8As (figure 3.15A). NOD SCID mice were injected with 100,000 PriGO8As transduced with the CD47 GPI construct. Two months after injection, brains were harvested and sectioned for immunohistochemistry of total cancer (STEM121) and CD47 expression. Analyses revealed that only a small proportion of the total tumour mass was CD47 positive (figure 3.15B). This suggests that the cells with a higher expression of the CD47 GPI construct proliferate less than those with a lower expression.

A.



B.



All PriGO8A (Stem121)

CD47

Figure 3.15. *In vivo* analyses of PriGO8A CD47 GPI construct. (A) Flow cytometry analyses of CD47 GPI expression shows a large variation of expression levels within the population. (B) Example of 2 mice demonstrating that the cells expressing high levels of the CD47 GPI construct make up a small proportion of the tumour mass.

4 – Discussion

In glioblastoma, macrophages have been shown to infiltrate the tumour environment and are often the dominant immune cell (36-38). Once in the tumour, the macrophages are re-educated to be supportive of the tumour microenvironment and promote glioblastoma progression (41, 42). For this reason, macrophages are a promising new area of research for cancer therapeutics. The aim of this study was to investigate the interactions between glioblastoma and macrophages. In order to do so, co-culture conditions of glioblastoma and macrophages were established. The use of primary glioblastoma cells acquired from surgical samples provides the most clinically relevant model of glioblastoma. These cells retain both the genetic characteristics and invasive potential of the original tumour (17-19). Primary glioblastoma cells express markers of neural stem cells and are able to differentiate along neuronal and astrocytic lineages (17-19). Primary macrophages were used in co-cultures by isolating monocytes from whole blood and differentiating them into macrophages. This procedure is well documented in the literature and results in a non-polarized macrophage (designated M_0 in current nomenclature) (84). The use of both primary glioblastoma and primary macrophages makes this model clinically relevant to study their interactions.

One of the better characterized protein interactions between cancer cells and macrophages is between CD47 on cancer and SIRPα on macrophages. We began studying these proteins for their role in phagocytosis but the initial co-culture experiments revealed that macrophages are able to stimulate PriGO8A cell proliferation. We also observed that addition of a blocking anti-CD47 antibody reduced macrophage

stimulated PriGO8A proliferation. We therefore hypothesized that macrophages promote glioblastoma cell proliferation through the CD47/SIRPα interaction. Furthermore, addition of a PI3Kβ inhibitor reduced PriGO8A proliferation in co-cultures which lead to the hypothesis that SIRPα on macrophages interacts with CD47 on glioblastoma which then signals through the Gβγ protein to activate the PI3Kβ pathway and promote proliferation.

4.1 TAMs promote glioblastoma proliferation independently of the EGFR pathway

TAMs have known roles in promoting tumour progression by effecting cell growth, angiogenesis and migration (28). This study is the first to provide direct evidence that macrophages effect glioblastoma proliferation as co-culture of PriGO8As and macrophages resulted in increased PriGO8A proliferation. This experiment was performed both in the presence and absence of EGF. In both cases, macrophage stimulated PriGO8A proliferation is similar despite the fact that EGF increases baseline PriGO8A proliferation. This suggests that macrophage stimulated PriGO8A proliferation occurs independently of the EGFR signalling pathway. This is an important finding as *EGFR* amplification is common in glioblastoma but current treatments targeting EGFR showed no significant benefit in clinical trials (16). This suggests that there is an alternative pathway which leads to glioblastoma proliferation and the potential for new a therapeutic target.

4.2 A novel role for the CD47/SIRPα interaction

In this study, the role of CD47/SIRPα was investigated in the context of macrophage stimulated glioblastoma proliferation. Results provide evidence that macrophages are able to stimulate glioblastoma proliferation through direct contact of SIRPα on macrophages and CD47 on glioblastoma as blocking with either anti-SIRPα or anti-CD47 reduced PriGO8A proliferation in the presence of macrophages. Previously, macrophages were known to play a role in glioblastoma progression through the release of a variety of factors and cytokines (45-47). This study is the first to show that increased glioblastoma cell proliferation is occurring through direct contact with macrophages, specifically through the CD47/SIRPα interaction. This novel finding could be beneficial in the creation of new treatment options for a variety of cancer types.

In recent years, the focus has been on studying the downstream signalling pathways of SIRPα and downstream signalling of CD47 has been forgotten. Our results demonstrate that the increase in proliferation is occurring through downstream signalling of CD47 and not SIRPα. Blocking SHP-1 and SHP-2, the downstream signalling molecules of SIRPα, resulted in no change in macrophage stimulated PriGO8A proliferation. Whereas blocking PI3Kβ resulted in a decrease in macrophage stimulated PriGO8A proliferation suggesting that signalling is occurring through the PI3Kβ pathway downstream of CD47. Previously, the CD47/SIRPα interaction has been of interest in the context of cancer as helping the tumour evade the innate immune system. When CD47, which has been shown to be overexpressed on many cancer types, binds to SIRPα on macrophages it prevents phagocytosis of the cancer cell by the macrophage. For this reason, anti-CD47 has been of interest as a potential

therapeutic. However, often anti-CD47 alone is not sufficient to promote phagocytosis of the cancer cell and a secondary pro-phagocytic antibody is needed. This study provides an alternative role for the CD47/SIRPα interaction in promoting tumour proliferation and provides more evidence for the use of anti-CD47 as a therapeutic.

Despite the well characterized role of CD47/SIRPα in phagocytosis, other potential roles for the protein interaction, especially those occurring downstream of CD47 have not been explored. One of the cytoplasmic binding partners of CD47 that has been determined is BCL2/adenovirus E1B 19kDa-interacting protein-3 (BNIP3) (85). CD47 signalling may play a role in regulating BNIP3 in autophagy and mitophagy but further investigation is needed (86). Two ubiquitin-related proteins; protein-linking integrin-associated protein and cytoskeletol-1 (PLIC1) and PLIC2 have also been shown to bind cytoplasmically with CD47 (87). This pathway may be involved in altering the vimentin intermediate filament in the cytoskeleton. Further studies also demonstrated that PLIC1 binds Gβγ and tethers the protein to CD47 (88). Indeed it was earlier shown that G_iαβγ co-immunoprecipitates with CD47 in a pertussis toxin-sensitive manner (56). Pertussis toxin sensitivity also implicates CD47 binding in regulating heterotrimeric G-protein signalling (89). PLIC1 may have other functions in CD47 signalling, such as: resistance to oxidative stress, regulation of cytoplasmic calcium levels and limitation of autophagosome formation (54). However, to date no studies have addressed the effects of CD47 signalling on cell function. The data presented here suggest a novel role of CD47 in that it stimulates glioblastoma proliferation.

The current model is glioblastoma releases factors and cytokines which result in chemoattraction of macrophages to the tumour environment. CD47 which has five

transmembrane regions is in contact with an integrin which has two transmembrane regions, this may create a seven transmembrane domain that is typical of Gβγ signalling. However, it remains unclear exactly how CD47 is able to activate G proteins. Our model suggests that when SIRPα on macrophages interacts with CD47 and its integrin on glioblastoma, it forms a seven transmembrane protein and signals through Gβγ to the PI3Kβ pathway to promote proliferation of glioblastoma (figure 4.1).

This model is supported by findings from a study that showed that CD47 increased proliferation of U87 and U373 atrocytoma cells but not normal astrocytes and that using blocking anti-CD47 reduced proliferation in the astrocytoma (90). They suggest that downstream signalling following CD47 activation involves Gβγ dependent activation of the PI3K/Akt pathway in astrocytoma cells but not in normal astrocytes (90). These findings broadly agree with the data presented. However, in their experiments they used cell lines which are not a good model of the disease compared to primary cell cultures used in this study. Furthermore, they only used a peptide to activate CD47 which has well documented activities that are independent of CD47 (91).

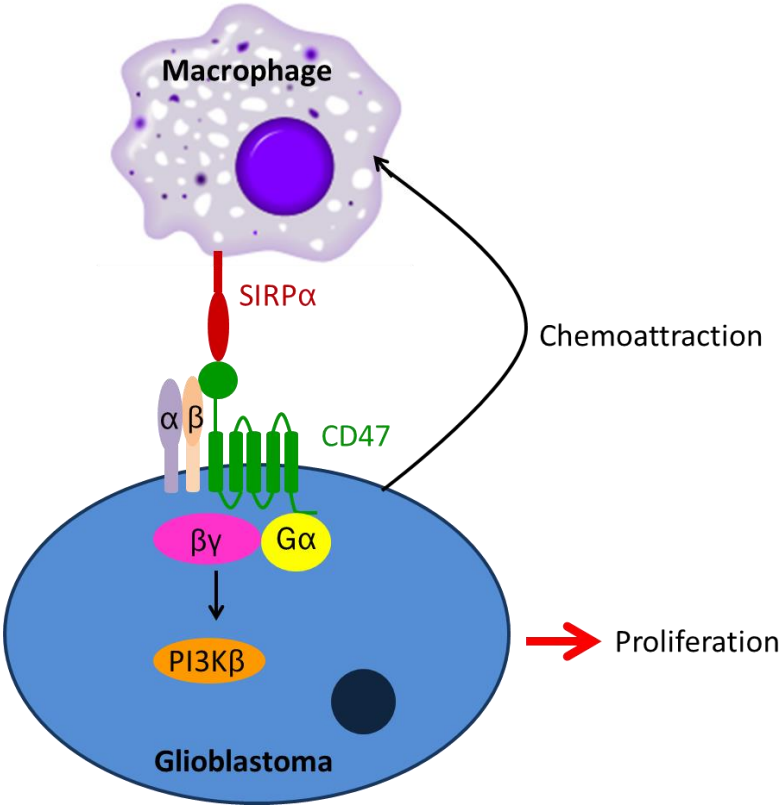

Macrophage
SIRPα
Chemoattraction
α β
CD47
βγ
Gα
PI3Kβ
Proliferation
Glioblastoma

Figure 4.1. Summary model of thesis.

To confirm that macrophage stimulated PriGO8A proliferation is occurring due to downstream signalling of CD47, a GPI linked CD47 protein was engineered. This modified version of CD47 has the external CD47 domain but instead of the internal domain, there is a GPI anchor to the cell membrane. This protein will still be able to bind to integrins and it will compete with endogenous CD47 to bind with SIRPα. This model will allow downstream signalling of SIRPα to remain unaffected while inhibiting any downstream signalling of CD47. This model is ideal for verifying the downstream signalling effects of CD47 because of the role CD47 plays in phagocytosis. Knockdown or knockout was not used as reduction in CD47 could result in an increase in phagocytosis and results will be skewed. Preliminary results demonstrate that the GPI linked CD47 expressing PriGO8As have reduced macrophage stimulated proliferation compared to control.

It is interesting to consider whether microglia, the resident macrophages of the brain are also able to stimulate glioblastoma cell proliferation. Microglia as well as macrophages have been shown to express SIRPα (92). Since microglia reside in the brain, the tumour will be exposed to them early in its development and can possibly use the microglia to aid in its proliferation. In this sense, anti-CD47 blocking antibody could be used as a therapeutic in glioblastoma patients that are detected early.

4.3 Heterogeneity of glioblastoma

Co-culture experiments were repeated using primary glioblastoma cells obtained from a different patient, PriGO9As. Co-culture of PriGO9As and macrophages did not result in increased PriGO9A proliferation. However, addition of cetuximab, an EGFR

blocking antibody, resulted in decreased PriGO9A proliferation both in the presence and absence of macrophages. This suggests that PriGO9As are more reliant on the EGFR signalling pathway for proliferation and thus, the co-culture with macrophages has no effect. Indeed it has been shown by a previous student that EGFR amplification is higher in PriGO9As compared to PriGO8As (50).

Co-culture experiments were also repeated with PriGO17As. Co-culture of PriGO17As and macrophages did not result in increased proliferation. However, addition of anti-CD47 resulted in a decrease in PriGO17A proliferation in the presence and absence of macrophages. This suggests that PriGO17As are reliant on a different pathway for proliferation and as such are not affected by the addition of macrophages. Microarray data acquired from another student shows that PriGO17As express more TSP-1 compared to PriGO8As or PriGO9As. Recall that TSP-1 is a ligand for CD47 and that binding of TSP-1 to CD47 blocks binding of SIRPα to CD47 (51). It is therefore likely that PriGO17A release TSP-1 which binds to CD47 and prevents SIRPα from binding. Furthermore, TSP-1 binding to CD47 must enhance cell proliferation as addition of anti-CD47 reduces PriGO17A proliferation.

These studies provide clear evidence of the heterogeneous nature of glioblastoma as experiments repeated using cells from three different patients yielded three different results. Single cell studies have demonstrated that as well as heterogeneity between patients, glioblastoma also has heterogeneity within a single tumour (15). Different cells within the tumour rely on different pathways for growth and in order to develop effective therapies we need a better understanding of these different pathways to be able to successfully target them. The use of anti-CD47 as a potential

therapeutic is still promising as it did reduce glioblastoma proliferation in two of the patient derived cell lines. Furthermore, it is possible that this therapy will target subsets of cancer cells within a variety tumours leading to better patient outcomes.

4.4 Anti-CD47 as a potential therapeutic

A concern with translating anti-CD47 as a therapeutic for humans is the risk of toxicity. CD47 is more highly expressed on glioblastoma cells but is also expressed at varying levels on normal healthy cells so there is a risk of toxicity to the normal cells (48). However, previous studies have demonstrated that administering therapeutic doses of anti-CD47 mAbs to normal C57BL/6 mice produced no significant toxic effect (75). This suggests that anti-CD47 antibodies may also be safe to use as a therapeutic in humans.

The experimental evidence presented provides the rationale for the use of anti-CD47 monoclonal antibodies to inhibit glioblastoma tumour proliferation as a monotherapy, beyond their previously described function in promoting phagocytosis. However, these antibodies may be more effective as part of a combination strategy. It is of interest to try using anti-CD47 in combination with an EGFR blocking antibody as many glioblastoma tumours have EGFR amplification. This combination will be more likely to affect a larger variety of glioblastoma cell types given the heterogeneous nature of the disease.

Reoccurrence of cancer is often caused by the inability to eliminate the cancer stem cells as most current treatments target the rapidly dividing cells. CD47 is expressed on both cancer cells and cancer stem cells making the CD47/SIRPα

interaction an excellent target (75). The use of anti-CD47 as a glioblastoma therapeutic could slow down tumour progression and lead to longer survival times which have not changed for glioblastoma at all in the past years.

4.5 Conclusions

By completing co-culture experiments, I have shown a novel role for the CD47/SIRPα interaction in that it can promote glioblastoma cell proliferation, likely through CD47 to the PI3Kβ pathway. These results establish an additional rationale, beyond the effects on phagocytosis, for considering the use of an anti-CD47 monoclonal antibody as a novel therapy for glioblastoma.

Future work will be to repeat these experiments in more cell lines to get a better picture of CD47 activity in other patients. Furthermore, *in vivo* studies will be done using the PriGO8A CD47 GPI linked cells in comparison to full length CD47 to assess whether they result in differential tumour development. Additional experiments will need to be done in order to provide more detailed information of the downstream signalling pathway of CD47. Finally, these experiments will be repeated using microglia to determine if they are also able to stimulate glioblastoma cell proliferation.

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Contributions of Collaborators

The content of this manuscript was written by Katelyn Badham with editorial suggestions from Dr. Ian Lorimer. All of the experiments presented in this manuscript are the work of Katelyn Badham with the exception of the CD47 GPI lentiviral construct which was designed by Ian Lorimer and created by Sylvie Lavictoire.

5 - Appendix

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atgtggcccctggtagcggcgctgttgctgggctcggcgtgctgcggatcagctcagcta
 M  W  P  L  V  A  A  L  L  L  G  S  A  C  C  G  S  A  Q  L
ctatttaataaaacaaaatctgtagaattcacgttttgtaatgacactgtcgtcattcca
 L  F  N  K  T  K  S  V  E  F  T  F  C  N  D  T  V  V  I  P
tgctttgttactaatatggaggcacaaaacactactgaagtatacgtaaagtggaaattt
 C  F  V  T  N  M  E  A  Q  N  T  T  E  V  Y  V  K  W  K  F
aaaggaagagatatttacacctttgatggagctctaaacaagtccactgtccccactgac
 K  G  R  D  I  Y  T  F  D  G  A  L  N  K  S  T  V  P  T  D
tttagtagtgcaaaaattgaagtctcacaattactaaaaggagatgcctctttgaagatg
 F  S  S  A  K  I  E  V  S  Q  L  L  K  G  D  A  S  L  K  M
gataagagtgatgctgtctcacacacaggaaactacacttgtgaagtaacagaattaacc
 D  K  S  D  A  V  S  H  T  G  N  Y  T  C  E  V  T  E  L  T
agagaaggtgaaacgatcatcgagctaaaatatcgtgttctcagagacaaactggtcaag
 R  E  G  E  T  I  I  E  L  K  Y  R  V  L  R  D  K  L  V  K
tgtgagggcatcagcctgctggctcagaacacctcgtggctgctgctgctcctgctctcc
 C  E  G  I  S  L  L  A  Q  N  T  S  W  L  L  L  L  L  L  S
ctctccctcctccaggccacggatttcatgtccctgtga
 L  S  L  L  Q  A  T  D  F  M  S  L  -
```

Figure 5.1. Final sequence of CD47 with a GPI anchor.

Curriculum Vitae

Education

M.Sc	Biochemistry Department of Biochemistry, Faculty of Medicine University of Ottawa Thesis title: Stimulation of glioblastoma proliferation by macrophages through CD47/SIRPα Supervisor: Dr. Ian Lorimer	Ongoing
B.Sc	Honours in Biomedical Science. *Magna cum laude.* Faculty of Science, University of Ottawa	2015
OSSD	Ontario Secondary School Diploma John McCrae Secondary School • Honour Role • Member of the Athletic Council	2011

Honours and Awards

University of Ottawa Admission Scholarship – Master's 2015, 2016
Scholarship awarded by the University of Ottawa to students with a CGPA of at least 8.0.

University of Ottawa Admissions Scholarship - Undergraduate 2014, 2013, 2012, 2011
Scholarship awarded by the University of Ottawa to applicants who excel in highschool. The scholarship is renewable as long as a CGPA of 8.0 is maintained.

AECOM- Founding Chairman Scholastic Recognition Program 2013, 2012, 2011
Scholarship awarded by the engineering company AECOM to university and college students with high GPAs and community involvement.

UROP- Undergraduate Research Opportunity Program 2014
Research grant awarded to students which allows them to work under a professor in their laboratory. This provides students with valuable research experience.

Research Experience

Master's Student	Ottawa Hospital Research Institute, Centre for Cancer Therapeutics Supervisor: Dr. Ian Lorimer • Established experimental conditions for the co-culture of primary glioblastoma cells and macrophages. • Discovered novel role for the CD47/SIRPα interaction in that it stimulates glioblastoma proliferation.	Sept 2015 - Ongoing
Research Assistant	Ottawa Hospital Research Institute, Centre for Cancer Therapeutics Supervisor: Dr. Ian Lorimer • Set up co-culture experiments between glioblastoma and THP1 cells. • Created PTEN inducible cell line and assessed MCP-1 expression.	Summer 2015
Honour's Project	Faculty of Science, University of Ottawa Supervisor: Dr. Marc Ekker • Lineage tracing of dopamine neurons in zebrafish.	Sept 2014 – Apr 2015
Research Assistant	Faculty of Science, University of Ottawa Supervisor: Dr. Rees Kassen • Developed DNA extraction protocol for *Aspergillus* • Planned and conducted pilot pheromone experiment	Summer 2014
UROP	Faculty of Science, University of Ottawa Supervisor: Dr. Rees Kassen • Studied condition-dependent sex in *Aspergillus.*	Sept 2014 – Dec 2014

Presentations

Katelyn Badham, Sylvie Lavictoire, Ian Lorimer. *The Interaction of Glioblastoma with Macrophages*. [Abstract accepted for poster presentation] In: 2017 Resident's Day. June 7th 2017; Ottawa, ON.

Katelyn Badham, Sylvie Lavictoire, Ian Lorimer. *The Interaction of Glioblastoma with Macrophages*. [Abstract accepted for poster presentation] In: 2016 Research Day. November 10th 2016; Ottawa, ON.

Katelyn Badham, Sylvie Lavictoire, Ian Lorimer. *The Interaction of Glioblastoma with Macrophages.* [Abstract accepted for poster presentation] In: 2016 Resident's Day. June 3rd 2016; Ottawa, ON.

Katelyn Badham, Sylvie Lavictoire, Ian Lorimer. *The Interaction of Glioblastoma with Macrophages.* Presentation at the University of Ottawa's department of biochemistry, microbiology and immunology poster day. May 12th 2016; Ottawa, ON.

Katelyn Badham, Cynthia Solek, Marc Ekker. *Lineage Tracing of GABAergic neurons in the zebrafish brain.* Presentation at the University of Ottawa's Biomedical Science Poster Day. April 2015; Ottawa, ON.

Katelyn Badham, Nicholas Rhode, Rees Kassen. *Condition-dependent sex in* Aspergillus nidulans. Presentation at the University of Ottawa's undergraduate research opportunity program poster day. March 2014; Ottawa, ON.