

Glioblastoma: multifaceted immunosuppression mediated through galectin family members

Item Type	Thesis or dissertation
Authors	Greywood, Rye
Citation	Greywood, R. (2023) Glioblastoma: multifaceted immunosuppression mediated through galectin family members. University of Wolverhampton. http://hdl.handle.net/2436/625627
Publisher	University of Wolverhampton
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Download date	2026-03-24 15:58:14
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Glioblastoma: Multifaceted immunosuppression mediated through galectin family members

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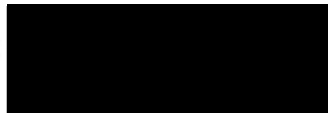
October 2023

A thesis submitted to the University of Wolverhampton
in partial fulfilment of the requirements for awarding
the degree of Doctor of Philosophy (Ph. D)

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ABSTRACT

Glioblastoma (GBM) is an aggressive brain cancer with a near-uniformly lethal prognosis. Anti-tumoural immunity remains low due to a highly suppressive tumour microenvironment. This project set out to identify immunomodulatory proteins secreted by glioblastoma, describe the distribution of intratumoural immune cells, and characterise the effects of secreted immunomodulatory on adaptive immune effector cells.

Mass spectrometry data identified galectin family members in the glioblastoma secretome. ELISA assays confirmed galectin presence in GBM-derived liquid samples; immunofluorescence confirmed their presence in tumour sections, as well as the presence of T-cells, B-cells and macrophages. Changes in cell surface marker expression and phagocytosis following exposure to recombinant proteins were assayed using flow cytometry and pHrodo-conjugated *Escherichia coli* respectively.

B-cells and macrophages were predominantly found in tumour bulk ($p=0.0053$ and 0.0087) with T-cells located in the perivascular niche ($p=0.0932$). Additionally, 22% of tumours contained T-cell aggregations. Galectins 1, 3, 4 and 7 were also found in the majority of GBM assayed; immune checkpoints galectin-9 and PD-L1 were found in 100% and 50% of assayed tumours, respectively. The perivascular location of galectin-1 approached significance ($p=0.0844$), whereas other galectins showed a more equitable distribution. Consistent, though insignificant, downregulation of MHCII and phagocytosis was observed in pro-inflammatory adult macrophages exposed to GBM-relevant galectin-1 concentrations.

These results suggested a T-cell-specific mechanism of suppression resulting in reduced infiltration, whereas B-cells and macrophages were able to effectively infiltrate GBM. Antigen presentation by macrophages may be inhibited by perivascular galectin-1, thus limiting T-cell retention; encounter with known immunosuppressive proteins such as galectins 1, 3, 9 and PD-L1 following effector extravasation may be a key mechanism through which GBM immunosuppression is mediated. These results posit the perivascular region as a uniquely immunosuppressive environment, and the presence of galectins as a potential key mediator of immunosuppression within glioblastoma.

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ACKNOWLEDGEMENTS

Many thanks to my supervisors Kesley Attridge and Tracy Warr, without whom this project would not exist, and who have given me invaluable insight and advice throughout. Additional thanks to Hafid Omar for further knowledge and discussion, Clare and Nadia for their expertise in confocal and flow cytometry, and everyone at The Walton and Brain Tumour North West tissue banks. Many thanks to The Colin Oliphant Charitable Trust for funding the project. Thanks also to Alexandra Elbakyan.

My friends inside and outside the lab: Athena, Amelie, Benjamin, Catryn, Chaos, Chris, Finch, Hannah, Jerrica, Kinza, Layne, Moira, Priya, Robyn, Sharne, Sofie, Stefan, Taylor. Harry and Joe – you should have been here a lot longer.

Special thanks to my grandad.

April and Sacha, for being my family.

Results herein are in part based upon data generated by The Cancer Genome Atlas research project, available at www.cancer.gov/tcga.

ABBREVIATIONS

µg – microgram

µL – microlitre

µM – micromolar

µm – micrometre

AID – activation-induced deaminase

AIDS – acquired immunodeficiency syndrome

AML – acute myeloid leukaemia

ANOVA – analysis of variance

Anti-CD3 – antibody against CD3, AlexaFluor 647 conjugate

Anti-CD19 – antibody against CD19, FITC conjugate

Anti-CD34 – antibody against CD34, FITC conjugate

Anti-CD56 – antibody against CD56, unconjugated

Anti-CD68 – antibody against CD68, unconjugated

Anti-CD80 – antibody against CD80, allophycocyanin conjugate

Anti-CD86 – antibody against CD86, FITC conjugate

Anti-galectin-9 – antibody against galectin-9, AlexaFluor 594 conjugate

Anti-GFAP – antibody against GFAP, unconjugated

Anti-LYVE1 – antibody against LYVE1, unconjugated

Anti-MHCII – antibody against MHCII, phycoerythrin conjugate

Anti- PDGFRβ – antibody against PDGFRβ, unconjugated

Anti-PD-L1 – antibody against programmed death ligand 1, AlexaFluor 594 conjugate

AP-1 – activator protein 1

APC – antigen-presenting cell

ATM – ataxia telangiectasia mutated

BAT3 – HLA-B associated transcript 3

BBB – blood-brain barrier

BCA – bicinchoninic acid assay

BCL-6 – B-cell CLL/lymphoma 6 protein

BCR – B-cell receptor

BM – bone marrow

BMDC – bone marrow-derived dendritic cell

BMDM – bone marrow-derived macrophage

BSA – bovine serum albumin

CAR – chimeric antigen receptor

CCGA – Chinese Cancer Genome Atlas

CCL – C-C motif chemokine ligand

CCN2 – cell communication network factor 2

CCR – C-C motif chemokine receptor

CD – cluster of differentiation

CD80-APC – antibody against CD80 with allophycocyanin conjugate

CD86-FITC – antibody against CD86 with fluorescein isothiocyanate conjugate

cDC – conventional dendritic cell

CDR – complementarity-determining region

CEACAM – carcinoembryonic cell adhesion molecule

CI – confidence interval

CLN – cervical lymph node

CLP – common lymphoid progenitor

CM – conditioned media

CNA – copy number alteration

CNS – central nervous system

CO₂ – carbon dioxide

COX2 – cyclooxygenase 2

CRC – colorectal cancer

CRD – carbohydrate recognition domain

CS – culture supernatant

CSF – cerebrospinal fluid

CSR – class-switch recombination

CT – computerised tomography

CTGF – connective tissue growth factor

CTLA-4 – cytotoxic T-lymphocyte associated protein 4

CXCL – C-X-C motif chemokine ligand

CXCR – C-X-C motif chemokine ligand

Cy3 – sulfo-cyanine 3

DAPI – 4',6-diamidino-2-phenylindole

DC – dendritic cell

DEG – differentially expressed gene

dgal-1 – dimeric galectin-1

dH₂O – distilled water

DMSO – dimethyl sulfoxide

DN – double-negative

DNA – deoxyribonucleic acid

DP – double-positive

DS – directly secreted

DSB – double strand break

DTT – dithiothreitol

EAE – experimental autoimmune encephalitis

EC – endothelial cell

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

EGFR – epidermal growth factor receptor

ELISA – enzyme-linked immunosorbent assay

EMT – epithelial-mesenchymal transition

ERK – extracellular signal regulated kinase

EV – extracellular vesicle

EZH1 – enhancer of zeste homolog 1

FBS – foetal bovine serum

FC – flow cytometry

FDA – Food and Drug Administration

FDR – false discovery rate

FFPE – formalin-fixed, paraffin-embedded

FITC – fluorescein isothiocyanate

FOXP3 – forkhead box P3

FSC – forward scatter

FYN – tyrosine protein kinase Fyn

Gal-1 – galectin-1

Gal-3 – galectin-3

Gal-4 – galectin-4

Gal-7 – galectin-7

Gal-9 – galectin-9

GATA-3 – GATA binding protein 3

GBM – glioblastoma

GC – germinal centre

GFAP – glial fibrillary acidic protein

GITR – glucocorticoid-induced tumour necrosis factor related protein

GSC – glioma stem cell

Gy – Gray

HC – heavy chain

HCC – hepatocellular carcinoma

HGG – high-grade glioma

HIGM – hyper IgM syndrome

HIV – human immunodeficiency virus

HLA – human leukocyte antigen

HMG – high mobility group

HNC – head and neck cancer

HSC – haematopoietic stem cell

HUVEC – human umbilical vein endothelial cell

IBA1 – ionised calcium-binding adapter molecule 1

IC – immune checkpoint

ICAM-1 – intercellular adhesion molecule 1

ICOS – inducible costimulator

ICOSL – inducible costimulator ligand

IDH – isocitrate dehydrogenase

IDO – indoleamine 2,3-dioxygenase

IF – immunofluorescence

IFN – interferon

Ig – immunoglobulin

IHC - immunohistochemistry

IL – interleukin

INK4 – cyclin-dependent kinase inhibitor 2A

iNOS – inducible nitric oxide synthase

IPEX syndrome – immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

IR – ionising radiation

IRF8 – interferon regulatory factor 8

IS – immunological synapse

ITAM – immunoreceptor tyrosine activation motif

ITIM – immunoreceptor tyrosine inhibition motif

ITSM – immunoreceptor tyrosine switch motif

iTreg – induced T regulatory cell

KCNQ1 – potassium voltage-gated channel subfamily Q member1

KD – knockdown

KDM3A – lysine demethylase 3A

KO – knockout

LAG-3 – lymphocyte activation gene 3

LC – light chain

LCK – lymphocyte-specific protein tyrosine kinase

LGG – low-grade glioma

LN – lymph node

LPS – lipopolysaccharide

LV – lymph vessel

LYVE1 – lymphatic vessel endothelial hyaluronan receptor 1

M – molar

MAPK – mitogen activated protein kinase

mDC – myeloid dendritic cell

MFI – mean fluorescence intensity

mg - milligram

mgal-1 – monomeric galectin-1

MGMT – O-6-methylguanine DNA methyltransferase

MHC – major histocompatibility complex

MHCI – major histocompatibility complex class I

MHCII – major histocompatibility complex class II

MHCII-PE – antibody against major histocompatibility complex class II with phycoerythrin conjugate

MIF – macrophage migration inhibitory factor

mL – millilitres

MLV – meningeal lymphatic vessel

mM – millimolar

MMP – matrix metalloproteinase

MRI – magnetic resonance imaging

mRNA – messenger RNA

MS – multiple sclerosis

NF1 – neurofibromin 1

NFAT – nuclear factor of activated T-cells

NFκB – nuclear factor kappa beta

ng – nanogram

NHA – normal human astrocyte

NHEJ – non-homologous end joining

NK – natural killer cell

nm - nanometre

nTreg – natural T regulatory cell

OS – overall survival

PAX5 – paired box 5

PBMC – peripheral blood mononuclear cell

PBS – phosphate-buffered saline

pDC – plasmacytoid dendritic cell

PDGFRα – platelet-derived growth factor receptor alpha

PDGFRβ – platelet-derived growth factor receptor beta

PDI – protein disulfide isomerase

PD-1 – programmed death 1

PD-L1 – programmed death ligand 1

PD-L2 – programmed death ligand 2

PFS – progression-free survival

pg – picogram

PI3K – phosphatidylinositol-3 kinase

PKC – protein kinase C

PMA – phorbol 12-myristate 13-acetate

PP2A – protein phosphatase 2

PRR – pattern recognition receptor

PS – phosphatidylserine

PTEN – phosphatase and tensin homolog

PV – perivascular

PVN – perivascular niche

RPMI-1640 – Roswell Park Memorial Institute-1640

RAG – recombination activating gene

RFP – red fluorescent protein

RNA – ribonucleic acid

ROR – RAR-related orphan receptor

RRX – rhodamine red-X

RSS – recognition signal sequence

RT – radiotherapy

RTEL1 – regulator of telomere elongation helicase 1

S100A7 – S100 family member A7

S1P1 – sphingosine-1-phosphate receptor 1

SEM – standard error of the mean

SHP – protein tyrosine phosphatase SHP

SHM – somatic hypermutation

slg – surface immunoglobulin

siRNA – small interfering RNA

SLO – secondary lymphoid organ

SNP – single nucleotide polymorphism

SP – single-positive

SSC – side scatter

STAT – signal transducer and activator of transcription

TAM – tumour-associated macrophage

TB – tumour bulk

T-bet – T-box transcription factor 21

Tc – T cytotoxic

TCA – trichloroacetic acid

TCGA – The Cancer Genome Atlas

TCR – T-cell receptor

TDT – terminal deoxynucleotidyl transferase

TERT – telomerase reverse transcriptase

Tfc – T follicular cytotoxic

Tfh – T follicular helper

TGF – transforming growth factor

Th – T helper

TIGIT – T-cell immunoreceptor with Ig and ITIM domains

TIL – tumour-infiltrating leukocyte

TIM-3 – T-cell and immunoglobulin mucin domain 3

TLS – tertiary lymphoid structure

TM – transport media

TME – tumour microenvironment

TMZ – temozolomide

TNF – tumour necrosis factor

TP53 – tumour protein p53

Treg – T regulatory cell

WHO – World Health Organisation

UK – United Kingdom

US – United States

VCAM-1 – vascular cell adhesion molecule 1

VDJ – variable, diversity, joining

VEGF – vascular endothelial growth factor

ZAP70 – zeta chain of T-cell receptor associated protein kinase 70

1: INTRODUCTION

1.1. Glioblastoma – an overview

1.1.1. Overview and characteristics

Gliomas are the most common type of malignant central nervous system (CNS) tumour and comprise a variety of diagnoses, depending on the cell with which they share histological similarities. Glioma is an umbrella term referring to astrocytomas, oligodendromas and ependymomas, which resemble astrocytes, oligodendrocytes and ependymal cells respectively; in addition, some gliomas show a mixed phenotype.

Glioblastoma, formerly known as glioblastoma multiforme (GBM), an astrocytoma, is the most severe of the gliomas, and is classed as a grade 4 cancer by the World Health Organisation (WHO). In addition to its aggression and rapid lethality, this is also one of the most common CNS tumours, accounting for nearly half of all malignant CNS tumours in the United States in the period 2008-12 (Ostrom et al., 2015). There are 3.19 age-adjusted diagnoses of GBM per 100,000 annually in the United States population (Ostrom et al., 2013).

Glioblastomas are characterised by proliferative cells, necrosis – including the unique formation of pseudopalisading necrosis: densely cellular regions organised around necrotic areas – and angiogenesis. However, they also show intra- and intertumoural heterogeneity at the histologic and molecular levels. As with many aggressive malignancies, the tumours are typically hypoxic, resistant to treatment,

and have a marked tendency to recur (Stupp et al., 2005). Additionally, GBM has a highly immunosuppressive tumour microenvironment (TME) which prevents the patient's immune system from mounting an effective anti-tumour response (for review see Himes et al., 2021). The combination of these factors makes glioblastoma very difficult to treat; thus, it represents a significant challenge to healthcare systems.

GBM has a near-universally poor prognosis. Its diffuse infiltration and anatomical location renders complete surgical resection unfeasible. The high level of intratumoural heterogeneity renders treatment less effective, resulting in recurrence in a treatment-resistant form and lethality. A study of Swiss patients diagnosed between 1980-94 found a one-year survival rate of 17.7% (Ohgaki et al., 2004); the median survival time after diagnosis is 14.6 months, and the 5-year overall survival (OS) is as low as 5% (Ostrom et al., 2013; Ostrom et al., 2015). There is a pressing need for the development of new and effective interventions.

Glioblastoma is almost always found in the supratentorial region of the brain, comprising the frontal, temporal, parietal and occipital lobes; cerebellar GBM is rare, accounting for under 1% of diagnoses between 1973 and 2009. However, cerebellar GBM may have a worse prognosis, with a 5-year survival rate of just 2%. Most GBM diagnoses occur in patients of European descent. It has been suggested that cerebellar GBM is more frequent in patients of other races, although these still comprise a minority of diagnoses (Adams et al., 2013).

Unlike other aggressive cancers, GBM rarely metastasises. Meta-analyses have observed, in each case, less than 100 examples in the medical literature (Smith et al., 1969; Pasquier et al., 1980; Anghileri et al., 2016), of which lung metastases were suggested to be the most quickly lethal (Lun et al., 2011). As this cancer is rapidly fatal, it is possible that patients simply die before metastases are established, as opposed to an inability of the tumour to metastasise.

1.1.2. Introduction to risk factors

1.1.2.1. Genetic

No GBM-specific mutations are known; at best, the current understanding of genetic risk factors more generally indicates risk of glioma, tumour syndromes or solid tumours.

Mutations in tumour suppressors *TP53* and *ATM* – respectively associated with Li-Fraumeni syndrome and ataxia telangiectasia – contribute to risk of developing GBM (Kleihues et al., 1997; Malmer et al., 2007; Bondy et al., 2008). *TP53* mutations may partially explain why a small percentage of gliomas cluster in families (Kleihues et al., 1997). Age and sex distribution does not seem to vary between familial and non-familial glioma (Sadetzki et al., 2013).

Other chromosomal regions have been implicated in CNS tumour development, including but not limited to: the *RTEL1* telomere maintenance helicase; the *INK4* tumour suppressor locus; the *KCNQ1* gene; and chromosomal regions 1q23, 1q25 and 15q25-26.3, the latter of which contains proto-oncogenes and genes linked to tumour syndromes or signalling pathways (Bahua et al., 1998; Paunu et al., 2002; Malmer et al., 2005; Wrensch et al., 2009; Enciso-Mora et al., 2013; Kitahara et al., 2014).

Many of these studies examine European populations. While GBM is more common in patients of European descent, it is not exclusive to this population, and the results may not be applicable to other populations. It bears mentioning that mutations can

have population-specific effects and caution is warranted when extrapolating the results of genetic studies to populations not included in the original research.

1.1.2.2. Environmental

Unlike other solid tumours, for which smoking and alcohol consumption are well-established risks, there is no conclusive evidence that GBM incidence is affected by alcohol or tobacco use (Gousias et al., 2009; Cote et al., 2021), although a study of almost 150,000 Californian adults identified smoking marijuana monthly or more often correlated with increased risk of brain tumour, as did smoking tobacco in women (Efird et al., 2004). Two other studies have associated unfiltered cigarettes with general brain tumour risk (Burch et al., 1987; Lee, Wrensch and Miike, 1997).

There is also very little evidence for dietary-related risk in GBM development. There are some implications that nitrites e.g. from cured foods may be a risk factor in females; the same paper noted no significant trends with regard to alcohol consumption, whereas others have noted significant trends for wine only (Burch et al., 1987; Lee, Wrensch and Miike, 1997) and yet other authors for liquor only, but not beer or wine (Efird et al., 2004). Another paper suggested high-protein diets as a risk factor for brain tumours in general, and noted increased incidence in patients with a high-fruit diet. For gliomas specifically, these authors noted reduced incidence with a high-fat or sodium diet and a possible preventative effect of high cholesterol intake alongside high N-nitroso compounds (Kaplan, Novikov and Modan, 1997). Evidence for occupational and chemical-related exposure risk is varied. For modern and historic review of risk factors, see Wen et al., 2020 and Wrensch et al., 2002, respectively.

It is worth noting that dietary studies have many well-known drawbacks, including recall bias and reliance on estimating and averaging dietary component intake, and

these drawbacks also apply to studies utilising self-reported tobacco and alcohol usage. Dietary categories can range from broad to very granular, making cross-study comparisons difficult. In addition, diet is often highly regionalised, making international comparisons unreliable. In the above studies, the Kaplan paper focused on Israeli patients, who are more likely to obtain significant amounts of dietary protein from non-animal sources compared to Americans. Lee, Wrensch and Miike's paper studied Californians and Burch's paper looked at Canadians; it can be inferred that Americans and Canadians will have similar diets, and indeed the authors found quite similar trends as compared to Kaplan's study. Inversely, diets and tobacco usage vary significantly around Europe, which may render comparisons between different European populations unreliable.

Clearly, results from this type of study must be interpreted with caution and cannot simply be extrapolated to other populations. In addition, without controlling for at least different ethnic origins and socioeconomic status, such comparisons will quickly become meaningless. Finally, many of these studies focus on brain tumours or gliomas in general, and thus any solid GBM-specific effects have yet to be conclusively identified.

1.1.2.3. Radiation

Ionising radiation (IR) exposure is a known risk factor for many solid cancers, including gliomas. Radiotherapy (RT) for intracranial tumours increases the risk of secondary CNS tumours in both children and adults, though not all go on to develop GBM (Nishio et al., 1998; Armstrong et al., 2009). Repeated computerised tomography (CT) scans of the head and neck, but not dental X-rays – which are much less powerful – are also associated with later glioma development, but only in patients with a family history of gliomas (Davis et al., 2011). Additionally, a Danish study found an association between radon exposure and brain tumour development, although the authors did not study GBM specifically (Bräuner et al., 2013). Overall, it is clear that IR exposure is a risk factor, but is rare enough that it cannot be regarded as one of the main causes.

Non-ionising radiation in the form of electromagnetic fields from long-term mobile phone use have been proposed as a cause of GBM; a 2017 meta-analysis showed an association between long-term (>10 years) mobile phone use and low-grade glioma (LGG), but no association with high-grade glioma (HGG), though the rapid progression of HGG may have masked any link in their analysis. As the authors were not able to correct for all confounding factors, a link between gliomas and mobile phone use currently seems tenuous at best (Yang et al., 2017). Modelling has found that many proposed risk level-induction time combinations are implausible, based on the Nordic population, and suggests instead that correlations between mobile phone use and glioma incidence are heavily influenced by reporting bias (Deltour et al., 2012).

1.1.3. Diagnosis and treatment

1.1.3.1. Diagnosis – historical and current

GBM diagnosis is not straightforward, due to its variable and non-specific symptoms. Patients typically present with headaches, cognitive impairment and seizures; GBM may not be the first diagnosis considered. Once a tumour is suspected, imaging such as CT or magnetic resonance imaging (MRI) can be used to confirm tumour presence and location. Diagnosis can then be completed using a histological and molecular data from a biopsy.

Historically, brain tumours were classified based only on histological features – in GBM, this included cellular resemblance to astrocytes. Histology is variable, reflecting the heterogeneity of these tumours, but generally shows hypercellularity, necrosis, vascularisation and atypical nuclei. The development and use of more advanced techniques has been hugely beneficial in glioma classification and diagnosis.

GBM used to be diagnosed as either a primary or secondary tumour, wherein a primary tumour arose from *de novo* mutations, and a secondary tumour developed from lower-grade astrocytoma (for review see Ohgaki and Kleihues, 2013). As early as 1940, Scherer suggested a clinical distinction ought to be made, after observing that astrocytomas may transform into glioblastomas and that primary glioblastoma was more aggressive. Later work confirmed that primary tumours were more common in males and older patients, with a mean age at diagnosis of 62 years. Secondary tumours were documented mainly in females and younger patients, with an average age of 45 (Louis et al., 1993; Ohgaki et al., 2004) and constituted

approximately 5% of GBM diagnoses (Ohgaki et al., 2004); thus, males and older patients are more heavily represented (Ostrom et al., 2013). Histological differences between primary and secondary tumours were noted, but were not always sufficient to distinguish the two, so secondary tumour diagnoses were often reliant upon clinical evidence of a previous lower-grade glioma; if no previous diagnosis was available, this would typically be from neuroimaging or histology (Homma et al., 2006; Ohgaki and Kleihues, 2007).

In 2007, the WHO Fourth Edition Classification of Tumours of the CNS (Louis et al., 2007) was published, for the first time incorporating genetic sequencing into diagnosis alongside histology. An update was published in 2016 (for summary see Louis et al., 2016), incorporating recommendations from the International Society of Neuropathology that highlighted the importance of molecular subtyping in diagnosis (Louis et al., 2014). From this point on, where histological and genetic results differ, the latter are considered more important.

Under this guidance, GBM was divided into three categories: isocitrate dehydrogenase (IDH) wild-type, IDH mutant, and not otherwise specified, used when IDH subtyping could not be performed. IDH wild-type was the most common, generally regarded as synonymous with the older primary classification; IDH mutant glioblastoma accounted for about 10% of cases and correlated with secondary tumours. There were some documented exceptions. Nobusawa et al. in 2009 observed a subset of secondary glioblastoma with wild-type IDH, but suggested these samples were misclassified. While IDH1 mutations were also detected in a

subset of primary glioblastoma, it was suggested that these tumours actually developed from undiagnosed astrocytomas (Balss et al., 2008).

A further update was published in 2021: the WHO Fifth Edition Classification of Tumours of the CNS, which further integrates molecular and histological characteristics into diagnosis. Under this guidance, only IDH wild-type tumours may be diagnosed as GBM; IDH mutant tumours may be grade 2, 3 or 4 astrocytoma, and grades are now described using Arabic instead of Roman numerals. In addition, three genetic markers are given a central role in diagnosis alongside histology: to be assigned grade 4, at least one of EGFR amplification, mutations at the TERT promoter, and co-occurring loss of chromosome 10 with gain of chromosome 7 must be observed within the tumour alongside necrosis or microvascular proliferation (Louis et al., 2021).

Additionally, in 2010, analysis of data from The Cancer Genome Atlas (TCGA) identified four molecular subtypes of GBM, referred to as classical, neural, proneural and mesenchymal. Subtypes were found to have different levels of proliferation, aggression and necrosis, and differential responses to treatment intensity. These data were published when IDH-mutant tumours were still considered GBM, and so this schema will not necessarily apply well to post-2021 GBM diagnoses; however, the authors identified that most secondary GBM in their dataset fit the proneural subtype (Verhaak et al., 2010). Later analysis showed subtype may vary across intratumoural regions (Gill et al., 2014). GBM heterogeneity and subtyping is reviewed in Lee E et al., 2018.

1.1.3.2. Treatment and prognosis

Where possible in the UK and US, treatment usually follows the Stupp protocol: a combination of maximal surgical resection, RT and chemotherapy (Stupp et al., 2005). In 2005, a phase 3 clinical trial showed patients who received both RT and temozolomide (TMZ) treatments had a median survival of 14.6 months, compared to 12.1 months for the RT-only control group. Patients receiving combined treatment also benefited from longer progression-free survival (PFS), and the two-year survival more than doubled from 10.4% to 26.5% (Stupp et al., 2005), resulting in Food and Drug Administration (FDA) approval. Later studies confirmed the beneficial effect of this treatment protocol (Stupp et al., 2009; Koshy et al., 2012), which remains the gold standard today.

In addition, patients may be prescribed corticosteroids such as dexamethasone to reduce oedema, and anti-epileptic drugs if they experience seizures (Johnson et al., 2015). In cases of recurrence, patients may be prescribed the monoclonal antibody bevacizumab, which inhibits angiogenesis by binding the pro-angiogenic protein vascular endothelial growth factor (VEGF). Bevacizumab is used in the treatment of other cancers and was approved by the FDA for recurrent GBM in 2009 (Cohen et al., 2009); it also reduces radiation-induced necrosis in healthy brain tissue (Gonzales et al., 2007; Wong et al., 2008).

Where possible, gross total resection shows the strongest benefit in terms of OS (Kuhnt et al., 2011; Johnson et al., 2015). However, unlike other cancers, additional tissue cannot be removed; in combination with GBM's diffuse infiltrating margin, this makes prevention of recurrence nearly impossible through surgical means alone.

Thus RT is also administered, typically to a total of 60Gy, although a 34Gy dose given in large fractions may be more beneficial (Malmström et al., 2012); doses over 60Gy do not appear efficacious (Wegner et al., 2019).

TMZ is typically given alongside and after RT. TMZ is a prodrug that is intracellularly converted to the methyl diazonium cation, which causes deoxyribonucleic acid (DNA) methylation, often resulting in cell death; however, such damage can be repaired by the DNA suicide repair enzyme MGMT (O-6-methylguanine DNA methyltransferase). For this reason, TMZ therapy is considered to be most beneficial in gliomas with a methylated MGMT promoter (Hegi et al., 2005; Stupp et al., 2009; Zhang, Stevens and Bradshaw, 2012). A 2000 study showed MGMT promoter methylation to be a more reliable predictor of response to a different alkylating agent, carmustine, than tumour grade or patient age. Methylated gliomas had a median average of 21 months PFS, as compared to 8 months for non-methylated gliomas (Esteller et al., 2000). However, some authors have found MGMT promoter methylation not to influence prognosis or response to TMZ (Costa et al., 2010; Kmiecik et al., 2013).

Despite these advances in treatment, OS remains low, and there is a pressing need for better interventions. Since at least 2003, GBM has been suggested as a target for molecular therapy, such as oncolytic viruses, kinase inhibitors or antibody therapy, due to its resistance to conventional treatment (Mischel and Cloughesy, 2003). This would also allow for the development of novel therapies tailored to molecular subtypes, and the heterogeneity of GBM offers a wide range of potential targets (for review see Wilson, Karajannis and Harter, 2014).

1.2. The GBM microenvironment

1.2.1. Overview

The GBM TME is highly important in understanding tumour biology and behaviour. Historically, the role of the TME was underplayed and transformed cells were assumed to be the sole relevant population. In recent decades, this perception has been replaced by the understanding that varied cell types and extracellular matrix (ECM) proteins in the TME may all influence cancer through direct and indirect interactions with transformed cells. The mammalian brain is already a unique environment with many cell populations – such as astrocytes and microglia – found nowhere else in the body, alongside a high neuron density. The brain is also subject to reduced – though not absent – immunosurveillance as compared to other sites in the body. Due to this, the GBM TME cannot always be directly compared to the TME of other solid tumours.

1.2.2. The extracellular matrix

As early as 1998, Friedberg et al. discerned that matrix metalloproteinases (MMPs) were secreted into the cerebrospinal fluid (CSF) of CNS tumour patients and correlated with tumour aggressiveness. These small soluble enzymes digest components of the ECM, the effects of which in tumour growth are twofold. Firstly, ECM degradation can reduce stiffness. Secondly, it can expose new binding sites on the ECM for tumour cell surface molecules to bind to. Through these mechanisms, secretion of MMPs in many solid tumours, including GBM, may facilitate tumour invasion. These authors found a significant increase in gelatinase expression in brain tumour patients compared to controls. Analysis of ECM components, adhesion- and invasion-related molecules shows a distinct expression signature in patients with below-average survival, with increased expression of MMP2 and adhesion-related molecules such as CD44 and integrins (Virga et al., 2019). These results illustrate the importance of ECM degradation and adhesion in GBM invasion, and its resultant effects on patient outcome.

1.2.3. Vasculature and necrosis

GBM tumours are rich in both vasculature and necrosis. Normal brain vasculature has a blood-brain barrier (BBB) comprised of endothelial cells (ECs) held together by tight junctions, resulting in non-fenestrated capillaries surrounded by pericytes and astrocyte endfeet. This highly specialised structure permits close regulation of molecular and cellular transport into the healthy brain, thus protecting it from inflammation and toxins while allowing nutrient uptake and, where necessary, immune effector extravasation. However, despite the high levels of angiogenesis in GBM, vasculature is often malformed and leaky, lacking an intact BBB (for review of BBB in glioma, see Dubois et al., 2014). Concurrently, rapid tumour growth leads to poor perfusion in many areas, resulting in hypoxia and eventually, necrosis (for review see Rong et al., 2006).

Vascular growth in GBM takes place through multiple mechanisms, including release of pro-angiogenic factors, recruitment of precursor cells that differentiate into new vessels, and differentiation of transformed cells themselves into vascular structures – known as vascular mimicry. As in other solid tumours, this vascular growth is poor: vessels lack an intact BBB and are leaky, often delivering an inadequate supply of oxygen. The result is hypoxic and eventually, necrotic, intratumoural regions. The angiogenic process is a double-edged sword in terms of novel therapies: anti-angiogenic approaches are widely trialled in an effort to starve the tumour of oxygen and nutrients, thus limiting its growth; however, the leaky, malformed vasculature also represents an opportunity to deliver cytotoxic drugs or immune effectors without affecting healthy brain tissue. Vessel normalisation may

also improve patient outcomes by reducing oedema. GBM angiogenesis and therapeutic implications are reviewed in Wang, Jain and Batchelor, 2017.

1.3. The mammalian immune system – an overview

1.3.1. Innate and adaptive immunity – an overview

The immune system is perhaps best thought of as a collection of processes that protect an organism from external threats such as pathogens, toxins and parasites. The mammalian immune system comprises two branches: innate and adaptive immunity.

Innate immunity is mediated primarily by cells such as macrophages, basophils, eosinophils and neutrophils, and depends on recognition of broadly conserved molecules secreted by various pathogens. This requires pattern recognition receptors (PRRs) encoded in the human genome and subject to little variation. It allows for an extremely rapid response to invading pathogens, but does not facilitate the development of long-term immunological memory and allows no scope for novel responses to unfamiliar antigens. Innate immunity is outside the scope of this project. For an overview see Beutler, 2004.

At birth, humans have only innate immunity. While adaptive immunity allows for a great diversity of immune responses and the ability to overcome a greater array of pathogens, its development takes time. Adaptive immunity allows for both the development of a specific immune response to novel antigens, and long-term, stable immune responses to previously encountered antigens. It can be directed against pathogens or malignant cells. The primary effectors of adaptive immunity are T-cells, which participate in either direct killing or cytokine secretion, and B-cells, which secrete antibodies cognate to a target antigen. In order for antigen-specific responses to develop, cells must first be primed to recognise the target antigen,

which requires contact between the effector and an antigen-presenting cell (APC). APCs may be macrophages, B-cells or dendritic cells (DCs); the site of contact is termed the immunological synapse (IS). Activated effectors can then migrate to the site of inflammation to resolve the problem.

1.3.2. Development and function of immune effectors

The primary effectors of adaptive immunity are T-cells, B-cells and monocytes. All three types of cells are leukocytes, the collective term for white blood cells. Both T- and B-cells are lymphocytes – cells commonly found in the lymphatic system, which is comprised of lymph vessels (LVs), lymph nodes (LNs) and specialised organs such as the thymus and spleen – whereas monocytes are myeloid cells. All blood cells derive from haematopoietic stem cells (HSCs) – although some heterogeneity may exist in this subset – and are renewed throughout life (for review see Benz et al., 2012).

Lymphocytes originate from common lymphoid progenitors (CLPs), which in turn are derived from HSCs via multipotent progenitors. CLPs engraft in the bone marrow (BM) during embryonic development; some remain in the BM to give rise to B-cells, whereas others migrate to the thymus to generate T-cells (Figure 1.01). Both T- and B-cells share some functional and developmental similarities. The third type of lymphocyte is the natural killer (NK) cell, which is part of the innate immune system and will not be discussed here. The existence of a CLP versus early lineage-specific commitment of HSCs was debated, with the matter beginning to be settled in 1995 by Galy et al, who identified CD10⁺ cells in human BM that could be differentiated to T-, B- and NK cells and DCs.

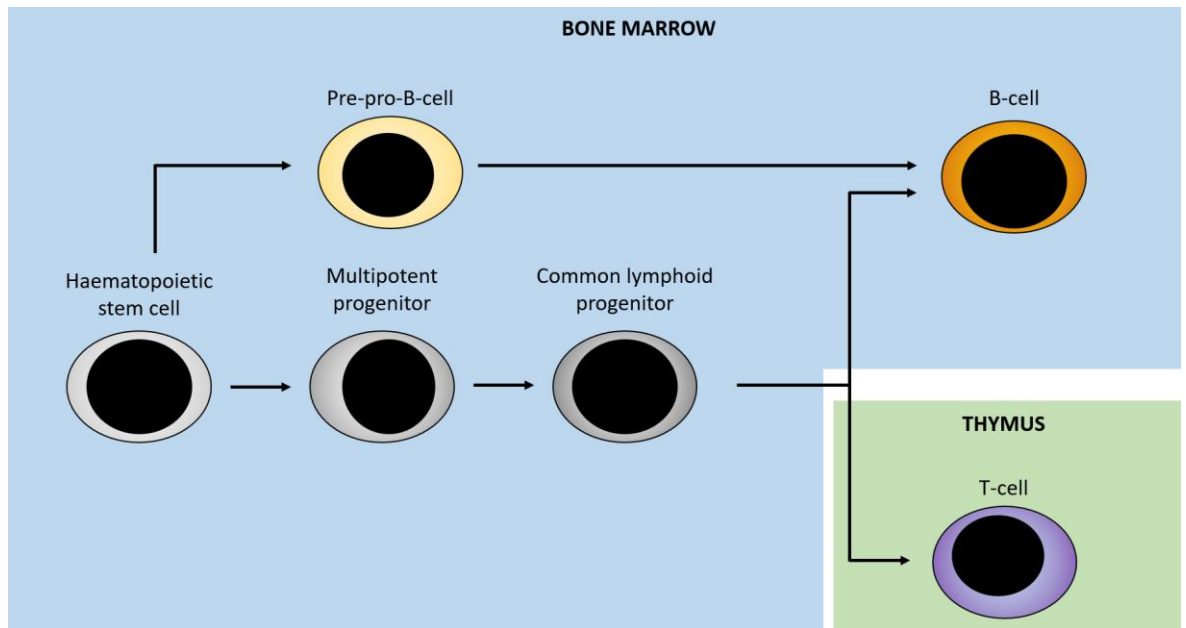


Figure 1.01. Lymphocyte development in thymus and bone marrow. HSCs differentiate to multipotent progenitors, then CLPs, in bone marrow. CLPs engrafting in bone marrow may differentiate to B-cells, whereas those migrating to the thymus differentiate to T-cells. B-cells may also arise from pre-pro-B-cells, which are derived from HSCs.

Both T- and B-cells progress through a series of tightly regulated developmental stages to become mature effector cells. Both lymphocytes must also be tolerant: able to avoid responding to self-antigens. When the immune system does recognise self-antigens and attack bodily tissues, this is termed autoimmunity; several regulatory mechanisms exist to limit development of autoimmunity. Finally, both T- and B-cells can also adopt a memory phenotype, allowing for rapid and effective immune responses when re-encountering a pathogen. T and B-cell development, phenotypes and function are discussed in sections 1.3.2.1 and 1.3.2.2. respectively.

In addition, the process of VDJ (variable, diversity, joining) recombination is unique to lymphocytes and is used to produce a functional T- or B-cell receptor (TCR or BCR). Briefly, genes encoding certain chains in the immunoglobulin (Ig) superfamily contain V, D and J regions (TCR β and Ig heavy chains) or V and J regions alone (TCR α and Ig light chains). There are multiple tandem repeats of each region, but the resultant protein only contains one of each, allowing for a large number of possible combinations. RAG1 and 2 (recombinase activating gene) proteins bind to recognition signal sequences (RSS) upstream of the gene segments and induce double-strand breaks (DSBs). These are then repaired by the error-prone process of non-homologous end joining (NHEJ), resulting in two gene segments placed in tandem, while the intervening DNA is excised from the genome. This occurs first between D and J segments, and next between V and the newly formed DJ segment where relevant. During the process, terminal deoxynucleotidyl transferase (TDT) may also insert random nucleotides at the break site, leading to even greater variation in the complementarity-determining regions (CDR) of the receptor. For a review of VDJ recombination, see Bassing, Swat and Alt, 2002. Mice lacking RAG1

or 2 fail to develop mature T- or B-cells (Mombaerts et al., 1992; Shinkai et al., 1992), showing that these proteins are indispensable for VDJ recombination, cannot substitute for each other, and further, selection of these cells during development requires creation of a functional receptor. In both cases, only lymphocytes were affected, demonstrating the highly specific nature of VDJ recombination.

Monocytes, along with lymphocytes, make up the agranulocytes; other leukocytes e.g. neutrophils, eosinophils and basophils contain granules within their cytoplasm, and are part of the innate immune system. Upon exposure to different environmental conditions, monocytes can differentiate to either a macrophage or a DC. These may be more accurately referred to as bone marrow-derived macrophages (BMDM) and conventional, myeloid or bone marrow-derived dendritic cells (cDC, mDC or BMDC) in order to distinguish them from the numerous types of tissue-resident macrophages and plasmacytoid dendritic cells (pDC). For the sake of clarity, this thesis will use the terms macrophage and DC to refer to these cells broadly, and will use BMDM, pDC and other specific abbreviations where required for clarity.

Macrophages are highly plastic and phagocytic cells with the ability to ingest pathogens or transformed cells, process the resulting antigens and present them on MHCII molecules (major histocompatibility complex class II) to allow T-cell priming. MHCII is restricted to professional APCs and presents exogenous antigens, whereas MHCI (major histocompatibility complex class I) is expressed by all cells and presents antigens from inside the cell, for example in the case of transformation or viral infection, to facilitate rapid killing by T-cells. In humans, these molecules are also termed HLA (human leukocyte antigen). Macrophages are highly responsive to

their environment and may promote further inflammation or inflammation resolution and wound healing. Along with DCs and monocytes, they form the mononuclear phagocyte system, which is discussed in 1.3.2.3. Finally, DCs are the main APCs of the immune system. mDCs are derived from circulating monocytes, whereas pDCs originate from HSCs. DCs continually survey their surroundings and present antigen efficiently to T-cells in lymph nodes.

In terms of basic immunology, it can be very hard to examine exactly what occurs in humans – many mouse studies rely on knockouts, transgenic mice, BM transplants and similar experiments which cannot be performed ethically in humans. As such, a lot of direct human knowledge is limited to what is known from naturally-occurring mutations and *ex vivo* experiments. Comparisons can often be drawn between the results of mouse knockout experiments and human clinical entities; however, this can also be fraught with misinterpretation as many genetic differences exist between mouse and human, such as gene duplication events allowing for redundancy in one species and not another. As will be discussed below, while the overall processes of immune cell development are the same in human and mouse, the different timing of key events can have massive implications resulting in entirely different results between organisms. Furthermore, mice have a much shorter lifespan, which may result in less need for long-term adaptive immune responses. Thus, while the current understanding of human basic immunology provides a reasonable framework, there are still large knowledge gaps. These are hard to address without relying on extrapolation to at least some extent, which problem is only compounded when studying interactions between the immune system and malignancies or other largely aging-related diseases.

1.3.2.1. T-cells

Mature T-cells perform multiple roles in adaptive immunity and typically fall into one of two broad categories: CD4⁺ T-helper (Th) cells and CD8⁺ cytotoxic (Tc) cells. CD8⁺ cells directly kill pathogens or transformed cells via release of perforins and granzymes, while CD4⁺ cells produce cytokines to recruit more cells and modulate the local immune response. CD4⁺ T-cells require antigen presentation via MHCII, so can only be activated by professional APCs. CD8⁺ T-cells respond to antigen on MHCI, which also enables them to kill transformed or virally-infected cells rapidly, as all cells constitutively express MHCI.

All T-cells have a unique TCR, engagement of which is required for activation. The TCR is a transmembrane protein complex, part of which is a heterodimer comprised of TCR α and β chains. The majority of T-cells are $\alpha\beta$, although a small percentage of $\gamma\delta$ T-cells exist (for review see Shiromizu and Jancic, 2018). The α , β , γ and δ chains, as with other members of the Ig superfamily, have a constant and variable region; the variable region is rearranged using VDJ recombination as discussed in 1.3.2. to create a unique TCR. CDRs in the $\alpha\beta$ or $\gamma\delta$ chains directly interact with peptides presented on MHC molecules; this interaction is stabilised by either CD4 or CD8. The TCR complex also contains one each of CD3 γ and δ , and two each of CD3 ϵ and ζ . Each member of the CD3 family contains intracellular immunoreceptor tyrosine activation motifs (ITAM) which are indispensable for signalling through the TCR, as the α and β chains have very short cytoplasmic domains.

1.3.2.1.1. T-cell development

As described above, T-cells originate from HSCs, which become CLPs; some of this population migrates to the thymus, where they are termed thymocytes. Thymocytes must create a functional TCR before entering the periphery.

Early thymocytes express neither CD4 nor CD8, and are termed double-negative (DN). These cells undergo VDJ recombination of the TRB gene. Upon production of a functional β chain, thymocytes begin to express both CD4 and CD8 co-receptor molecules, making them double-positive (DP). They proceed to rearrange the α chain which complexes with the β chain at the cell surface to form a TCR.

DP cells begin to move through the thymic cortex where they undergo positive selection, via the presentation of common self-antigens on cortical thymic epithelial cells. DP cells that lack a functional TCR cannot respond to these MHC-antigen complexes, and die by neglect or undergo receptor editing. Engagement of MHCI or MHCII restricts cells to become single-positive (SP) CD8+ or CD4+, respectively; exactly how and why this occurs is the subject of some debate, with several models proposed (Singer, Adoro and Park, 2008). From this point in selection, their future interactions are restricted to the appropriate molecule.

Cells that respond too potently to self-MHC complexes at the DP stage are killed through a process known as clonal deletion to limit the development of autoimmunity; however, the precise location and timing of this event is a matter of debate. In mice, it seems to occur at the DP-SP transition in the thymic cortex. Alongside this is the process of clonal diversion, wherein self-reactive thymocytes

enter the Treg development program, which mouse experiments suggest takes place in the thymic medulla rather than the cortex (for review of tolerance, see Xing and Hogquist, 2012).

Surviving SP cells are then presented with further antigen-MHC complexes in the thymic medulla, this time on medullary thymic epithelial cells or DCs. Tissue-restricted antigens are also displayed here to maximise self-tolerance. Cells that respond to self-antigens are killed to prevent autoimmunity. The resultant naïve T-cells then exit the thymus in favour of secondary lymphoid organs (SLOs) such as the lymph nodes and spleen, to which they are guided by the CCR7 cell-surface receptor, which enables them to respond to CCL19 and CCL21. Murine experiments have shown that thymic exit is dependent on expression of S1P1, a receptor for sphingosine-1-phosphate (Matloubian et al., 2004). For comprehensive reviews of T-cell development, see Klein et al., 2014; Lancaster, Li and Ehrlich, 2018. This process is illustrated in Figure 1.02.

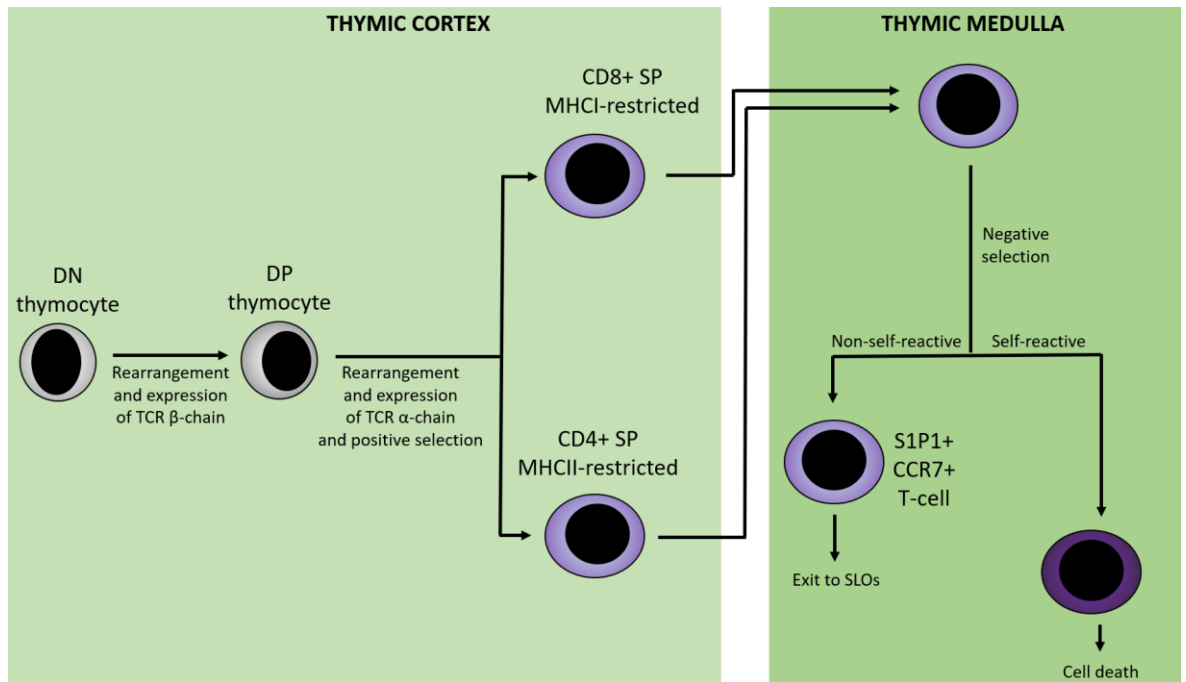


Figure 1.02. T-cell development. DN thymocytes create a functional TCR β -chain using VDJ rearrangement and express both CD4 and CD8 to become DP. DP cells undergo VJ rearrangement of the TCR α -chain. The functional TCR engages either MHC I or MHC II, which restricts them to become either CD8+ or CD4+ SP, respectively. SP cells proceed to the thymic medulla, where self-reactive cells die by apoptosis following negative selection. Non-self-reactive cells upregulate S1P1 and CCR7 and exit the thymus.

Although many immunological studies are carried out on mice and the results are often readily extrapolated to humans on the assumption that all mammalian immunology is broadly similar, T-cell development is one area in which such extrapolation can be unreliable. In humans, T-cells enter the thymus as early as 9 weeks gestation, whereas in mice, this occurs towards the end of gestation. Thus, post-birth thymectomy in mice results in impaired development of immune responses, which cannot be recovered by the peripheral compartment (Dalmaso et al., 1963). (Haynes et al., 1984). Post-birth thymectomy in humans does not affect T-cell function (Wells et al., 1998), and in patients with DiGeorge syndrome – resulting in the complete absence of a thymus – transplants are able to reconstitute T-cell presence (Li et al., 2011). The human naïve T-cell population is largely sustained by peripheral proliferation, whereas in mice, it depends on thymic output, although there is some evidence that in thymectomised mice, peripheral naïve T-cells do begin to proliferate to compensate (den Braber et al., 2012). Thus, T-cell development within mammals is not always readily comparable, even when mechanisms are broadly similar.

Some T-cells have a memory phenotype, and exactly how this fits into T-cell development is currently unclear. Memory T-cell lineage is debated, with some researchers proposing that memory cells form from effectors following antigen encounter and proliferation, while others suggest they develop as naïve cells and are the precursors of activated effectors. There are several memory T-cell subsets with varied localisation and effector function; for review of memory T-cells, see Farber, Yudanin and Restifo, 2014.

As aging proceeds, immunosenescence begins. The thymus shrinks, the CD4+:CD8+ ratio decreases and T-cells show reduced proliferation in response to antigens, resulting in higher vulnerability to infectious and malignant disease (Pera et al., 2015; Pawelec, 2017). After approximately 40 years, thymic output declines, and DP cells drop from over 60% to under 15% of thymocytes. Due to peripheral proliferation, naïve T-cells are still present in adult lymphoid tissues and blood, despite having the most age-related decreases; these are primarily CD8+ and they are retained better in non-spleen organs, so it appears that self-renewing mechanisms vary by phenotype and compartment (Thome et al., 2014; Thome et al., 2016b). Conversely, memory cells increase and are more often found in the mucosa and other tissues (Thome et al., 2014). Humans switch from developing responses to new antigens to depending on rapid expansion of memory cells as unfamiliar pathogens are encountered less frequently.

1.3.2.1.2. T-cell phenotypes and function

CD4⁺ and CD8⁺ T-cells can be further subdivided based on their specific roles, expression of cell-surface and secreted molecules, and transcription factors, which allow them to exert different functions and be retained in different compartments. These are often referred to as lineage-defining transcription factors and the roles are frequently described as absolutes; however, it is worth bearing in mind that cells often retain considerable plasticity and can shift between phenotypes (for review see Geginat et al., 2014). It is perhaps better to regard these T-cell subsets as a spectrum of polarisation and function, rather than permanently committed lineages.

1.3.2.1.2.1. CD4+ T-cells

CD4+ cells can differentiate into multiple subsets depending on which cytokines they are exposed to during APC interaction. Th cell phenotypes have defining transcription factors, and under some circumstances, cells of one phenotype may differentiate to another, showing some flexibility. In the oldest model, T-helper cells were classed as Th1, Th2 or T-regulatory (Treg) phenotype.

When exposed to interferon (IFN) γ and IL-12, naïve T-cells upregulate the transcription factor T-bet and adopt a Th1 phenotype. They secrete IFN γ , TNF α and IL-2 to promote the cytotoxic activity of CD8+ T-cells and macrophages. Conversely, Th2 cells express the transcription factor GATA-3, are induced by IL-4, and secrete the cytokines IL-4, 5, 10 and 13 to support B-cell antibody production (Mosmann et al., 1986).

Tregs exert suppressive effects through constitutive expression of the inhibitory molecule CTLA-4, disruption of which causes lethality in mice due to myocarditis as self-reactive cells infiltrate the heart (Wing et al., 2008). This highlights their essential role in maintaining tolerance. Tregs also scavenge IL-2 through high CD25/IL-2R α expression to prevent effector T-cell proliferation (de la Rosa et al., 2004), and under certain conditions, may secrete immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF) β . For review of Treg function, see Yamaguchi, Wing and Sakaguchi, 2011; Wing and Sakaguchi, 2012. They may develop in the thymus (nTregs) or be induced in the periphery (iTregs) (Lathrop et al., 2011). Tregs recirculate and reenter the thymus using the CXCR4 receptor. In mice, this could inhibit the development of new Tregs in thymic cell cultures; in

humans, the authors could not conclusively show that recirculation inhibited further Treg development. However, it does seem that Treg development is subject to negative regulation, likely in order to prevent excessive Treg generation resulting in decreased immunity. The authors also note that peripheral Tregs proliferate upon encountering their cognate antigen, and their self-suppressing role may serve to prevent dilution of “useful” clones in the periphery (Thiault et al., 2015). For a review of Tregs, see Wing and Sakaguchi, 2010. Loss of the Treg lineage-defining transcription factor FOXP3 results in IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome as the organism becomes unable to suppress self-reactive effector cells (Bennett et al., 2001). Infants have more Treg cells, and these Tregs are found primarily in mucosal sites as compared to adults, where Tregs are found primarily in LNs. This may occur as adults are more dependent on memory effector populations, whereas in the infant, a repertoire of effector cells is still being established, and exposure to new pathogens often happens at mucosal sites (Thome et al., 2016a).

This simple model was disrupted by the discovery of further CD4⁺ effectors such as Th0 cells, which secreted both Th1 and Th2-associated cytokines (Mosmann and Sad, 1996).

The Th17 lineage was first described in 1999, when clones were isolated from rheumatoid arthritis patients to identify the source of pro-inflammatory IL-17. These authors found IFN γ secretion in some IL-17⁺ clones, suggesting some interrelationship between Th1 and Th17 cells; no Th2 clones produced IL-17. The authors suggested IL-17-secreting cells may be a novel subset or may be early in

their differentiation paths (Aarvak et al., 1999). It was later shown that Th17 cells are specified by the transcription factor ROR γ t, and in mice, ROR α (Ivanov et al., 2006; Yang et al., 2008).

IFN γ is also able to suppress Th17 development (Mangan et al., 2006), suggesting cross-inhibition of two closely-related developmental paths. The interrelationship between IFN γ and IL-17 secretion later led to much discussion about the interrelationship between Th1 and Th17 lineages (for review of Th17 cells, see Peck and Mellins, 2010). Antagonism between Th17 and Treg cells was also noted in mice: Tregs are specified by FOXP3 following TGF β exposure, whereas Th17 cells require IL-6 and TGF β (Bettelli et al., 2006; Mangan et al., 2006). Later experiments showed that the murine models of Th17 development and cytokine interactions were also applicable to humans (Annunziato et al., 2007).

Around the same time, T follicular helper cells (Tfh) were discovered. These cells express CXCR5, which allows them to migrate to the germinal centre (GC) in SLOs. Here, they express ligands essential for B-cell development to support antibody secretion (Breitfeld et al., 2000). In line with their specialised function, Tfh cells were found to have a distinct transcriptional profile and may be specified by the BCL6 transcription factor (Chtanova et al., 2004). Also in the early 2000s, Th22 cells were described as a tissue-specific subset, secreting IL-22 to promote epithelial immunity and wound healing, and playing a role in inflammatory skin diseases (Eyerich et al., 2009). Concurrently, Th9 cells were characterised as murine cells that secrete IL-9 without IL-4 and are specified by TGF β with IL-4. These cells appeared to begin differentiating along a Th2 pathway but altered their phenotype on exposure to

TGF β , even after the point at which they were restricted out of Th1 development (Veldhoen et al., 2008). For a review of T-cell subsets and plasticity, see Caza and Landas, 2015. These subsets are illustrated in Figure 1.03.

To conclude, CD4⁺ T-cells have diverse phenotypes and functions, many of which are tissue-specific and highly specialised. Some debate exists over to what extent these are discrete phenotypes as opposed to naturally occurring variation within broader phenotypes. While newer subsets such as Th17 and Th22 have been primarily characterised using human cells, the archetypal Th1 and Th2 subsets were largely studied in mice, and thus cross-subset comparisons relying on established knowledge of Th1 and 2 subsets must be made cautiously, utilising human-applicable knowledge. Furthermore, many of these experiments take place primarily *ex vivo*, and even when the subsets described are clearly also applicable *in vivo*, function may vary in unforeseen ways. T-cell diversity is not static, but changes with age, in ways that also appear to be compartment-specific. Therefore not only is it erroneous to make rapid inferences between mouse and human, but it may also be poor practise to do so between child and adult, or younger and older adults. The effect of aging on the immune system likely has significant implications for development of many diseases.

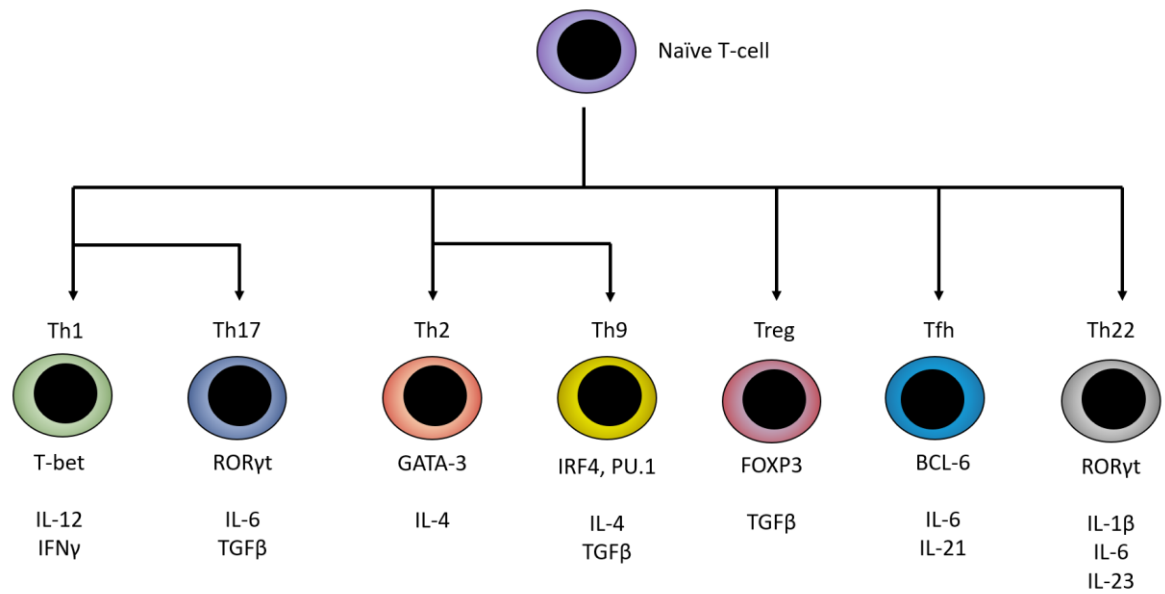


Figure 1.03. CD4⁺ T-cell subsets. Naïve T-cells can adopt a variety of phenotypes, depending on which cytokines they are exposed to during antigen encounter. For each subset, the lineage-defining transcription factor or factors are shown immediately below, followed by the specifying cytokine or cytokines.

1.3.2.1.2.2. CD8+ T-cells

While most subtyping work focuses on CD4+ T-cells, there is also some literature devoted to characterising cytotoxic T-cell subsets. However, in most contexts cytotoxic T-cells are regarded as a relatively homogenous population with little phenotypic diversity.

In the mid-nineties, murine Tc1 and Tc2 subsets were described. Like Th1, Tc1 cells are specified by exposure to pro-inflammatory cytokines such as IL-12 and IFN γ , and go on to secrete these cytokines; Tc2 are induced by IL-4 and secrete Th2-associated cytokines, though in both cases, Tc cells secreted less total cytokine than their Th counterparts. Functional assays revealed that neither phenotype assisted B-cell antibody production, and both were cytotoxic (Croft et al., 1994; Sad, Marcotte and Mosmann, 1995). Furthermore, both Th1 and Tc1 are specified by the T-bet transcription factor, and both Th2 and Tc2 by GATA-3 – which is completely understandable as these transcription factors are downstream of the specific lineage-determining cytokines (Sullivan et al., 2003).

In addition, CD8+ counterparts also exist for Th9, Th17, Tfh and Th22 CD4+ cells. Tc17 can be induced by TGF β with either IL-21 or IL-6, and unlike other Tc subsets, has impeded cytotoxic behaviour due to a lack of granzyme B and IFN γ , which has been shown both *in vitro* and *in vivo* using the experimental autoimmune encephalomyelitis (EAE) murine model of multiple sclerosis (MS) (Huber et al., 2009). Follicular cytotoxic, or Tfc, cells, like Tfh, use CXCR5 to enter B-cell follicles and appear to depend on this specialised environment for their continued survival, at least in mice. These cells are suggested to perform immunosurveillance and

eliminate infected Tfh and B-cells, which may be important for maintaining humoral immunity (for review of Tfc cells, see Yu and Ye, 2018). Finally, a CD8⁺ Treg subset has been identified (for review of CD8⁺ Tregs, see Yu et al., 2018). For the sake of clarity, Tregs will be assumed to be CD4⁺ in the rest of this thesis unless otherwise specified.

1.3.2.1.3. T-cell activation

T-cell activation is often described using the two-signal model. The first signal is the interaction of TCR and MHC-antigen complexes. Following TCR ligation, T-cells also require a costimulatory signal, without which they enter a hyporesponsive state termed anergy.

The second signal is generally transmitted via CD28 on the T-cell, which is constitutively expressed and binds a costimulatory ligand – either CD80 or CD86 – on the APC. A second member of the CD28 family exists: ICOS, or inducible costimulator, which binds ICOSL on APCs. ICOSL was identified in mouse tissues and B-cells as a CD80/86 homolog that bound an unspecified receptor to promote T-cell proliferation (Swallow, Wallin and Sha, 1999). The ICOS receptor itself was discovered on human cells in the same year. Unlike CD28, ICOS is not constitutively expressed and is only detectable at the cell surface after TCR engagement, when T-cells are activated (Hutloff et al., 1999). ICOS ligation promoted IL-4, IL-5, IFN γ and TNF α , but not IL-2, secretion; given that ICOSL is induced by TNF α , this may form a feedback loop promoting continued T-cell stimulation. Mouse experiments suggest that ICOS signalling is required for memory T-cell expansion and survival (Burmeister et al., 2008) and for Tfh cells to support antigen-specific antibody production by B-cells, in concordance with its expression in GCs (Hutloff et al., 1999; Akiba et al., 2005). In mice, ICOS can substitute for CD28 to maintain normal immune function and antibody production (Linterman et al., 2009).

Following ligation of the TCR and CD28, a signalling cascade is initiated via the kinase LCK, which is bound to the intracellular region of CD4 or 8. LCK

phosphorylates ITAM motifs in the intracellular CD3 ζ chain to promote signalling through the kinase ZAP70, as does the kinase FYN. CD45 phosphatase is required to keep LCK in a conformationally active state, although it can also prevent LCK activation, and excluding CD45 from signalling regions may be required for appropriate signalling (Douglass and Vale, 2005; Furlan et al., 2014). Recruitment of scaffold proteins and further signalling follows, resulting in cytoskeletal rearrangement to permit T-cell mobility and alterations in gene expression via the transcription factors NF κ B, NFAT and AP-1 (for review see Bernard, Lamy and Alberti, 2002).

It is clear at this point that TCR signal transduction is temporally regulated; there also appears to be a spatial component to this regulation. Tight control prevents prevent aberrant T-cell activation, which is at best a waste of energy and resources, and at worst actively damaging through increased inflammation. However, there are large parts of the process which require further study. It is often stated that the TCR requires its CD4 or CD8 coreceptor to bind MHC; coreceptors stabilise the interaction, but initial TCR-MHC interactions and subsequent signalling occur independently of coreceptors. LCK is reputed to be bound to intracellular CD4 or CD8 and to create ZAP70 binding sites, but it can also be targeted directly to the membrane, and another kinase may be relevant, allowing ZAP70 to bind before LCK is involved (for review, see Gascoigne et al., 2011). Spatially, LCK and CD4 are contained in sphingolipid- and cholesterol-rich membrane microdomains; the TCR may be excluded from these outside of MHC engagement to limit erroneous T-cell stimulation (Janes, Ley and Magee, 1999), in keeping with the theory that membrane segregation is a key regulator of signalling cascades (Simons and

Ikonen, 1997; Otáhal et al., 2010). Other work suggests that protein-protein interactions also localise specific molecules to discrete regions of the membrane (Janes, Ley and Magee, 1999; Douglass and Vale, 2005). There is now more focus on integrating these seemingly disparate pieces of information into a more comprehensive model of TCR signalling (Filipp, Ballek and Manning, 2012). Furthermore, the general consensus is that the IS is required for full T-cell activation; however, LCK, ZAP70 and the TCR itself vanish from the IS before post-activation division begins (Lee et al., 2002); these authors suggest that the IS may be required for non-TCR signalling or to promote TCR internalisation. Overall, despite the general consensus that T-cell activation and signalling is well understood, the truth is that many questions remain about the sequence of events and the molecules responsible.

Once activated, T-cells proliferate and undergo clonal expansion, resulting in many T-cells all able to recognise the same antigen. T-cells may then enter peripheral circulation and exit the vasculature at sites of inflammation. They express integrins and selectins, which bind to ICAM-1 and VCAM-1, or glycosylated selectins, on ECs. Following this tethering process, T-cells roll along the capillary wall, allowing them to respond to chemokine signalling. They then crawl along the endothelium to reach an appropriate site for extravasation (for review, see Engelhardt and Ransohoff, 2012). After migrating to the periphery, T-cells require recognition of their cognate antigen to be retained in tissue; otherwise, they will return to the vasculature.

1.3.2.1.4. T-cell inhibition: immune checkpoints

Uncontrolled T-cell activation can result in chronic inflammation. Furthermore, thymic mechanisms to establish tolerance are not perfect, and some T-cells do begin to recognise self-antigens, leading to autoimmunity, which can be life-threatening. Thus the mammalian organism requires mechanisms to inhibit uncontrolled T-cell activation and proliferation, which include Tregs and immune checkpoints (ICs). IC molecules are upregulated following T-cell activation and can be used to identify exhausted cells with low levels of activity. Some of the best-known ICs – PD-1, CTLA-4, TIM-3 and LAG-3 – are detailed below in a specifically immunological context. HIV studies have identified PD-1, TIM-3 and LAG-3 as markers of T-cell exhaustion and dysfunction (Jones et al., 2008; Okazaki et al., 2011; Huang et al., 2015; Tian et al., 2015), and the latter two are expressed on distinct subsets from PD-1. It remains to be seen whether LAG-3 and TIM-3 together define a second functionally abnormal subset, or three or more exhausted T-cell phenotypes are present in chronic viral infection. Regardless, all three markers are likely also to indicate T-cell exhaustion in malignancy and have been suggested as targets for immunotherapy, both in isolation and combination, as tumours often exploit these brakes on immunity by expressing IC ligands to avoid destruction. (Huang et al., 2017).

1.3.2.1.4.1. PD-1

Human PD-1 was identified in 1994 (Shinohara et al., 1994), with its ligands following shortly thereafter. PD-L1 was identified based on similarity to CD80 and CD86 (Dong et al., 1999) and quickly shown to be a PD-1 binding partner (Freeman et al., 2000). PD-L2 was identified by two groups in 2001. Latchman et al. identified it in mouse cells, where it inhibited TCR-stimulated proliferation and cytokine secretion. They saw both PD-1 ligands induced cell cycle arrest after TCR engagement of murine CD4⁺ cells without promoting apoptosis. PD-1 was more markedly upregulated on T-cells in response to low levels of antigen, which the authors noted may limit weak antigen responses, autoimmunity or unnecessary inflammation (Latchman et al., 2001). In the same year, Tseng et al. identified PD-L2 in human and mouse DCs, but not macrophages, and noted its sequence similarity and proximity to PD-L1. Notably, they described PD-L2 as a costimulatory molecule expressed on DCs: with suboptimal CD3 ligation, PD-L2 induced proliferation and IFN γ secretion (Tseng et al., 2001).

Today, the interaction of PD-1 with its ligands is known to inhibit T-cell proliferation and cytokine secretion. Both ligands have been shown to suppress cytokine secretion *in vivo* and *in vitro* (Latchman et al., 2001; Keir et al., 2006). PD-L1 is upregulated following exposure to IFN γ (Freeman et al., 2000). PD-1 was upregulated on T-cells and PD-L1 on splenocytes in chronic viral infection; blocking the PD-1/PD-L1 or 2 interaction was sufficient to restore some T-cell function, including CD8⁺ proliferation, degranulation and IFN γ production, even in the absence of support from CD4⁺ cells (Barber et al., 2006). Both ligands may also be expressed in murine brain tissue. In mice with EAE, astrocytic PD-L1 correlated with

disease severity, and anti-PD-L1 facilitated T-cell expansion and cytokine secretion, whereas anti-PD-1 or -PD-L2 worsened disease (Salama et al., 2003). Thus it appears that PD-1 signalling plays a role in maintaining self-tolerance and that PD-1 signalling disruption may have adverse clinical outcomes in autoimmunity.

Structurally, PD-1 contains an immunoreceptor tyrosine inhibition motif (ITIM) and immunoreceptor tyrosine switch motif (ITSM), allowing the recruitment of phosphatases SHP-1 and 2 to dephosphorylate CD3 ζ , thus inhibiting ZAP70 activity; the ITSM appears to be most important for this (Latchman et al., 2001; Chemnitz et al., 2004; Sheppard et al., 2004; Yokosuka et al., 2012). PD-1 can also inhibit other signalling pathways. By interacting with the murine TCR *in vitro*, it destabilises the IS and blocks CD28 from interacting with the kinase PKC θ , which is necessary for activation of T-cell transcription factors AP-1, NFAT and NF κ B (Yokosuka et al., 2012). PD-1 engagement can also inhibit the MAPK-ERK and PI3K/AKT signalling paths in T- and B-cells (Okazaki et al., 2001; Sheppard et al., 2004; Parry et al., 2005).

PD-1/PD-L1 signalling can disrupt the interactions between T-cells and DCs by countering the DC-induced reduction in T-cell movement to promote APC interaction; blockade can reverse this effect and lead to diabetes in susceptible mice (Fife et al., 2009). Two groups have demonstrated that pancreatic PD-L1 expression is required to prevent diabetes (Fife et al., 2006; Keir et al., 2006). Furthermore, PD-1 knockout in BALB/c mice results in early death through cardiomyopathy; heart tissue typically expresses PD-L1 and so it seems likely that death in these mice is through a failure of self-tolerance (Nishimura et al., 2001). Clearly, tolerance in the

periphery is maintained through several mechanisms, and continued PD-1 engagement is required to suppress self-reactive T-cells; PD ligand expression only on APCs is not sufficient. This explains the widespread expression of PD-L1 in nonlymphoid tissues (Dong et al., 1999; Latchman et al., 2001).

1.3.2.1.4.2. CTLA-4

CTLA-4 (cytotoxic T-lymphocyte associated protein 4) is a coinhibitory receptor related to CD28 and ICOS, expressed on the T-cell surface following activation.

CTLA-4 binds the costimulatory ligands CD80 and CD86 with a higher affinity than CD28 (Chen, 2004). *In vitro* experiments showed that CTLA-4 removes costimulatory ligands from the APC surface, resulting in their endocytosis and degradation in the T-cell. The affected APC is then more likely to induce anergy than full activation when interacting with subsequent T-cells, until more ligands can be brought to the membrane. These authors found a 1:8 ratio of CTLA-4:CD86 to be sufficient for depletion, but did not perform the same experiments with CD80 (Qureshi et al., 2011).

Blockade of CTLA-4 can also prevent diabetes in susceptible mice, but does not affect the DC-mediated reduction in T-cell movement, illustrating that PD-1 and CTLA-4 play slightly different roles in tolerance (Fife et al., 2009). Furthering this is the fact that both proteins inhibit the PI3K/AKT signalling pathway, but while CTLA-4 signalling acts through PP2A phosphatase, PD-1 utilises PTEN (Parry et al., 2005; Patsoukis et al., 2013), suggesting potential synergistic function between the two molecules.

1.3.2.1.4.3. TIM-3

The T-cell immunoglobulin and mucin domain 3 (TIM-3) signalling axis is widely mentioned in the context of cancer immunotherapy. The main ligand for TIM-3 is the soluble carbohydrate-binding protein galectin-9. TIM-3 is a transmembrane protein with an extracellular Ig fold and mucin domain (Monney et al., 2002) and is able to modulate T-cell function through several mechanisms.

TIM-3 appears to be a greater regulator of Th1 than Th2 cells. In mice, galectin-9 treatment depletes antigen-specific IFN γ -producing cells while sparing Th2 cells; this is sufficient to reduce EAE severity, which was worsened by anti-TIM-3 antibodies as these induced macrophage activation (Monney et al., 2002; Zhu et al., 2005). Accordingly, it is expressed more on human Th1 and Th17 than Th2 cells, although it is present on all three phenotypes; in humans, it is associated with naïve cells, and in mice, with memory CD4 $^{+}$ cells (Zhu et al., 2005; Hastings et al., 2009). Blockade of TIM-3 alongside CD3/CD28 stimulation promoted Th1- but not Th2-associated cytokine secretion (Hastings et al., 2009). In Jurkat cells, TIM-3 ligation suppresses IL-2, and its overexpression results in NFAT and AP-1 inhibition (Lee et al., 2012). TIM-3 $^{+}$ cells isolated from HIV patients were typically Tc1/Th1 and dysfunctional, but function could be rescued with TIM-3 blockade; the same paper found that, in these patients, TIM-3 positivity correlated with T-cell activation, except in the case of viral controllers (Jones et al., 2008). Another group found CD8 $^{+}$ TIM-3 $^{+}$ cell function to be restored with blockade in HIV patients, but not in other viral infections (Sakhdari et al., 2012).

TIM-3 is further involved in apoptosis by heterodimer formation with CEACAM1, which transmits an apoptotic signal on galectin-9 binding; both proteins are upregulated after T-cell activation, and the TIM-3+CEACAM1+PD-1+ signature is associated with T-cell exhaustion (Huang et al., 2015). These effects extend to macrophage function. Phosphatidylserine (PS) is exposed on the outer membrane leaflet during apoptosis; TIM-3-transfected cells can bind apoptotic cells, and TIM-3 variants with higher PS affinity result in more efficient phagocytosis of apoptotic thymocytes. Human macrophages, but not monocyte cell lines, express TIM-3 (DeKruyff et al., 2010).

This protein also translocates to the IS to inhibit T-cell activation, and blockade enables CD8+ cells to form a more stable IS. TIM-3 may recruit galectin-9 to the IS, which can bind CD45 and CD148 phosphatases to suppress TCR signalling, supported by the lack of colocalisation between TIM-3 and conformationally active LCK (Clayton et al., 2014). Intracellularly, TIM-3 binds BAT3, which promotes T-cell function through its interaction with LCK, but only when galectin-9 is absent (Rangachari et al., 2012). Thus, it appears that TIM-3 and galectin-9 interact with numerous proteins both intra- and extracellularly to inhibit TCR signalling.

1.3.2.1.4.4. LAG-3

First identified in 1990, LAG-3, or lymphocyte activation gene 3, is a four-domain MHCII-binding protein which likely shares a common ancestor with CD4, but has an additional loop in the extracellular domain (Triebel et al., 1990; Baixeras et al., 1992). LAG-3 also binds galectin-3, FGL-1, α -syn and LSECtin. LAG-3 is typically membrane-bound but a soluble isoform does exist. The two most membrane-distal domains are required for MHCII binding, and stable interactions with MHCII appear to require oligomerisation (Huard et al., 1997).

LAG-3 has a higher affinity for MHCII than CD4 does, but it was quickly shown that LAG-3 likely does not outcompete CD4 to inhibit T-cell activation (Huard et al., 1996a; Huard et al., 1996b). Instead, LAG-3 preferentially binds peptide-associated MHCII and accumulates at the IS, where it can suppress self-reactive T-cells by signalling through its cytoplasmic domain. It is possible that LAG-3 and the TCR are sequestered in different membrane microdomains and only interact during MHCII binding (Hannier et al., 1998; Hannier and Triebel, 1999; Maruhashi et al., 2018).

LAG-3 crosslinking inhibits T-cell proliferation, proinflammatory cytokine secretion and can downregulate surface TCR levels (Hannier et al., 1998). Like other inhibitory molecules, it is not constitutively present but upregulated 48 hours post-stimulation, which is promoted by IL-2 and -12 (Baixeras et al., 1992). It is preferentially expressed on Th0 and Th1 over Th2 clones (Annunziato et al., 1996). LAG-3 deficient Tregs are functional in vitro but fail to prevent colitis in vivo, possibly due to their inability to prevent Th proliferation and cytokine secretion (Do et al., 2016). LAG-3-null mice shown increased T-cells, which appear unresponsive to

inhibitory signals as they continue to proliferate and secrete IL-2 and IFN γ post-stimulation (Workman and Vignali, 2003; Workman and Vignali, 2005). LAG-3 deletion has varied effects on different murine backgrounds: in the non-obese diabetic (NOD) model, it results in accelerated development of diabetes as self-reactive T-cells infiltrate the pancreatic islets (Bettini et al., 2011; Okazaki et al., 2011); in the BALB/c strain, autoimmune Th1 cells infiltrate the heart, resulting in early death from myocarditis (Okazaki et al., 2011). These experiments conclusively show that LAG-3 suppresses autoimmunity by limiting T-cell replication.

In HIV patients, LAG-3 expression correlates negatively with survival and positively with increased viral load; blockade promoted HIV-specific T-cell proliferation (Tian et al., 2015). It was preferentially expressed on memory cells (Tian et al., 2015), which dovetails with the finding in mice that LAG-3 functions in regulating the memory cell pool size post-viral infection, although whether CD4 $^{+}$ or CD4 $^{+}$ and CD8 $^{+}$ subsets were affected appeared to vary with the type of virus (Workman et al., 2004).

Of note, two groups have noted that PD-1 and LAG-3 were expressed on different T-cell subsets and suggested blockade of both receptors could act synergistically to restore T-cell functions (Okazaki et al., 2011; Tian et al., 2015).

The cytoplasmic domain contains a unique KIEELE motif and EX repeats, conserved across human and mouse; the KIEELE motif has been suggested to be key for its suppressive functions (Workman, Dugger and Vignali, 2002; Workman and Vignali, 2005; Tian et al., 2015). Another group, however, were unable to

confirm the absolute requirement observed by Workman and Vignali for the KIEELE motif and instead observed a requirement for an FXXL sequence and the distal EX repeat to inhibit T-cell stimulation as determined by IL-2 secretion. However, it is worth noting that the FXXL-disrupted sequence used in these experiments had a complete KIEELE motif, which the EX-disrupted sequence did not (Maeda et al., 2019). Thus, the finer details of LAG-3-mediated suppression are still unknown.

1.3.2.2. B-cells

B-cells develop in the mammalian bone marrow and are the effectors of humoral adaptive immunity; however, they also function in innate immunity through secretion of broad-specificity IgM antibodies termed natural antibodies, which is outside the scope of this thesis. B-cells can also act as professional APCs, and like T-cells, use VDJ recombination to produce a functional BCR. The BCR is an Ig fixed to the cell surface, with two heavy and two light chains (HC and LC). HCs encode the constant region to determine antibody isotype, which may be IgA, IgD, IgE, IgG or IgM; various IgA and IgG subtypes exist. The BCR is bound to a CD79a and b (Ig- α and - β) heterodimer, without which signal transduction cannot occur. The antibody secreted by plasma cells is a soluble version of the membrane-bound BCR.

1.3.2.2.1. B-cell development in bone marrow

Human B-cells, like T-cells, pass through several developmental stages. They originate either from CLPs, or from pre-pro-B-cell precursors arising from CD34+ stem cells. Either population can give rise to mature B-cells (Sanz et al., 2010).

Pro-B-cells are committed to the B-cell lineage by the transcription factor PAX5 (for review of PAX5 function, see Medvedovic et al., 2011). A functional IgM HC is produced by VDJ recombination and complexes with a surrogate LC to form a pre-BCR (Wang et al., 1998; for review, see Almqvist and Mårtensson, 2012); both stages are essential for B-cell maturation (Yel et al., 1996; Minegishi et al., 1998). The pan-B-cell marker CD19 is expressed from this point onwards (Nadler et al., 1984; Wang et al., 1998). Pre-B-cells undergo VJ recombination at the LC locus to produce a functional BCR, and begin to upregulate MHCII (Carsetti, Köhler and Lamers, 1995). Self-reactive immature B-cells can undergo receptor editing, resulting in expression of IgD instead of IgM. Immature B-cells then exit the BM and navigate to SLOs, where they are termed transitional B-cells. As with T-cells, BM egress depends on S1P1 expression, without which mature B-cell levels will be depleted (Allende et al., 2010).

In SLOs, transitional B-cells can undergo somatic hypermutation (SHM) and class-switch recombination (CSR) in germinal centres (GCs) in order to become terminally-differentiated plasma cells that secrete high-affinity antibodies. Although cells at the transitional stage express a functional BCR, SHM of the variable region improves antigen binding, and CSR at the constant region allows production of IgA, IgE and IgG antibodies. Thus, B-cells must proceed through another round of selection in the SLO once antigen presentation occurs. This is shown in Figure 1.04.

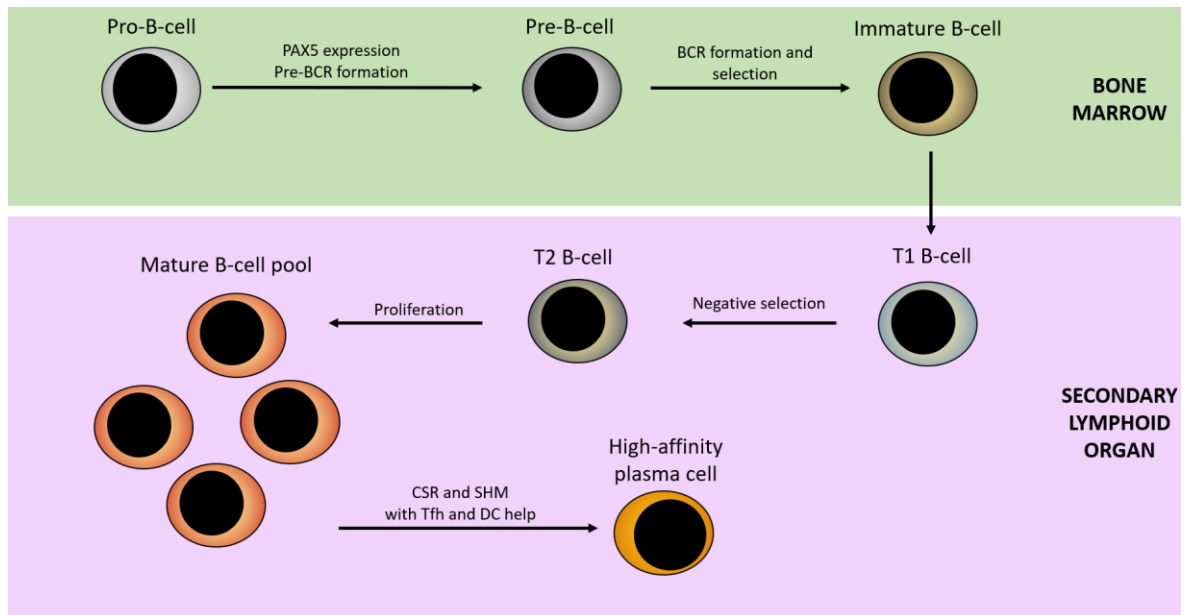


Figure 1.04. B-cell development. Pro-B-cells undergo VDJ recombination of the Ig heavy chain, which forms a pre-BCR in complex with a surrogate light chain. A BCR is formed following VJ recombination of the light chain in the pre-B-cell. Immature B-cells enter an SLO and become transitional B-cells, progressing through rounds of negative selection and proliferation to establish mature B-cells. These undergo CSR and SHM, followed by further rounds of selection, to become antibody-secreting plasma cells.

1.3.2.2.2. *Germinal centres*

At the point of entry to SLOs, B-cells are described as transitional (T1 and T2) B-cells. Negative selection occurs to maintain tolerance, and targets cells through IgM crosslinking, which they are protected from in the BM stroma (Tiegs, Russel and Nemazee, 1993). T1 cells are negatively selected; conversely, T2 cells should have established self-tolerance and proliferate on BCR engagement. This positive selection establishes a pool of mature B-cells (Su and Rawlings, 2002). Receptor editing may also occur in SLOs, followed by clonal deletion if self-reactivity is still unresolved (Tiegs, Russel and Nemazee, 1993). After this initial antigen encounter, a GC can be established to facilitate plasma cell development.

In the GC, immature B-cells interact with follicular DCs, which present antigen, and Tfh cells, which support their proliferation and differentiation into memory and plasma cells (MacLennan, 1994). This requires B-cells to obtain antigen from DCs and present it to T-cells using MHCII; higher-affinity clones may obtain more antigen, thus receiving more support. Tfh cells express CD154, which interacts with CD40 on B-cells to promote anti-apoptotic gene expression and cytokine secretion, promoting further T-cell and DC recruitment. CD154 mutation results in hyper-IgM syndrome (HIGM) type 1, wherein defective CSR results in immunodeficiency via reduced IgA, IgE and IgG antibodies (Allen et al., 1993; Fuleihan et al., 1993). T-cell assistance and CD40/CD154 interactions are indispensable for correct B-cell development. CD40 blockade is not sufficient to completely inhibit plasma cell differentiation, though antigen presentation blockade is. A two-signal model of plasma cell maturation has been proposed, wherein the first step is BCR engagement and the second is Tfh cell help (Kräutler et al., 2017).

1.3.2.2.3. SHM, CSR and plasma cell maturation

SHM and CSR generate high-affinity antibodies, and both depend on activation-induced cytidine deaminase (AID). Mutations of this enzyme result in HIGM2, characterised by reduced SHM and CSR and abnormal GC anatomy (Revy et al., 2000). For an overview of the role of AID, see Pavri and Nussenzweig, 2011; for genetic elements of SHM and CSR, see Chi, Li and Qiu, 2020.

SHM is most prevalent in the CDRs, but in broadly-neutralising anti-HIV antibodies, non-CDR framework regions are also mutated and confer higher binding avidity and neutralising ability; in some clones, this also confers greater thermal stability (Klein et al., 2013). In CSR, antibody isotype is altered by replacement of the IgM gene segment with a new HC constant region. AID was confirmed to be the enzyme responsible for CSR in murine experiments in 2000 (Muramatsu et al., 2000). It is worth noting that some researchers have recently suggested that CSR occurs before SHM and takes place outside GCs. These authors identified class-switched antibody production prior to GC formation (Roco et al., 2019). As with T-cells, there is still much that is unknown about B-cell biology.

Following SHM and CSR, B-cells can develop either to a memory phenotype or become antibody-secreting plasmablasts, the precursors to plasma cells. Plasma cells are often regarded as short-lived non-proliferating cells, but a long-lived population does exist. Long-lived plasma cells are dependent on specific tissue niches for their survival.

Plasma cell differentiation is limited to high-affinity cells; low-affinity cells persist in the GC without dying (Phan et al., 2006). A further population of plasma cells is found in BM. These cells often have fewer mutations than splenic plasma cells, suggesting selection for long-term plasma cells can occur early in the GC reaction (Smith et al., 1997). Memory B-cells are also generated in the GC reaction and may derive from lower-affinity clones (Shinnakasu et al., 2016). For a review of plasma cell biology, see Radbruch et al., 2006.

At this point, it is also worth noting the existence of tertiary lymphoid structures (TLS), which recapitulate the function of the GC outside SLOs and can develop at the site of long-term inflammation in solid tumours (for review see Jacquelot et al., 2021).

1.3.2.3. The mononuclear phagocyte system

Mononuclear phagocytes comprise monocytes, macrophages and DCs. Monocytes are circulating myeloid cells derived from the BM and can differentiate into BMDMs and BMDCs. Macrophage itself is also a broad term: there are several subsets of tissue-resident macrophages with specialised functions, such as brain-resident microglia, alveolar macrophages and many others (Chazaud, 2014). Unlike T- and B-cells, they do not undergo multiple developmental steps in specific organs, and they do not recognise specific antigens, meaning there is no requirement for tolerance, selection and clonal expansion. Instead, monocytes and macrophages respond to chemokines and pro- and anti-inflammatory molecules in their vicinity, resulting in high levels of plasticity. As a result, they play roles in innate immunity, but also function in adaptive immunity through their ability to secrete cytokines, thus modulating local immune responses, and their role as professional APCs.

1.3.2.3.1. BMDM and microglial development and activation

Circulating monocytes originate from HSCs in the BM. In mice, HSCs give rise to common myeloid progenitors, then a granulocyte/macrophage progenitor (Akashi et al., 2000), which further differentiates to a monocyte and DC-restricted progenitor (Fogg et al., 2006). In humans, the granulocyte/monocyte/dendritic cell progenitor gives rise to separate monocyte- and DC-restricted progenitors (Lee et al., 2015). A further human monocyte-generating population that does not pass through a stage with DC differentiation potential also seems to exist (Kawamura et al., 2017). The transcription factor IRF8 is required for monocyte and DC maturation; IRF8-mutant patients lack both cell types and show severe immunodeficiency (Hambleton et al., 2011).

Circulating human monocytes are a heterogeneous population. Three phenotypes exist: classical CD14⁺⁺CD16⁻, intermediate CD14⁺⁺CD16⁺, and non-classical CD14⁺CD16⁺ (Passlick, Flieger and Zeigler-Heitbrock, 1989), of which classical are the most common, at approximately 90% of circulating monocytes. Deuterium labelling revealed that monocytes proceed from the classical to non-classical phenotype in sequential fashion. In these experiments, classical monocytes were short-lived and persisted in circulation for approximately one day; intermediate monocytes for four days; and non-classical cells for up to a week before dying or exiting the vasculature. Peripheral monocyte populations are subject to homeostasis and their numbers are replenished quickly from BM precursors, again in sequential fashion, following depletion (Patel et al., 2017).

Furthermore, each subset has differential gene and surface marker expression (Patel et al., 2017). Classical monocytes express pro-inflammatory and wound-healing molecules (Wong et al., 2011) and have high phagocytic ability, as does the intermediate subset (Mukherjee et al., 2015); intermediate and non-classical cells also have strong antigen-presentation ability (Wong et al., 2011; Mukherjee et al., 2015). Wong et al. noted that non-classical monocytes appear to be strongly phagocytic, whereas Mukherjee et al. found the inverse. A third group also described this CD14⁺CD16⁺ population to be weakly phagocytic with low levels of antigen presentation and cytokine secretion. Instead, these authors found them to engage in patrolling behaviour and function better in response to viruses, but it is worth noting that these experiments transferred human monocytes into mice and may not be applicable to humans (Cros et al., 2010). Some of these conflicting results may be explained by the fact that analysis of peripheral blood mononuclear cells (PBMCs) versus purified monocytes resulted in different CD14 and CD16 expression intensity as well as altered ratios between the three subpopulations (Mukherjee et al., 2015).

Monocyte subsets also appear to be temporally regulated: the non-classical subset becomes more prevalent with age, and presence of surface molecules such as MHCII and various chemokine receptors also changes with age; so does production of the potent monocyte chemoattractant CCL2, which may compensate for reduction in its receptor CCR2 (Seidler et al., 2010). Monocytes isolated from elderly individuals show increased secretion of other cytokines, which may contribute to inflammation and predispose some individuals to disease (Verschoor et al., 2014).

Altogether, these results illustrate both the heterogeneity and essentiality of this population.

It was described as early as 1939 that monocytes can exit vasculature and differentiate into macrophages (Ebert and Florey, 1939). They are drawn to sites of inflammation by various chemokines (for review see Shi and Pamer, 2011). Upon extravasation, monocytes are exposed to a complex mixture of molecules, including damage-associated molecules, cytokines and chemokines, and potentially, pathogen-associated molecules. Each of these may influence the phenotype adopted by the mature macrophage.

Additionally, several populations of tissue-resident macrophages exist throughout the body. Brain-resident macrophages are termed microglia; they account for perhaps 10% of CNS cells and were originally described by del Río-Hortega (translated in Sierra et al., 2016). Microglia are effectors of innate immunity which constantly survey their surroundings and exhibit phagocytic behaviour upon activation (for review of microglial innate immunity, see Rivest, 2009). In the steady state, microglia are responsible for phagocytosis of debris and apoptotic cells, thus preventing unnecessary CNS inflammation. Additionally, they play a role in synapse formation and elimination (reviewed in Andoh and Koyama, 2021).

Both microglia and other tissue-resident macrophages are myeloid immune effectors, and historically, it was assumed that the microglial compartment was renewed by influx and differentiation of monocytes. Earlier research did show invasion of macrophage cells into the developing murine brain and subsequent

differentiation into microglia (Perry, Hume and Gordon, 1985). These infiltrating cells were later determined to be primitive macrophages derived from the embryonic yolk sac, and this paper also showed that the majority of murine microglial development took place after establishment of the BBB, providing strong evidence that early microglia do not derive from circulating monocytes (Alliot, Godin and Pessac, 1999). However, mammalian microglial ontogeny was still debated for several more years. In 2007, Ajami et al. showed that microglia self-renew and the compartment is not maintained by peripheral monocytes; work by Ginhoux et al. in 2010 then showed that microglia originate from yolk sac macrophages that enter the brain during embryonic development. For the purposes of clarity, both cell types will be collectively referred to as macrophages unless distinction is necessary, when they will be referred to as BMDMs and microglia.

1.3.2.3.2. *The M1/M2 model*

The historic dichotomy of macrophage activation proposed two phenotypes, based on *in vitro* differentiation of murine monocytes and reflective of the Th1/Th2 model, with which it was associated. Pro-inflammatory M1, or classically activated, macrophages, were induced by IFN γ , TNF α and bacterial products such as LPS (lipopolysaccharide); and anti-inflammatory M2, or alternatively activated cells, were induced by Th2 cytokines (Mills et al., 2000). M1 macrophages were regarded as phagocytic IL-12-producing cells, whereas M2 were thought to promote wound healing and modulate immune responses via anti-inflammatory IL-10; the initial M1/M2 dichotomy has been a useful tool in a variety of pathologies and is still widely used today.

Since much early macrophage polarisation work was carried out *in vitro*, experiments typically used one or two distinct stimuli – hardly reflective of the many chemokines macrophages *in vivo* would encounter. Similarly, M1/M2 macrophages were distinguished using limited numbers of markers, often one or two per phenotype, when large panels of multiple markers offer far more insight into various behaviours. However, in the early days of flow cytometry (FC), such experiments were not always practical, and they still are not today for non-flow cytometric techniques.

However, as outlined by Mantovani et al., 2004 and Mosser and Edwards, 2008, the alternately activated macrophage category encompasses cells polarised by a wide variety of stimuli. They note that M1 and M2 are not discrete categories, but extreme ends of a spectrum of plasticity. Mantovani et al. proposed several further subsets:

M2a cells induced by IL-4 or -13; toll-like receptor stimulation- or immune complex-induced M2b cells; and M2c, resulting from IL-10 or glucocorticoid stimulation (for review of macrophage subsets, see Mantovani et al., 2004). Mosser and Edwards also identified the same oversimplification and suggested functional categorisation of macrophages based on pro-inflammatory, wound-healing and regulatory behaviour (Mosser and Edwards, 2008).

More recently, transcriptomic analysis using 29 conditions including non-M1/M2-associated stimuli, such as fatty acids or chronic inflammation-related molecules, further disrupted the M1/M2 dichotomy. These authors described a spectrum model of macrophage activation with 10 distinct transcriptional programs induced by different stimuli. While this still does not recapitulate the complexity of an *in vivo* model, it may be a closer approximation (Xue et al., 2014). Eventually, the volume of terminology around macrophage subtyping led to efforts to create a more rigorous, standardised framework for macrophage nomenclature and experimental procedures (Murray et al., 2014).

1.3.2.3.3. *Phagocytosis and antigen presentation*

While macrophages play roles in innate immunity, they also function in adaptive immunity by presenting antigen, thus allowing activation and expansion of T-cells. This necessitates phagocytosis such that peptides can be obtained and processed into presentable antigens, which also has the concurrent benefit of clearing pathogens or transformed cells from the site of inflammation.

Regulation of phagocytosis is a complex area of biology, and is mediated in a cellular context both by pro-phagocytic signals such as PS and protective molecules such as CD47. For this reason, multiple surface receptors are involved in target recognition, which may range from apoptotic cells to opsonised bacteria. Upon binding of multiple receptors, actin remodelling takes place, allowing engulfment of the target in a structure called the phagosome. As the phagosome matures, it merges with endosomes and lysosomes and becomes highly acidic, resulting in degradation of the target. For a review of phagocytosis, see Rosales and Uribe-Querol, 2017. Resultant peptides can then be loaded onto MHC molecules and trafficked to the cell surface.

While multiple APC populations exist, several differences are present between them. One commonly-cited difference between macrophages and DCs is cross-presentation: the ability to present exogenous antigens on MHCI instead of MHCII, thus activating CD8⁺ T-cells, which was historically thought to be unique to DCs. Intriguingly, recent work has found that macrophages can also cross-present antigen both *in vitro* and *in vivo*. This ability extends to murine tissue-resident macrophages as well as human tumour-associated macrophages (TAMs), although

the latter did not express costimulatory ligands, resulting in a lack of T-cell activation (reviewed in Muntjewerff, Meesters and van den Bogaart, 2020). Further differences also exist in terms of efficiency – DCs are more efficient – and location. T-cell priming by DCs almost exclusively occurs in LNs close to the site of inflammation, whereas macrophages can present antigen at the site itself. This is common to both tissue-resident macrophages and BMDMs, and is essential for T-cell retention in the tissue, as activated T-cells will typically return to the vasculature if their cognate antigen is not present.

Cell-cell interactions that occur during phagocytosis can also have further effects on macrophage biology. T-cells express CD154, which ligates CD40 at the macrophage surface, resulting in upregulated of antigen-presentation machinery and pro-inflammatory cytokines such as IL-1 β and IL-12, which further support T-cell function. CD40 is itself upregulated by pro-inflammatory stimuli such as LPS and IFN γ , thus permitting efficient T-cell activation at sites of inflammation; in the worst-case scenario, this can lead to development of autoimmunity (for review, see Suttles and Stout, 2009). Phagocytosis itself can also alter macrophage function. Ingestion of myelin and PS result in alterations to transcriptional profile and lipid metabolism alongside reduced IL-6 and nitric oxide secretion in rodent macrophages (Bogie et al., 2012; Bogie et al., 2013).

We can conclude that the plasticity of this cell type extends far beyond the extremes of the M1/M2 activation spectrum. Indeed, macrophages are affected by a variety of extracellular factors, engulfed molecules and direct cellular interactions even past the point of differentiation from monocytes. *In vitro* experiments to analyse

macrophage function are unlikely to recapitulate this highly complex set of stimuli; however, *in vivo* experiments are often limited to mouse work for practical and ethical reasons. Thus, it is difficult to assess behaviours that may be unique to human monocytes and macrophages. This may be particularly important in the context of aging – wherein extrapolation from mice to humans may be particularly fraught – and especially in the context of cancers.

1.3.2.4. Natural killer cells

Though they form part of the innate immune system, natural killer (NK) cells play a role in anti-tumour immunity. They perform similar functions to CD8⁺ T-cells: namely, destruction of pathogen-infected or cancerous cells using granzymes and perforins, alongside secretion of pro-inflammatory cytokines – particularly IFN γ – and as such, merit a brief discussion.

These cells are defined by their expression of CD16 – the Fc γ RIII scavenger receptor – and CD56, and are strongly represented in the blood, bone marrow, lung and spleen; conversely, in tonsil and gut, they may account for under 2% of lymphocytes. They can be further stratified into the less numerous CD56-bright and more numerous CD56-dim populations, which subsets appear to favour different tissue niches (Dogra et al., 2020). CD56 appears to be lost as the cells continue to mature; within the CD56-dim population, CD57 serves as a marker of terminal differentiation and has been associated with reduced proliferative capacity and shortened telomeres (Björkström et al., 2010). Both subsets show distinct transcriptional profiles: CD56-bright cells appear to be associated with tissue homing and produce greater levels of cytokines when stimulated *in vitro*, whereas CD56-dim counterparts express higher levels of cytolytic molecules; additionally, further tissue-specific transcriptional programs are also apparent (Cooper et al., 2001; Dogra et al., 2020).

Like T-cells, B-cells and mononuclear phagocytes, NK cells ultimately derive from HSCs and progress through a series of stages during development. The first of these is the CLP, also common to T- and B-cells. From this stage, cells become NK

precursors by acquiring CD122 and CD132: two of the three molecules forming the IL-15 receptor. These cells can respond to IL-15 even though they lack the high-affinity CD215 chain; a pre-NK precursor population that lacks CD122 has also been described. While IL-15 signalling plays a crucial role in NK cell maturation, human cells at this stage are not exclusively committed to the NK lineage, and can be differentiated to T-cells or DCs *in vitro*; mice also have a T/NK precursor cell that is restricted out of the B-cell lineage, showing close interrelationship between the T-cell and NK cell developmental pathways (Douagi et al., 2002). The importance of IL-15 signalling is underscored by the discovery that aberrant IL-15 signaling in humans results in a severe combined immunodeficiency (SCID) phenotype characterised by absent NK cells and dysfunctional T- and B-cells (Gilmour et al., 2001). For review of IL-15 in NK cells, see Becknell and Caligiuri, 2005. NK precursors then express further lineage-specific molecules such as NKG2D, killer immunoglobulin receptors, and CD16 and CD56. Memory NK cells also exist; much like T-cells, their precise origin is a matter of debate. NK cells appear to mature in lymphoid tissue. For review of NK cell development, see Yu, Freud and Caligiuri, 2013.

Whereas T- and B-cells produce functionally unique receptors using VDJ recombination as described in 1.3.2., NK cells express encoded receptors. Many of these provide an inhibitory response to MHCI, such that NK cells function using what is termed the “missing-self response”: cytolytic killing of cells lacking MHCI. The MHCI-responsive KIR receptors can be expressed in variable combinations; a KIR-null population also exists, but perhaps contrary to expectations, is hyporesponsive to stimuli. It has been theorised that NK cells are licensed by prior inhibitory

signalling through KIR receptors, and without this, cannot become fully functional (Anfossi et al., 2006).

1.4. The immune system in GBM

1.4.1. Overview

GBM is generally regarded as an immunologically “cold” tumour, although it is known to be infiltrated by various immune effector populations, including T-cells, B-cells, BMDMs and others. Despite sometimes-extensive infiltration, development of anti-tumoural immunity remains limited and ineffective.

In other tumours, multiple methods of anti-tumoural immunity become evident: the generation of antibodies by B-cells; engulfment of transformed cells and resultant antigen presentation by macrophages; direct cytolysis and support activity by T-cells. Strongly immunogenic tumours such as melanoma, which has higher levels of mutational burden than GBM, can elicit an initial adaptive immune response. However, even immunogenic cancers quickly acquire methods to evade such attack. These can include downregulation of specific antigens and MHC1, upregulation of protective secreted and cell-surface molecules, and co-option of non-transformed cells to support tumour growth. Immune escape in the context of melanoma is reviewed in Marzagalli, Ebelt and Manuel, 2019; many of these same mechanisms are present in GBM, a less immunogenic tumour, and an overview will be given here.

1.4.2. Immunity in the CNS

It was historically assumed that the BBB rendered immune effector extravasation into the mammalian brain impossible, and therefore the brain was described as an immune-privileged organ. The modern understanding of immune privilege is not one where peripheral populations cannot enter the CNS, but rather one in which entry of peripheral effectors and CNS immunity is heavily regulated. Inflammation and oedema could easily cause a damaging rise in intracranial pressure even in the absence of haemorrhage. Neurons are highly sensitive cells that may be damaged by inflammation, and tissue damage cannot be as easily repaired as in non-CNS tissue. Finally, a failure to maintain tolerance to CNS self-antigens can result in debilitating autoimmune conditions, as is seen in EAE when myelin is targeted by the immune system. With this in mind, the necessity of tight control of immune responses in the CNS is obvious, and yet immune activity cannot be dispensed with, positioning the CNS as a unique environment.

1.4.2.1. Immune infiltration

Experiments in both human and mouse brain have confirmed the presence of adaptive immune cells. Mass cytometry of the mouse brain found that approximately 80% of immune effectors were CNS-resident myeloid cells, likely microglia. The other 20% comprised DCs, monocytes and BMDMs, B- and T-cells. These cells were mostly absent from the brain parenchyma, instead being detectable in the meninges and choroid plexus; furthermore, different effectors appeared subject to selective regulation in terms of their infiltration into the brain, illustrating the highly regulated nature of CNS immunity (Korin et al., 2017). Analysis of the human brain identified the same adaptive effectors, and furthermore, that their presence – particularly that of CD8⁺ T-cells – correlated negatively with aging in several areas (Nevalainen et al., 2022). In both cases, antigen-experienced B- and T-cells were more prevalent. Clearly, mechanisms do exist to allow peripheral entry to the CNS compartment.

1.4.2.2. CNS entry and lymphatics

Historically, proposed routes of immune cell entry to the CNS included trafficking across the choroid plexus into the CSF, and under some circumstances, extravasation across the BBB (Bartholomäus et al., 2009; reviewed in Marchetti and Engelhardt, 2020).

However, the existence of murine intracranial LVs was recently described by two groups (Aspelund et al., 2015; Louveau et al., 2015), with the latter also identifying the same structure in human specimens. These vessels do not enter the brain parenchyma but rather exist in the meninges, providing a clear mechanism through which antigen can leave and effectors can enter the CNS. The location of such vessels also explains the high presence of effectors in the meninges, but not parenchyma, described by Korin et al. in their 2017 paper. Later work showed that meningeal lymphatic vessels (MLVs) drain CSF and facilitate T-cell migration from the CSF to the cervical lymph nodes (CLN), allowing for the development of CNS-specific autoimmune responses in the context of EAE (Louveau et al., 2018). More recently, the MLV network was mapped in humans and mice (Jacob et al., 2022), and there is much speculation about the biological and therapeutic relevance of MLVs in various CNS pathologies (Olate-Briones et al., 2022).

1.4.2.3. CNS antigen presentation

The existence of fluid drainage from the CNS has been described for decades, as has the ability of intracerebrally-administered antigens to provoke an immune response (reviewed in Cserr, Harling-Berg and Knopf, 1992). CNS antigens are typically presented in the CLN within hours of intracranial administration and are cleared from the parenchyma within days (Ling et al., 2003). In this context, presentation by DCs appears indispensable for promoting CD8⁺ T-cell immunity (Ling et al., 2003), including in CNS viral infection or cancer (Malo et al., 2018; Tritz et al., 2020), and DC-mediated antigen presentation drives relapses in EAE, in which pathology peripheral DCs accumulate in the CNS (Bailey et al., 2007). In experimental cerebral malaria, DC antigen presentation can promote CD8⁺ cytotoxicity to the extent of lethal BBB disruption (Malo et al., 2018). Thus, DCs are extremely potent inducers of T-cell function in CNS pathologies.

Within the parenchyma, microglia are the primary antigen-presenting cell. Murine experiments have shown antigen presentation is necessary for CD8⁺ T-cell infiltration and retention, even if BBB disruption permits initial extravasation (Malo et al., 2018; Goddery et al., 2021). MHCII⁺ cells in the perivascular (PV) and subarachnoid spaces interact with T-cells to permit activation and pro-inflammatory cytokine secretion (Bartholomäus et al., 2009; Kivisäkk et al., 2009). During viral infection, CNS-resident myeloid cells upregulate MHCI and MHCII and associate with vasculature, possibly to facilitate such interactions (Goddery et al., 2021).

Additionally, numerous other CNS populations have some antigen presentation ability, including ECs, which express MHCII during inflammation; B-cells, which

appear to play a role in multiple sclerosis (MS) and EAE; and astrocytes, which also upregulate MHCII in MS, although whether they express costimulatory molecules is less clear (reviewed in Waisman and Johann, 2018). While results cannot be extrapolated directly from MS/EAE to GBM, it is possible that any or all of these populations also act as APCs in GBM, or in CNS pathologies more broadly, which merits further study.

1.4.3. Risk and protection – immunodeficiency and autoimmunity

It is clear that the brain has high levels of immune activity, derived from both CNS-resident and peripheral effectors. In combination with the potential ability of the immune system to eliminate transformed cells and small tumours before they reach clinical relevance, it then follows that this process may be aberrant in GBM – and that some patients may be uniquely protected. The consensus is that some autoimmune conditions or hypersensitivity do seem to confer protection against glioma, although the mechanism is currently unclear, and not every study examines GBM specifically. Conversely, if immunosurveillance confers protection, immunodeficiency should confer risk of GBM development; there is some evidence that HIV and transplant patients are at greater risk, although this has not been conclusively proven to be a result of immunosuppression. Supposing that pre-existing immune surveillance is sufficient to destroy brain tumours before symptoms develop, it can be assumed that anti-tumoural immune activity, especially in smaller tumours, is highly effective; thus lifting of immunosuppression in GBM patients may be beneficial.

1.4.3.1. Atopy

An inverse correlation between atopy – IgE-mediated hypersensitivity, typically manifesting as asthma, eczema and allergy – and glioma has been documented for decades, and does not apply to several other cancers (for review see Cui and Hill 2016).

This protective effect has been described for asthma, eczema and allergy individually, as well as asthma-associated single nucleotide polymorphisms (SNPs) in some interleukin genes (Hochberg et al., 1990; Brenner et al., 2002; Wiemels et al., 2004; Schwartzbaum et al., 2005; Brenner et al., 2007; Amirian et al., 2016); the effect is specific to tumour type, not anatomical location, as confirmed by a meta-analysis comparing glioma and meningioma (Linos et al., 2007). Serum IgE and eczema have also been found to correlate negatively with GBM specifically (Schwartzbaum et al., 2012; Disney-Hogg et al., 2018). Allergy has been found to correlate with better prognosis as well as reduced incidence (Lehrer et al., 2019).

The mechanisms through which these conditions may confer protection is not yet understood, but it cannot be explained by use of antihistamines (Amirian et al., 2016). It has been found that higher IgE levels correlate with reduced incidence at least two decades before glioma diagnosis, and it is possible that the correlation may be due to effective immune surveillance in atopy sufferers (Schwartzbaum et al., 2012).

1.4.3.2. Diabetes

Diabetes also appears to be inversely correlated with glioma specifically in males (Kitahara et al., 2014; Seliger et al., 2016; Ni, 2018; Wang Y et al., 2019). Medication does not appear to be responsible (Seliger et al., 2016). Separate studies found no protective or contributory effect of type 2 diabetes, or acquired insulin resistance, whereas type 1 is an autoimmune condition and is the less common of the two (Barami, Lyon and Conell, 2017; Disney-Hogg et al., 2018). Others have associated diabetes with a worse prognosis after surgery due to hyperglycaemia, which effect disappears when diabetes is well-managed (Chambless et al., 2012; Barami, Lyon and Conell, 2017) and would presumably be a prognostic risk for non-GBM-related surgeries as well. Schwartzbaum et al. noted an inverse correlation between hyperglycaemia and glioma in their 2017 paper. They suggested that hyperglycaemia inhibits neural stem cell proliferation and diabetes may impair cerebral blood flow, which could limit tumour growth.

The thread potentially linking diabetes and atopy is long-term immune activation and possibly greater immune surveillance. If this were the sole responsible factor, an effect would be expected for type 1 diabetes only, an autoimmune disease. It is also possible that type 2 diabetes confers separate protective effects. Many recent studies focus on type 2 diabetes or make no distinction, and thus provide no evidence from the immunological side. As with dietary studies, many papers also refer to glioma in general and thus GBM-specific effects may not be uncovered, although Kitahara et al. in their 2014 paper found the effect was true for GBM specifically as well as glioma more broadly.

1.4.3.3. Multiple sclerosis

To examine the occurrence of GBM with greater CNS immunosurveillance, MS may be a useful model. The occurrence of both MS and glioma in patients is accepted to be rare; a 1974 paper noted 12 cases in the literature since 1938 and described three more. Of these 15 patients, nine had GBM; five of those were previously diagnosed with MS. While noting the rarity of co-occurrence, the authors also note that autopsy was rarely performed in MS patients, and that if tumours arise from MS lesions, the lesions themselves would be masked (Currie and Urich, 1974). These analyses are further complicated by the occasional tumour-like appearance of some MS lesions, although MRI-based methodologies exist to distinguish the two (French et al., 2022). Multiple mechanisms have been proposed for the development of MS lesions into primary tumours, including reactivation of developmental pathways during remyelination or a causative agent – whether genetic, epigenetic or exogenous – common to both (for review see Plantone et al., 2015).

Other analyses have found a reduced overall incidence of cancer in MS patients, which is suggested to be due to increased levels of immune surveillance (Bahmanyar et al., 2009). However, this could be at least partially explained by MS patients having increased scans and indeed, this and a later study found brain tumours were diagnosed two to five years earlier in patients with MS (Bahmanyar et al., 2009; Montgomery et al., 2013). The latter paper also confirmed the rarity: of over 20,000 MS patients in the cohort, a little over 100 were diagnosed with brain tumour, and high-grade tumours were less common in the MS cohort (Montgomery et al., 2013).

In terms of GBM more specifically, many papers are limited to case reports describing a co-occurrence in one or two patients. Because of the small numbers of MS and brain tumour or glioma co-occurrence, analysis of a specific subtype is therefore limited to very small numbers. Given the rarity of such co-occurrences, it does seem plausible that the high levels of immune activity within MS confer some protective effect; however, no definitive conclusion has been reached. In support of this hypothesis is the documentation of a small number of MS patients who develop GBM during or after treatment with immunosuppressive drugs (Sharim et al., 2016; Sierra Morales et al., 2017; London et al., 2020; Alkabie et al., 2021).

Overall, while there is some implication that greater immune surveillance of the CNS in MS specifically may confer some protection against GBM, there is currently no clear evidence, and it is certainly possible that in some patients, increased inflammation or common causative agents may worsen risk.

1.4.3.4. HIV

HIV is one of the leading causes of immunodeficiency, and results in widespread lymphopaenia and acquired immunodeficiency syndrome (AIDS). While a correlation between HIV and brain tumour is generally described, it focuses largely on the high prevalence of the AIDS-related primary central nervous system lymphoma; however, there is a large body of literature examining the interrelationship between HIV/AIDS and GBM.

A retrospective analysis covering 34 glioma patients across 34 years found a median survival of nine months, and a mean of 16. These authors noted a reduced age at diagnosis of 38-39 years compared to HIV-negative glioma patients (Wang et al., 2018); the same trend is seen in GBM patients (Choy et al., 2016). For GBM specifically, two separate meta-analyses of 17 and 24 HIV-positive patients found OS to be approximately eight months from the point of diagnosis – considerably lower than the overall average of 14.6 months in patients receiving the Stupp protocol (Choy et al., 2016; Mendez Valdez et al., 2022). The former noted that OS was approximately doubled in patients receiving anti-retroviral therapy. In almost all combined GBM-HIV patients, tumour progression rather than HIV/AIDS-related complications is the cause of death (Vannemreddy et al., 2000; Choy et al., 2016).

The co-occurrence of GBM and HIV appears to be particularly lethal, but surprisingly rare. An inverse correlation between the two pathologies has been described, although these authors also noted that some areas with high HIV incidence also have reduced healthcare infrastructure and thus the regional incidence of CNS cancer may be underreported (Mendez Valdez et al., 2022).

However, a study of Zimbabwean glioma patients found HIV to have an anti-glioma effect; this has also been seen in Mexican and Brazilian patients, where the reported HIV/GBM co-occurrence is in the single digits (Cedeno-Laurent and Trujillo, 2011; Jokonya et al., 2018). Breast, prostate and colorectal cancers are also reduced in the HIV+ population (Coghill et al., 2018); however, the increased incidence of solid tumours overall, especially virally-mediated tumours (Chiao et al., 2021), shows that HIV certainly cannot be regarded as broadly anti-tumourigenic. It has been suggested that HIV patients, as with MS, might be subject to more frequent neuroimaging, resulting in a higher rate of brain tumour diagnoses (Vannemreddy et al., 2000). However, this does not seem to be the case.

HIV proteins are suggested to have oncogenic effects through effects on metabolism, cell cycle progression, signalling pathways and induction of oxidative stress, and may facilitate infection with other oncogenic viruses. Furthermore, HIV appears able to directly potentiate the effects of such viruses (for review see Isagulants et al., 2021). Reduced immunosurveillance certainly seems to predispose HIV/AIDS patients to malignancy. However, the differential rates of different tumours show that other factors are likely at play. Whether these are tissue-specific effects of HIV remains to be seen. It appears that in the context of GBM, HIV likely does not predispose patients to a significantly greater risk, but when both co-occur, OS is markedly reduced.

1.4.3.5. Transplant recipients

Due to the allogeneic nature of many transplant tissues, transplant recipients are often given immunosuppressive medication for the rest of their life to prevent transplant rejection. It was historically assumed that, due to the rarity of CNS tumour metastases, transplant from such patients was safe. This assumption was challenged in 2000, when Detry et al. described 13 previous cases in which a CNS tumour was transferred during transplant and estimated a transmission risk of 0-3%. It is worth noting that in every case of GBM transfer, the recipient's tumour is located in the transplanted organ, not the CNS (reviewed in Jimsheleishvili et al., 2014). Other authors have found brain tumour transmission to be absent over a 17-year period in the UK (Greenhall et al., 2023) and how to balance the risk of tumour transfer with the risk of patients dying while waiting for a transplant is debated.

Separately, at least two groups have noted GBM arising intracranially following liver, lung, kidney and heart transplant (Schiff et al., 2001; Salvati et al., 2003). In these cases, the tumours do not appear to be transferred from the donor. Salvati et al. hypothesised that the immunosuppressants administered to transplant patients may facilitate GBM growth, emphasised the need for further investigation, and noted that post-transplant non-donor GBM may be underreported due to either rapid mortality or short follow-up times (Salvati et al., 2003). However, the topic does not appear to have been researched since, and certainly merits further investigation. Overall, it appears that transplants may be a risk for GBM development both through transfer of very small metastases and through reduced immunosurveillance.

1.4.4. Immune effector presence in GBM

Non-resident immune effectors can enter the CNS in a variety of ways, by crossing the choroid plexus, the BBB or the walls of MLVs. Tumour-infiltrating leukocytes (TILs) are documented to be present in GBM; however, this infiltration is heterogenous, with some tumours showing extensive presence of immune effectors and others a relative lack.

GBM contains more T-cells and macrophages than healthy brain (Mitsdoerffer et al. 2022). Infiltration is affected by tumour type: astrocytomas are less heavily infiltrated than brain metastases from non-CNS cancers (Friebel et al., 2020). Infiltration also varies with intratumoural location. A study of nine GBM found greater effector infiltration in bulk tumour than the peritumoural area. The authors suggest recruitment may be stronger in the tumour bulk (Rahimi Koshkaki et al., 2020), although it is also possible that the lack of an intact BBB in the tumour bulk permits effector extravasation.

Variation may also exist across subtypes: CD8+ T-cells and macrophages are most prevalent in the mesenchymal subtype, where they correlated with better prognosis; macrophage infiltration in the proneural subtype conferred worse survival (Beier et al., 2012; Kaffes et al., 2019). These data are illustrative of GBM's intratumoural heterogeneity, and show that there may still be low levels of anti-tumoural adaptive immunity that are able to extend patient lifespan but are insufficient for full recovery. Additionally, as well as genuine differences between tumours, sampling site will likely also affect observed infiltration. For a general review of immune infiltration and suppression in GBM, see Chen and Hambardzumyan, 2018.

1.4.4.1. Presence of T-cells and B-cells

T-cells are present within GBM, but their prevalence and distribution vary by paper. In 1997, Leung et al. found T-cells in 16 of 22 GBM, four of which contained moderate or extensive infiltration. At the other end of the scale, a more recent paper observed T-cell positivity in only 18% of 115 GBM by immunohistochemistry (IHC) (Lee KS et al., 2018). Berghoff et al. in 2015 found 66.7% of 117 GBM to contain T-cells; 44.4% had CD8+ T-cells. Another IHC study found CD8+ cells in all and CD4+ cells in 98% of grade 4 glioma – this dataset comprised six gliosarcomas and 52 GBM. This paper also found greater CD4+ infiltration in higher-grade tumours (Heimberger et al., 2008). Lohr et al. in their 2011 paper also identified T-cells of both subsets to be more present in grade 4 than other gliomas, and overall, T-cells have been found to account for 3-23% (Friebel et al., 2020), 0.1-19.1% (Orrega et al., 2018) and 16.4% (Fu et al., 2020) of intratumoural cells – a consistent range. The type of analysis performed – IHC, FC, or sequencing data – may return different results due to sensitivity, and thus a true estimate of T-cell positivity within GBM is hard to approach. However, it does appear that higher-grade tumours are the richest in T-cells.

CCL2 is widely regarded as a monocyte chemoattractant, but also attracts T-cells, and a correlation exists between the two (Leung et al., 1997). T-cells are primarily found in areas with leaky vasculature (Lohr et al., 2011). T-cells have been described as preferentially present in the PV region as compared to the infiltrating margin or tumour bulk; this study found a more equitable distribution of CD8+ T-cells between the PV and tumour bulk, although they were still reduced in the infiltrating margin (Berghoff et al., 2015). CD8+ TILs have been found to be equally present in the

tumour core and peritumoural area (Yang et al., 2010). Other work has found differential distribution of CD4+ and CD8+ TILs, with CD4+ cells present in perivascular and necrotic areas, and CD8+ cells absent from necrotic regions but present in perivascular and general tumour tissue (Mitsdoerffer et al., 2022). Thus, as with overall infiltration, T-cell distribution may be hard to ascertain, and is affected by the heterogeneity within this cell type.

1.4.4.2. Presence of tumour-associated macrophages

Some papers observe great consistency in certain results, despite differences in sampling site, methodology and diagnostic criteria. One of these consistent areas is the prevalence of TAMs.

These cells have been observed by many to be the most prevalent immune effector phenotype in GBM, sometimes accounting for a significant proportion of the tumour. Kmiecik et al., in 2013 found macrophages to account for 54% of tumour-infiltrating immune cells in eight patients; mass cytometry has returned a similar value of 59.1% (Fu et al., 2020). Some authors have noted a lower percentage: Orrega et al. in 2018 found about 9% of cells within GBM to stain positive for CD68, an internal macrophage marker. When using HLA-DR, an MHCII molecule, over 90% of tumours have been found to contain TAMs (Berghoff et al., 2015). As described above, the mesenchymal subtype is most heavily infiltrated by TAMs, and subtype-specific differences in infiltration may be masked by analyses that do not account for this.

High levels of TAMs correlate with CCL2, a potent monocyte chemoattractant that has been documented to be present at high levels within GBM for decades. CCL2 is secreted by both TAMs and transformed cells (Leung et al., 1997), which in combination with elevated circulating peripheral monocytes in GBM patients (Harshyne et al., 2016), may lead to a positive feedback loop driving macrophage infiltration.

1.4.5. Axes of immunosuppression within GBM

As seen above, the CNS and GBM itself contains immune effectors of various phenotypes. However, local and systemic immunosuppression in GBM patients is well-documented (for historic review see Dix et al., 1999). This suppression is mediated through varied means, ranging from recruitment of macrophages to support tumour growth to secretion of immunosuppressive molecules. The immune microenvironment of GBM is unique due to its location in the CNS and the potent suppression within. Some of the key axes will be outlined in this section.

1.4.5.1. Mononuclear phagocytes

While GBM contains many macrophages, these cells – like in many solid tumours – appear to facilitate tumour growth and further immunosuppression.

Though it has long been assumed that the GBM TAM population is composed of both BMDMs and microglia, evidence of monocyte infiltration was only described relatively recently. In 2016, Bowman et al. used lineage tracing to show monocyte infiltration across the BBB and subsequent differentiation in murine glioma. In this case, microglia and BMDMs showed differential gene expression, though both upregulated PD-L1. It has been found that the majority of GBM TAMs are BMDMs (Zhou et al., 2015) and that BMDMs are more heavily represented in aged (>65 years) patients (Wu et al., 2023); as most GBM occurs in older patients, these results are consistent. Wu et al.'s paper also noted that greater BMDM representation correlates with worse prognosis.

Peripheral monocytes isolated from GBM patients express elevated levels of the M2-associated marker CD163; GBM patient sera is sufficient to induce CD163 expression (Harshyne et al., 2016). GBM patient-derived monocytes show increased CD80 and reduced MHCII expression; monocytes lacking MHCII failed to stimulate T-cell proliferation (Gustafson et al., 2010). It is possible that GBM's effects on peripheral immunity extend to priming circulating monocytes towards a tumour-supporting phenotype even before their extravasation, thus limiting even further the likelihood of anti-tumoural macrophage activity, although such a hypothesis would require much further work to confirm.

Further gene expression analysis reveals complexity beyond any assumption that TAMs adopt an unambiguous M2-like phenotype. TAMs isolated from murine glioma express both M1- and M2-related genes, alongside differential regulation of multiple immunity pathways as compared to naïve cells (Szulzewsky et al., 2015). The same has also been seen in human gliomas of various grades (Zeiner et al., 2019). Even within the assumption that most or all TAMs adopt an M2-like phenotype, considerable heterogeneity exists. Mass cytometry has revealed 13 subsets based on differential surface marker expression in GBM (Fu et al., 2020). As discussed above (1.3.3.3.2.), the M1/M2 dichotomy likely does not apply well *in vivo*. In the context of GBM, macrophages may be better described as M1 or M2-like.

Higher levels of CD163+, CD206+ or CD68+ TAMs in the tumour core are associated with prolonged survival in human patients (Zeiner et al., 2019); a beneficial effect of increased IBA1 expression has also been documented (Sørensen et al., 2018). However, this paper also revealed high levels of macrophage markers conferred worse prognosis in combination with MGMT promoter methylation. TAM presence in the infiltrating margin did not correlate with survival (Zeiner et al., 2019). In conclusion, GBM TAMs are a phenotypically and possibly functionally heterogeneous population, and their impact on patient survival is likely more complex than a pure positive or negative indication.

However, TAMs are likely unable to stimulate T-cells, and do show signs of functional impairment. The BMDM compartment may be able to oppose IL-1 signalling through upregulation of the IL-1 decoy receptor and the secreted IL-1 receptor antagonist (Bowman et al., 2016) as well secreting reduced IL-1 β (reviewed

in Dix et al., 1999). Only a minority of TAMs express CD40, CD80 or CD86, without which stimulation T-cells encountering antigen would become anergic (Kmieciak et al., 2013). TAMs express high levels of the IC TIM-3 (Guo Q et al., 2022), which may render them sensitive to gal-9-mediated reduction of CD86 (Ni et al., 2022). Some TAMs also express PD-L1, allowing them to suppress T-cells as well as failing to activate them (Fu et al., 2020).

DCs isolated from the CSF of GBM patients also exhibit reduced expression of CD40, CD86 and MHCII molecules, implying impaired cross-presentation and thus reduced CD8⁺ T-cell function. Their function could be impaired by GBM-derived extracellular vesicles (EVs), which may enter peripheral circulation, thus removing a requirement for DCs to enter the tumour to become suppressed (Wang et al., 2020).

Additionally, these cells likely support GBM growth through various means. TAMs secrete TGF β and promote angiogenesis and MMP activity, alongside secretion of TGF β and various other growth factors (Bowman et al., 2016; for review of TAMs in GBM growth, see Grégoire et al., 2020).

1.4.5.2. T-cell infiltration and dysfunction

Despite clear evidence of T-cell infiltration, TILs isolated from GBM are often dysfunctional. These cells express high levels of ICs, which are upregulated following activation; this has come to be known as an exhaustion-associated signature. Additionally, they show defects in cytokine secretion, proliferation and activity.

Early work identified low expression of CD25 – an activation-associated molecule that forms part of the high-affinity IL-2 receptor – on GBM TILs, thus lowering their response to IL-2 (reviewed in Dix et al., 1999). A comparison with T-cells infiltrating brain metastases suggests that many GBM-infiltrating T-cells never reach full activation, instead becoming anergic (Friebel et al., 2020). T-cells in recurrent GBM have weaker expression of activation-related markers than their counterparts in brain metastases (Sun et al., 2023).

TILs can also – and often do – express multiple ICs, which is a strongly exhaustion-associated signature. PD-1+TIM-3+LAG-3+ TILs have even more severely impaired cytokine production than other TILs, and are overrepresented in murine glioma antigen-specific cells (Woroniecka et al., 2018). Human CD8+ TILs do not undergo clonal expansion *ex vivo* (Woroniecka et al., 2018), and the PD-1+TIM-3+CD4+ subset has been separately described as replication-deficient while retaining the ability to secrete some pro-inflammatory cytokines (Goods et al., 2017). GBM TILs show reduced TCR signalling (reviewed in Dix et al., 1999). T-cells isolated from the tumour show upregulated IC molecules, including PD-1, TIM-3, LAG-3, and reduced IL-2 production (Fu et al., 2020; Mohme et al., 2018). Intriguingly, PD-1, TIM-3 and

LAG-3 are all expressed more strongly on double-positive intratumoural T-cells than any other phenotype; this population also expressed IL-10 and TGF β (Fu et al., 2020). When cultured *ex vivo*, TILs fail to react to externally-validated tumour antigens or patient-specific antigens identified using microarray data (Lu et al., 2022); thus, GBM-induced immunosuppression persists after removal from the TME.

Although peripheral and TIL populations have differentially regulated expression programs (Goods et al., 2017), GBM-induced immunosuppression is also seen in the peripheral compartment; lymphopaenia and reduced splenic volume are observed in these patients (Gustafson et al., 2010; Kmiecik et al., 2013; Chongsathidkiet et al., 2018). Overall white blood cell count is reduced (Mohme et al., 2018) and T-cells specifically are lacking due to a loss of the S1P1 receptor and resultant sequestration in the BM; this is not unique to GBM but rather appears to be an artifact of intracranial tumours (Chongsathidkiet et al., 2018). Remaining peripheral T-cells produce less IL-12 and IFN γ but increased IL-10 (reviewed in Dix et al., 1999). CD4 positivity is decreased and peripheral T-cells in GBM patients lack CD154, the CD40 co-receptor – thus inhibiting the ability of T-cells to activate APCs (Kmiecik et al., 2013) – but are more likely to be PD-1+ (Mohme et al., 2018). Clearly, GBM immunosuppression extends beyond the intracranial compartment. It is possible that pre-existing suppression impedes the ability of T-cells to act against the tumour even before their extravasation.

In terms of T-cell prognostic impact, results are varied. Some authors have found T-cell infiltration to confer no benefit to OS (Lee KS et al., 2018; Orrego et al., 2018);

others have found the opposite (Lohr et al., 2011). Detail may be necessary: Kmiecik et al. in their 2013 paper found a protective effect with CD3+ cells generally and CD8+, but not CD4+, cells specifically. Another paper found proliferating CD8+ cells to confer favourable prognosis (Mauldin et al., 2021). Further classification of CD8+ T-cells using single-cell RNA (ribonucleic acid) sequencing data reveals that specific subpopulations are beneficial (Sun et al., 2023). CD8+ cells also correlate with longer survival in recurrent GBM (Lin et al., 2021) and are more present in long-term (>403 days) survivors (Yang et al., 2010). Thus, cytolysis of transformed cells may be the primary T-cell-related indicator for survival. However, this is likely impeded by reduced MHCI expression on glioma cells (Zagzag et al., 2005).

In terms of CD4+ cell phenotype, the Th2 subset appears to be favoured in at least some patients. Those with a high monocyte count also show elevated IL-4 and IL-13 (Harshyne et al., 2016). The IL-5:IFN γ ratio indicates a Th2 bias among CD4+ TILs (Shimato et al., 2012). Compared to healthy brain, GBM is Th1-poor and Th2-rich. High Th2 gene expression scores correlate with reduced survival, and patients with low Th2 scores also showed reduction in expression of the PD-1 signalling axis. While these data imply a beneficial role for Th1 immunity, survival was longest in patients with low Th1 and Th2 signatures (Takashima et al., 2018). Gene set enrichment analysis has also revealed overrepresentation of the Th17 phenotype (Mitsdoerffer et al., 2022).

Naïve T-cells have been associated with long-term (>five years) survival (Mohme et al., 2018). Friebel et al. suggested in their 2020 paper that CD4+ and CD8+ central memory T-cells may confer better OS in glioma. However, most CD8+ TILs have

been found to have an effector memory phenotype (Woroniecka et al., 2018), and recurrent GBM is heavily infiltrated by activated CD8+ and effector memory CD4+ phenotypes (Lin et al., 2021).

There are many permutations of T-cell subtype, naïve and memory phenotype, and GBM subtype that may make specific favourable correlations difficult to identify. Regardless, it is clear that in some cases, infiltrating effectors do retain some function which may be sufficient to extend patient lifespan without being curative.

1.4.5.3. Treg cells

Treg cells have been the subject of much interest in GBM as a potential means of immunosuppression. While studies such as Kaffes et al., 2019, have identified Treg cells to be more present in GBM than healthy brain, the general consensus is that Tregs are not present in all GBM. At least three research groups have found similar results: Lohr et al. in their 2011 paper found Tregs in just under half of GBM surveyed, where they accounted for under 1% of intratumoural T-cells. Two years later, Tregs were detected in three of five (60%) GBM patients and accounted for approximately 2% of CD3+ TILs (Kmieciak et al., 2013), and were just under 4% of T-cells and present in 47% of GBM according to Thomas et al., 2015. Heimberger identified Tregs in approximately half of GBM surveyed, and furthermore, found their presence to increase with astrocytoma grade. High Treg infiltration was exclusive to high-grade glioma (HGG) in this paper (Heimberger et al., 2008). Overall, approximately 50% of GBM are consistently found to be T-cell positive.

In terms of prognosis, three of the above papers did not find Treg presence to correlate with reduced survival (Heimberger et al., 2008; Lohr et al., 2011; Thomas et al., 2015). Other work has found a higher effector T-cell:Treg ratio to associate with better survival (Sayour et al., 2015). Tregs may also impact prognosis in conjunction with certain therapies: decreased Treg count following DC vaccination was found to be favourable (Fong et al., 2012). It is possible that the ratios of different immune cells can shed far more light on intratumoural behaviour and have greater clinical relevance than cell presence or density; additionally, Treg relevance may extend beyond their effect in isolation and should be considered in conjunction with therapies.

1.4.5.4. Immune checkpoints

IC molecules have become a topic of interest in GBM, as their upregulation may facilitate tumour immune escape by granting the ability to suppress T-cell responses. One of the most widely studied ICs in this context is the interaction of PD-1 with its ligands PD-L1 and PD-L2, which is detailed in 1.3.2.1.4.1. However, reports into its prevalence are varied, and the cellular source of intratumoural PD-L1 is still debated. Alongside, the checkpoints TIM-3 and CTLA-4 may be of relevance in this context. Compared to PD-1 and TIM-3, much less data about CTLA-4 in GBM is available, although a reduction in CTLA-4 expression on TILs is predictive of survival following DC vaccination, which in this study was not found to be true for PD-1 (Fong et al., 2012).

1.4.5.4.1. PD-1 and its ligands

PD-1 itself has been variously described to be present on a minority of TILs (Rahimi Koshkaki et al., 2020), on over 70% of TILs – except for patients with survival over five years, for whom it was lower – (Mohme et al., 2018), and on 98% of CD8+ TILs, implying high levels of functional exhaustion in the cytotoxic subset (Woroniecka et al., 2018). Some of this disparity may be reflective of different methodology: Rahimi Koshkaki et al. used IHC, whereas the latter two papers used FC, which is more sensitive. Conversely, work by Lu et al. in 2022 found PD-1 to be the most highly expressed IC molecule on TILs, and after *ex vivo* expansion, was more present on CD4+ than CD8+ cells, suggesting greater exhaustion in this subset; the CD8+ T-cells in these experiments were more able to downregulate PD-1 during *ex vivo* expansion. PD-1+ TIL density correlates with the density of CD3+ and CD8+ TILs, indicating that it is largely expressed on infiltrating T-cells (Berghoff et al., 2015). The picture of PD-1 positivity is overall not clear: it may vary with tumour subtype or other parameters not fully accounted for by current experiments, or may simply reflect intertumoural heterogeneity. Additionally, while CD8+ T-cells may be more able than CD4+ counterparts to downregulate PD-1 *ex vivo*, this has not been shown to occur *in vivo* and thus may have limited relevance for therapeutics that do not involve *ex vivo* expansion of patient-derived T-cells.

Several groups using datasets ranging from 99 to 163 tumours have observed PD-L1 in the majority of GBM using IHC (Berghoff et al., 2015; Nduom et al., 2016; Knudsen et al., 2021), although another group found it in 32% of a 115 GBM dataset (Lee KS et al., 2018). Spatially, Berghoff's paper did not find it to be preferentially distributed in either PV or perinecrotic areas, but Knudsen's paper found PD-L1

largely in pseudopalisading areas; whether these can be directly compared to Berghoff's perinecrotic regions cannot be readily ascertained.

Multiple analyses have found PD-L1 not to correlate with poor survival (Berghoff et al., 2015; Takashima et al., 2018; Knudsen et al., 2021) although Berghoff et al. found increased PD-L1 in the mesenchymal subtype, which typically has a worse prognosis, and Takashima's paper found a negative prognostic impact of PD-1 itself. As always, the picture is not entirely clear, as other groups report reduced OS with increased PD-L1 (Liu et al, 2013; Nduom et al., 2016; Lee KS et al., 2018); however, a recent meta-analysis incorporating several of the above studies found PD-L1 to not inform OS or PFS (Guo X et al., 2022). It is likely other factors are involved, as trends towards longer and shorter survival in PD-L1-high tumours with methylated and unmethylated MGMT promoters, respectively, have been noted (Knudsen et al., 2021). Furthermore, PD-L1 expression on tumour-adjacent neurons induces glioma cell apoptosis, resulting in improved survival (Liu et al., 2013). Thus, PD-L1 cellular source, as well as presence, merits study.

TAMs have been suggested to be the main PD-L1+ cells, although the use of IHC for these experiments makes this hard to definitively state (Rahimi Koshkaki et al., 2020). A FC-based analysis found just under 3% of cells within GBM to be PD-L1+, many of which were lymphocytes; in these experiments, lymphocytes, especially the CD4+ subset, were often PD-1+PD-L1+, and glioma stem cells (GSC) lacked PD-L1 (Nduom et al., 2016), although PD-L1 has been detected on GSCs after culture *ex vivo* (Ricklefs et al., 2018). Thus, it appears that the majority of PD-L1+ cells may

be infiltrating immune effectors that are co-opted by the tumour into suppressing other effectors.

1.4.5.4.2. TIM-3 and galectin-9

In addition to PD-1/PD-L1 interactions, the TIM-3 immunosuppressive axis may also be highly relevant. Some authors have described greater TIM-3 expression intensity, but not percentage positivity, on GBM TILs compared to peripheral T-cells (Woroniecka et al., 2018). Other researchers have found CD8+ TILs to upregulate TIM-3, but not PD-1 (Mitsdoerffer et al., 2022).

Multiple analyses have found high gal-9 to associate with poor survival using TCGA and Chinese Cancer Genome Atlas (CCGA) analysis and IHC (Yuan et al, 2020; Wang et al., 2020). Galectin-9 (gal-9), a TIM-3 ligand, was found in every tumour of a 163-GBM dataset, particularly in areas of microvascular proliferation. In these experiments, TAMs were often gal-9+, and a positive correlation was noted between gal-9 and PD-L1 positivity, suggesting that multiple ICs may act in concert to suppress immunity. This analysis found that gal-9 expression informed poor survival in TCGA data, but not in a patient cohort (Knudsen et al., 2021); however, the prevalence of the TIM-3 receptor was not studied in these experiments. Gal-9 correlates with the presence of M2 TAMs and other ICs in GBM (Yuan et al, 2020), and appears to facilitate tumour growth through its immunosuppressive effects in a mouse model (Wang et al., 2020; Ni et al., 2022). T-cells can induce gal-9 secretion from immortalised GBM cells *in vitro* and *in vivo*, resulting in further T-cell immunosuppression (Schlichtner et al., 2023), and PTEN-null GBM cells also secrete gal-9 (Ni et al., 2022).

TIM-3 itself is most prevalent in mesenchymal GBM, as is gal-9, and both these proteins also correlate with the macrophage marker CD68, as well as reduced

survival (Ni et al., 2022; Guo Q et al., 2022). TIM-3 may be more highly expressed than PD-1, PD-L1/2, CTLA-4 and other immune checkpoints, and is found in immortalised cell lines, freshly isolated GBM cells and neurospheres, as well as infiltrating immune effectors (Guo Q et al., 2022).

TIM-3 expression has been found to correlate with IC molecules PD-1 and LAG-3 using publicly-available mRNA sequencing data; it was found to be more highly expressed in GBM than healthy brain tissue, and to be present in nearly three-quarters of an 84-tumour dataset. In these patients, low TIM-3 expression and MGMT methylation tended to correlate, and conferred better prognosis (Zhang et al., 2020).

1.4.5.5. Extracellular vesicles

Direct secretion of immunosuppressive molecules by glioma has been characterised for decades (for historic review see Dix et al., 1999). These include IL-10, which is also elevated in GBM patient sera (Harshyne et al., 2016); IL-6, which promotes an M2 phenotype in macrophages while inhibiting B- and T-cell infiltration, and is associated with reduced OS (Yang et al., 2021); and in non-cytokine terms, secreted gal-9 can promote M2 macrophage polarisation (Ni et al., 2022).

More recently, attention has also turned to the presence of extracellular vesicles (EVs). These small lipid-based particles allow GBM to deliver molecules into other cells by vesicle uptake and modulate immunity beyond the range of juxtacrine or paracrine signalling, as EVs can enter the CSF; this also gives them potential as biomarkers (reviewed in Figueroa and Carter, 2018). GBM-derived EVs can further tumour growth through a range of mechanisms, including promotion of angiogenesis and chemotherapy resistance, co-option of astrocytes, and promotion of invasion. These occur through delivery of both proteins and micro-RNAs. For review of the role of EVs in modulating the GBM TME, see Yekula et al., 2020.

Unsurprisingly, EVs can also affect the function of immune effectors. GSCs and patient-derived cell lines express surface PD-L1 and IDO1 in response to IFN γ , which may be PBMC-derived. Resultant PD-L1+IDO1+ EVs can suppress T-cell function and cause monocytes to adopt a non-classic phenotype or become myeloid-derived suppressor cells; these monocytes inhibit T-cell proliferation *in vitro* more effectively than counterparts exposed to non-IFN γ -stimulated cell lines. Thus, GBM may be able to suppress T-cell function both directly and indirectly following

IFN γ exposure, in an example of transformed cells responding to pro-inflammatory signals with suppressive mechanisms (Ricklefs et al., 2018; Jung et al., 2022). However, other work has not shown a role for PD-L1+ GBM EVs in T-cell suppression (Himes et al., 2021).

U87- and primary culture-derived EVs downregulate MHCII on monocytes and promote CD163 expression even under M1-promoting conditions *in vitro*. Microglial MHCII and CD163 were unaffected by EV exposure, but these cells did upregulate the matrix metalloproteinase MT1-MMP. Mesenchymal GBM appears to secrete more EVs than other subtypes (de Vrij et al., 2015). In the following years, several other authors confirmed that GBM EVs promote an M2-like phenotype and anti-inflammatory cytokine secretion *in vitro* through transfer of miRNAs and potentially the M2-associated transcription factor STAT3 (van der Vos et al., 2016; Gabrusiewicz et al., 2018; Zhao et al., 2022). Gabrusiewicz et al. found in their 2018 paper, in contrast to de Vrij et al., that MHCII is not downregulated with EVs, which may be reflective of the use of different cell lines. Notably, this paper also found that EVs upregulated monocyte PD-L1. TAMs then produce further EVs, which interact with transformed cells to maintain stemness, resulting in reduced mouse survival (Zhao et al., 2022).

Thus, it appears that GBM-derived EVs exert effects on the mononuclear phagocyte compartment to facilitate T-cell suppression and possibly invasion via ECM degradation.

1.4.5.6. Myeloid-derived suppressor cells

T-cell suppression in GBM is also furthered by myeloid-derived suppressor cells (MDSCs). This heterogeneous population is often defined as CD33⁺ cells that lack markers associated with mature monocytes and granulocytes; they expand under inflammatory conditions to promote immunosuppression without fully maturing, and have historically been referred to as immature myeloid cells, which are today regarded as a separate and non-pathological population. The MDSC name was coined in 2007 by Gabrilovich et al., in an attempt to further clarity. Their function overlaps with immunomodulatory monocytic and granulocyte functions, making them hard to definitively identify and characterise. Monocyte-like (M-MDSC) and granulocyte-like (G-MDSC) subsets exist, and a third subset, termed lineage-negative, has been identified in human GBM patients (Raychaudhuri et al., 2011). MDSC development has been suggested to follow a two-stage pathway wherein immature cells are initially stimulated to expand, and are then pathologically activated, often by low-level chronic inflammation such as that seen in cancer. For review of MDSC biology, see Veglia, Perego and Gabrilovich, 2018.

Adoptive transfer of M-MDSCs drives murine glioma (Bayik et al., 2022), and mouse experiments suggest they may be drawn into the tumour by CCL2 and MIF (Zhu et al., 2011; Chang et al., 2016). The same mechanism may also occur in human GBM, which expresses the MIF receptor CD74; the MIF-CD74 interaction then promotes CCL2 expression (Alban et al., 2020).

MDSCs are significantly overrepresented in GBM compared to lower-grade gliomas (Alban et al., 2018), and have been found by flow cytometry to account for

approximately 15% of all immune cells with GBM (González-Tablas Pimenta et al., 2021). Furthermore, they are more prevalent in the blood of GBM patients than of other cancer patients or healthy controls, and G-MDSCs have been found to be more common than their monocytic counterparts in GBM (Raychaudhuri et al., 2011; Dubinski et al., 2016). MDSC presence is indicative of reduced survival following recurrence (Alban et al., 2018). Additionally, they are more prevalent in patients who develop lymphopaenia following treatment. MDSCs isolated from lymphopaenic patients more efficiently suppressed T-cell activation and proliferation than MDSCs from non-lymphopaenic patients. These authors found that cranial irradiation promoted MDSC expansion in murine models (Ghosh et al., 2023a).

Murine experiments have suggested that M-MDSCs rely more on NO synthase, whereas G-MDSCs utilise reactive oxygen species (ROS) and peroxynitrites (Movahedi et al., 2008; Raber et al., 2014). Patient-derived T-cells showed improved proliferation and IFN γ secretion *ex vivo* following MDSC depletion. Furthermore, elevated arginase was detected in patient sera, and it appears possible that MDSCs upregulate arginase to suppress T-cell function by depleting arginine from the TME (Raychaudhuri et al., 2011). MDSCs have been found to be IFN γ -responsive, which may enable them to respond to pro-inflammatory T-cells (Movahedi et al., 2008). Furthermore, GBM-derived MDSCs express the immunosuppressive ligand PD-L1 and are able to induce PD-1 expression on CD4 $^{+}$ cells (Dubinski et al., 2016) while suppressing T-cell response to tumour-associated antigens (Kusmartsev, Nagaraj and Gabrilovich, 2005). Other work has shown G-MDSC presence to correlate with reduced IFN γ -producing T-cells and increased levels of ARG1 and iNOS. The presence of these cells also correlated with reduced T-cell proliferation and earlier

disease recurrence (Chai, Zhang and Li, 2019). Thus, MDSCs may suppress T-cell function within GBM through several mechanisms.

1.4.6. Modern therapeutic concepts

As stated in 1.1.3.2., current best practice dictates maximal surgical resection with adjuvant RT and chemotherapy, which has improved survival in GBM patients but is not sufficient for long-term recovery. Thus, in recent decades, attention has turned to various immunotherapies, which has been aided by much of the recent work characterising the GBM immune microenvironment in both human and pre-clinical models, some of which has been outlined in 1.4.5. A brief overview of modern therapeutic concepts will be given here.

1.4.6.1. Immune checkpoint blockade in mice

Murine models have shown survival benefits with blockade of various immune checkpoints.

PD-L1 can be targeted by antibody or small interfering (si)RNA, the effect of which is enhanced by prior RT (Kim et al., 2017; Tian et al., 2022). Combined checkpoint blockade is even more effective. Anti-PD-L1, anti-TIM-3 and RT resulted in 100% survival at day 100 post-implantation, compared to less than 30 days' survival in untreated mice (Kim et al., 2017); inhibition of TIM-3 and its ligand CEACAM1 has also been found to have therapeutic benefit (Li et al., 2017). Combined PD-1 and TIGIT blockade also confers improved OS (Hung et al., 2018). Another study found murine glioma to be unresponsive to combined anti-PD-L1 and anti-CTLA-4 therapy, to which they could be sensitised with IL-6 inhibition and CD40 stimulation (Yang et al., 2021), whereas others have found it to be responsive, including with chemical inhibition of the tumour-expressed checkpoint molecule IDO (Wainwright et al., 2014). Other work has found combined PD-L1/CTLA-4 blockade to be effective and promote CD8⁺ T-cell infiltration in murine melanoma brain metastases, but only in the presence of an extracranial tumour (Taggart et al., 2018). Anti-CTLA-4 has been found to be effective with prior RT, although all mice died before 50 days post-implantation; earlier administration or concurrent 4-1BB stimulation prolonged survival beyond 100 days (Belcaid et al., 2014).

In all of these studies except Wainwright et al.'s, increased T-cell infiltration and activity is observed during or following treatment, and in some cases, an improved effector:Treg ratio, leading to the observed effects on survival. Additionally, three

papers found combined IC blockade to facilitate the development of immunological memory, allowing tumour rejection on rechallenge (Belcaid et al., 2014; Kim et al., 2017; Li et al., 2017).

Reprogramming Tregs to IFN γ -secreting CD4⁺ effectors has also been achieved using anti-PD-1 and anti-GITR antibodies; anti-GITR abrogated the ability of Tregs to suppress CD8⁺ effectors, facilitated tumour rejection, and synergised with TAM depletion. This resulted in a minority of mice surviving beyond 30 days post-injection, especially in combination with resection, RT and TMZ (Amoozgar et al., 2021). It is worth noting that, in these models, Tregs accounted for 30-40% of CD4⁺ TILs. As discussed in 1.4.4.1., T-cells often account for around 15-20% of cells in GBM, but as mentioned in 1.4.5.3., Tregs have been found to account for perhaps 1-2% of cells; thus, unless the CD8⁺:CD4⁺ ratio is heavily skewed, the proportion of Tregs in murine glioma is likely considerably higher than in human patients.

Further insights that may be relevant to human patients have also come from these studies: Yang et al.'s treatment reduced IL-10 and TGF β secretion from TAMs, thus alleviating some immunosuppression; Tian's paper noted that RT upregulated PD-L1 expression, which if it also applies to the human brain, should be considered in the therapeutic context; and adjuvant TMZ may decrease the effectiveness of anti-tumoural immunity (Wainwright et al., 2014). An additional problem exists in human patients, namely, treatment with the immunosuppressive steroid dexamethasone. Dexamethasone reduces the efficacy of concurrent anti-PD-1 in murine glioma and depletes TILs; in human patients undergoing anti-PD-L1 therapy, it reduced OS by several months (Iorgulescu et al., 2021).

Thus, mouse models validate the therapeutic potential of IC blockade – particularly combined blockade – and offer a variety of potential targets, as well as potential clinical considerations. However, these studies used the murine glioma GL261 line, for which there are conflicting reports about the efficacy of PD-1 blockade (for review, see Tritz, Ayasoufi and Johnson, 2021); furthermore, GL261 has a high mutational load, constitutively expresses MHCI, and upregulates MHCII in response to IFN γ . The SB28 line, which does not express either MHC and has a lower mutational burden, thus recapitulating human GBM better, is resistant to anti-PD-1 and anti-CTLA-4 combination therapy, and has been suggested to be a better pre-clinical model for trialling immunotherapy (Genoud et al., 2018). Additionally, rechallenge in these models uses the same line. Recurrent GBM often expresses different markers than its pre-treatment counterpart, and so the development of immunological memory is not enough. Rather, there is a need for the development of new adaptive immune responses. The real test of novel approaches is, of course, whether they permit improved survival in human patients in a clinical trial – and in this setting, the results are considerably less positive.

1.4.6.2. Immune checkpoint blockade in humans

The success of PD-1/PD-L1 inhibition in pre-clinical models and other solid tumours, such as lung or melanoma brain metastases (Goldberg et al., 2016), has led to considerable interest in its use in GBM – both individually and in combination with other therapies.

Multiple phase 3 clinical trials have found no benefit with use of the anti-PD-1 antibody nivolumab. Nivolumab did not improve OS and reduced PFS compared to the anti-VEGF therapy bevacizumab in recurrent GBM patients (Reardon et al., 2020); in primary GBM and gliosarcoma patients, nivolumab and RT reduced survival time compared to TMZ and RT in patients with an unmethylated MGMT promoter (Omuro et al., 2023). Another phase 3 trial compared these treatments to an RT and TMZ-only condition, finding no improvement in OS or PFS with nivolumab; this trial also found no differences in survival when stratified by PD-L1 positivity (Lim et al., 2022). PD-1 blockade alone does not improve the proliferation of exhausted T-cells from healthy donors (Goods et al., 2017), and IC blockade in recurrent GBM has been found not to promote T-cell infiltration, but rather to reduce myeloid cell presence, and in these samples, blockade did not appear to enrich tumour-specific CD8+ T-cell scores (Sun et al., 2023).

Retrospective analysis of anti-PD-L1 therapy revealed that PTEN mutation status, not PD-L1 expression, predicted better response: patients with wild-type PTEN showed longer OS, although only by some months (Zhao et al., 2019). One study of neoadjuvant pembrolizumab – a PD-1 blocking antibody – in recurrent HGG patients found improved PFS and OS compared to adjuvant administration, and

increased T-cell and IFN γ -related pathway representation. PD-L1 and CTLA-4 was upregulated in these patients in response (Cloughesy et al., 2019). Lee et al. observed similar results in recurrent GBM patients treated with neoadjuvant pembrolizumab in 2021. Based on mass cytometry and single-cell RNA sequencing data, they suggested a feedback loop wherein anti-PD-1 promotes activation and infiltration of peripheral T-cells, which show improved proliferation and IFN γ secretion in recurrent GBM. This promotes further T-cell and DC recruitment, resulting in increased anti-tumoural immunity, but also increased myeloid cell infiltration and resultant immunosuppression (Lee et al., 2021). Clearly, expression of PD-L1 on glioma cells alone is insufficient to predict response. Indeed, several parameters may be required to identify patients with the potential to respond to checkpoint blockade, such as molecular subtype, mutational burden, replication stress and others (for review see Arrieta et al., 2023).

The immunosuppressive CTLA-4 and TIGIT pathways appear to be upregulated in response to anti-PD-1 therapies, and so combined blockade may be effective in GBM patients (Lee et al., 2021; Sun et al., 2023). A phase 1 trial investigating combined PD-1 and CTLA-4 blockade in recurrent GBM patients is now underway (Wen, 2021). A phase 2 clinical trial has shown this treatment to be efficacious in treatment of intracranial melanoma (Tawbi et al., 2018), and pembrolizumab is beneficial in patients with non-small cell lung cancer brain metastases, which correlated with tumour PD-L1 positivity (Goldberg et al., 2020).

1.4.6.3. Other immunotherapy approaches

Other approaches besides checkpoint blockade also have merit and are under investigation. These include, amongst others, the well-known chimeric antigen receptor (CAR)-T-cell therapy, gene therapy and personalised tumour vaccines. In addition, tocilizumab, an anti-IL-6 therapy, has been suggested for use in GBM as IL-6 is expressed by murine glioma cells in response to glioma cell-expressed TIM-3 ligation patients (Guo Q et al., 2022). Several of these approaches have recently progressed to clinical trials, and will be outlined here.

CAR-T-cell therapy equips T-cells with an engineered receptor, the outer portion of which is usually an antibody-derived chain specific to a tumour antigen. The intracellular portion contains the CD3 ζ chain and costimulatory domains such as the intracellular portion of CD28 or 4-1BB. This allows the T-cell to bypass the requirement for both MHC-mediated antigen presentation and a costimulatory signal, such that it is instead able to bind directly to the target antigen and facilitate anti-tumoural immunity. CAR-T-cells are then expanded *ex vivo* and transferred into the patient. This approach has shown some benefit. A phase I trial using the epidermal growth factor receptor (EGFR) mutant EGFRvIII as an antigen found reduced EGFRvIII post-transfer and evidence of CD8⁺ T-cell proliferation; however, increased Treg and immunosuppressive molecule presence was also observed (O'Rourke et al., 2017). EGFRvIII-specific CAR-T-cells can persist in patients for over two years and may permit polyclonal T-cell infiltration (Durgin et al., 2021).

However, the effectiveness of CAR-T-cell therapy is limited by the immunosuppressive environment and heterogeneity of GBM – if the antigen is not

expressed on all tumour cells, eradication may well be incomplete, and if it is lost on recurrence, the CAR-T-cells will be ineffective. An example was seen in a patient with multifocal GBM who received IL-13R α 2-targeted CAR-T-cells. Administration directly into the CSF allowed control of tumours throughout the brain and spinal cord, but eventually tumours recurred and appeared to have reduced IL-13R α 2 expression (Brown et al., 2016). Thus, with CAR-T-cell therapy there is a risk of immune escape, and patients may then face the same issues as they do today: recurrence of a more aggressive and treatment-resistant tumour with resultant mortality. Exploration of multiple other antigens in clinical and preclinical studies is underway (reviewed in Maggs et al., 2021).

Other modern approaches are in early-stage clinical trials. A phase 1 gene therapy trial with 31 recurrent HGG patients used IL-12 to stimulate production of IFN γ and promote T-cell function; the treatment appeared to promote T-cell infiltration and was found to be tolerable (Chiocca et al., 2019). Another approach is the generation of personalised tumour vaccines. A phase 1 trial of eight patients found this approach to be tolerable and to confer an OS of nearly 17 months, though administration of dexamethasone during the priming vaccinations prevented the development of immunity (Keskin et al., 2019). A DC vaccine using cells loaded with tumour lysate was found to stimulate peripheral cytotoxic T-cell activity and IL-2 production, which were not sufficient to improve patient survival. However, patients with Th1 and CD8+ T-cell-associated markers had significantly improved PFS and OS post-vaccination (Erhart et al., 2018). Depletion of immunosuppressive cells is another approach: myeloid-derived suppressor cell depletion using the drug tadalafil was found to be safe and effective in a phase 1 trial of 16 patients, but it did not improve OS or PFS

over the control group (Ghosh et al., 2023b). Many further options exist, including oncolytic viruses and antibody-drug conjugates: tumour-directed antibodies conjugated to cytotoxic drugs (for review, see Maggs et al., 2021).

In conclusion, modern immunotherapeutic approaches are diverse and numerous, and many avenues for improvement of patient OS are open. However, many of these approaches cannot protect against recurrence. Lifting of the immunosuppressive environment is likely of high importance to improve patient survival and quality of life: doing so would hopefully allow development of native adaptive immunity responses, including in the case of GBM recurrence, to provide long-term anti-tumoural immunity. Thus a greater understanding of the immunosuppressive GBM TME may be of great benefit in terms of finding new synergistic therapeutic targets.

1.5. Aims and objectives

This project aimed to identify candidate immunosuppressive proteins secreted by human GBM tumours using mass spectrometry data, and then to ascertain their presence in GBM transport media. Concurrently, the intratumoural presence and distribution of these proteins alongside adaptive immunity effectors was studied to ascertain whether cell-protein interactions took place. Confirmation of cell-protein interactions would then allow characterisation of any immunosuppressive effects using *in vitro* assays to determine cellular function. This work was performed using three datasets, covering both the liquid fraction and the tumour itself. These are outlined in Table 1.01.

Table 1.01. Datasets used for experimental work

Dataset	Sample type	Patient number	Analyses performed
Mass spectrometry output (Gkanatsiou, 2022)	Transport media	30	Bioinformatics
Brain tumour liquid samples	Transport media and culture supernatant	23 (GBM), 7 (LGG)	BCA and ELISA
GBM slides	Tumour sections	50	Immunofluorescence staining

Table 1.01. Datasets used for experimental work.

2: METHODOLOGY

2.1. Cell culture

2.1.1. Cell culture

U251 cells were a gift from Professor Bigner (Duke University, USA). U373 cells were a gift from Dr Vinodh Kannapans (University of Wolverhampton, UK). THP-1 and Monomac-6 cells were a gift from Rei Hassanen (University of Wolverhampton, UK). Human umbilical vein endothelial cells (HUVECs) and normal human astrocytes (NHA) were a gift from Professor Angel Armesilla (University of Wolverhampton, UK).

HUVECs and NHAs were placed directly on glass slides (see 2.4.2.) but were not passaged or otherwise cultured. All cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Sigma) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Sigma) and 2mM L-glutamine (Sigma). Cells were passaged every 48-72 hours and discarded before the tenth passage. Cells were grown in a 37°C 5% CO₂ incubator and cell culture work was carried out in a Class II biological safety cabinet using pre-warmed reagents, unless otherwise stated.

2.1.2. Cryopreservation, revival and passaging

Adherent cells were cryopreserved in FBS supplemented with 10% dimethyl sulfoxide (DMSO) (v/v) in 1mL cryovials. Suspension lines were cryopreserved in 70% complete media, 20% additional FBS and 10% DMSO (v/v) in 1mL cryovials. All cell lines were stored at -80°C and additional cell banks were established in liquid nitrogen for long-term storage.

For revival, vials were thawed in a 37°C water bath. Cell suspension was added dropwise to 5mL pre-warmed media and centrifuged at 2000 x *g* for five minutes. Media was aspirated and the pellet was resuspended. Adherent cell suspension was then transferred to a flask pre-filled with warm media. Suspension cells were centrifuged and resuspended again, then transferred to a pre-filled flask. Cells were evenly distributed by gently shaking the flask and their presence was confirmed using a light microscope; the flask was then placed in a 37°C 5% CO₂ incubator.

Adherent cultures were passaged upon reaching 70% confluence or greater. Media was aspirated, cells were dissociated using 0.05% trypsin-EDTA (ethylenediaminetetraacetic acid) (Sigma). After minimum five minutes' incubation, flasks were agitated and cell dissociation was confirmed using a light microscope. Trypsin was neutralised using complete media and the cell suspension was centrifuged at 2000 x *g* for five minutes. Media was aspirated and the pellet was resuspended. Cell suspension was transferred to three new flasks unless seeding at a specific density was required.

Suspension cultures were passaged when cultures reached 1×10^6 cells/mL or greater. 2-5mL cell suspension was transferred to a new flask pre-filled with warm media. In all cases, cells were evenly distributed by gently shaking flasks and their presence was confirmed using a light microscope; the flasks were then placed in a 37°C 5% CO₂ incubator.

For cell counting, 50µL cell suspension was mixed with an equivalent volume of 0.4% trypan blue solution (Sigma). 10µL was pipetted into each chamber of a Countess™ Cell Counting Chamber Slide (ThermoFisher); cell counts and viability were determined using a Countess I or II instrument.

2.1.3. Supernatants

U251 and U373 cells were seeded into T-75 flasks containing 10mL complete media at a density of 1.8×10^5 cells/mL, as the maximum seeding density of 2.0×10^5 cells/mL could not be achieved. Supernatant was removed from flasks at 24, 48, 72 or 96 hours post-seeding and passed through a $0.22\mu\text{m}$ filter to remove cells. Aliquots were stored at 5°C or frozen at -20°C for long-term storage.

Immediately following surgery at the Walton hospital, Liverpool, United Kingdom, tumour sections were placed into 10mL serum-free Ham's F10 media (Gibco) (transport media, TM) and transported to University of Wolverhampton in a cooled, insulated container. Primary cultures were established within 24 hours of surgery, at which point TM were frozen at -80°C for long-term storage. Tumour sections were mechanically dissected in a 10cm dish and grown in 10mL serum-free Ham's F10 media. Initial culture supernatants were removed 24 hours after dissection and frozen at -80°C for long-term storage. Primary culture establishment, transport media and culture supernatant work was carried out by Dr Farjana Rowther; this work was approved by the Walton Tissue Research Bank under the title "Derivation of short-term cell cultures from brain tumours for rapid screening of novel therapies", reference number WRTB 13_02.

2.1.4. Monocyte differentiation and polarisation

Monocytes were seeded into 12-well plates containing 0.5mL media per well at a minimum density of 5×10^5 cells/mL and grown in media containing 20ng/mL phorbol 12-myristate 13-acetate (PMA) for 24 hours. This protocol has previously been found sufficient for THP1 differentiation (Valle and Guiney, 2005). Adherence was confirmed using a light microscope. Differentiated macrophages were then washed with phosphate-buffered saline (PBS) and grown for a further 24 hours in 1mL media without PMA. For M1 polarisation, macrophages were treated with 100ng/mL lipopolysaccharide (LPS) for a further 24 hours. Timing constraints relating to NHS Blood and Transport ethical approval necessitated the use of monocyte cell lines instead of patient-derived monocytes.

2.1.5. Treatment with recombinant proteins

Recombinant human proteins were reconstituted in PBS without carrier protein according to the manufacturer's instructions. Aliquots were stored at -20°C until required. Details are given in Table 2.01. Recombinant proteins were added to complete media containing 1mM dithiothreitol (DTT) (Sigma) to maintain a reduced state; to block protein activity, 0.1M lactose (Sigma) was used. Differentiated macrophages were cultured in media containing recombinant proteins for 24 hours and washed once in PBS before use in any further assays.

Table 2.01. Recombinant proteins used for cell treatment

Protein	Supplier	Source	Reconstitution (µg/mL)
CTGF	R&D Systems	NS0 mouse myeloma cell line	500
Galectin-1	Novus Biologicals	E. coli	100
Galectin-3	Peprtech	E. coli	100
Galectin-4	R&D Systems	E. coli	100
Galectin-7	R&D Systems	E. coli	200

Table 2.01. Recombinant proteins used for cell treatment.

2.1.6. Phagocytosis assays

pHRodo red-conjugated *Escherichia coli* were resuspended in sterile PBS at 2mg/mL and vortexed for a minimum of one minute. This stock was diluted tenfold in sterile PBS to produce a working solution. Media was aspirated from differentiated macrophages and the cells were washed once with PBS; media was replaced with 0.5mL phagocytosis assay reagent per well of a 12-well plate. Negative controls used an equivalent volume of PBS. Plates were placed in a non-CO₂ incubator for two hours, then imaged on an Evos FL Auto 2 using transmitted light and the red fluorescent protein (RFP) channel. Total cells and cells containing red fluorescence were manually counted using ImageJ with the Cell Counter plugin (NIH, Maryland, USA).

2.2. Flow cytometry

2.2.1. Staining of cell surface antigens

Cells were washed in PBS and harvested using a cell scraper into 1mL cold 0.22µm-filtered PBS 5% FBS; scraping was used to avoid potential degradation of surface markers by proteolytic enzymes. Suspensions were incubated with antibodies at 4°C for 30 minutes at a ratio of 3µL antibody per 100µL cell suspension unless otherwise specified. Cells were then pelleted in a benchtop microcentrifuge at 350 x *g* for five minutes. Supernatant was aspirated and the pellet was washed once with PBS. Cells were resuspended in 100µL cold, 0.22µm-filtered PBS 5% FBS.

2.2.2. Sample acquisition

Data were collected using a BD Accuri C6 flow cytometer equipped with 488nm and 647nm lasers using BD Accuri C6 cytometer software (BD Biosciences).

2.2.3. Antibodies used in flow cytometry

Antibodies used in immunofluorescence experiments are listed in Table 2.02. All antibodies were stored according to the manufacturer's instructions at 5-8°C.

Table 2.02. Antibodies used in flow cytometry

Specificity	Conjugate	Host	Clone	Isotype	Supplier	Catalog number
CD80	Allophycocyanin	Mouse	2D10	IgG2a κ	BioLegend	305219
CD86	FITC	Mouse	BU63	IgG1 κ	BioLegend	374203
MHCII	Phycoerythrin	Mouse	Tü39	IgG1 κ	BioLegend	361715

Table 2.02. Antibodies used in flow cytometry.

2.3. ELISA and BCA assays

2.3.1. ELISA overview

Sandwich enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer's instructions. ELISAs are summarised in Table 2.03. 96-well plates were coated with capture antibody as needed. Plates were blocked for one hour using 1% bovine serum albumin (BSA) in PBS (w/v). Samples were incubated in the plate, followed by incubation with the detection antibody, for two hours at ambient temperature or one hour at 37°C. Two further incubations of streptavidin-conjugated horseradish peroxidase and 1-Step™ Ultra TMB-ELISA Substrate Solution (ThermoFisher) were performed in the dark for 20 minutes each at ambient temperature. Reactions were stopped with 2N hydrochloric acid and plates were measured immediately. After blocking, and between each incubation, wells were washed with PBS 0.05% Tween-20 (v/v) three times; excess liquid was removed by blotting plates against clean paper towels followed by aspiration. Standards and samples were run in duplicate. Optical density at 450nm and a correction at 540nm were recorded using a Multiskan Ascent plate reader (ThermoFisher). Two technical replicates were performed for each standard and sample. Cell line supernatants were analysed using three biological replicates, each of which derived from a separate revival.

Table 2.03: Summary of ELISA kits

Target	Type	Supplier
CTGF	Sandwich ELISA	R&D Systems
CEACAM1	Sandwich ELISA	R&D Systems
Galectin-1	Sandwich ELISA	R&D Systems
Galectin-3	Sandwich ELISA	R&D Systems
Galectin-4	Sandwich ELISA	R&D Systems
Galectin-7	Sandwich ELISA	R&D Systems
S100A7	Sandwich ELISA	ThermoFisher

Table 2.03: ELISA kits used for supernatant assays.

2.3.2. BCA overview

2.3.2.1. Protein precipitation

Proteins were precipitated from supernatants using 100% trichloroacetic acid (TCA) (w/v) in distilled water (dH₂O). TCA solution was added to supernatants at a ratio of 1:4 for a final TCA concentration of 20%; proteins were allowed to precipitate on ice for 30 minutes. Samples were centrifuged at 18,600 x *g* for 5 minutes and supernatant was aspirated. The remaining pellet was washed with 100-200µL cold acetone, centrifuged again at 9500 x *g* if the pellet dissolved, and dried at room temperature or in a 56°C heat block. Protein was resuspended in a volume of PBS equal to the initial supernatant volume. Samples were used within 24 hours.

2.3.2.2. BCA assay

Bicinchoninic acid (BCA) assays were performed using the Pierce™ BCA Protein Assay Kit (ThermoFisher) according to the instructions. Standards were diluted as per the instructions in PBS. Working reagent was produced using a 50:1 mixture of reagents A and B respectively. 25µL of resuspended protein and 200µL working reagent was added per well of a 96-well plate and incubated at 37°C for 30 minutes. Optical density at 540nm was recorded using a Multiskan Ascent plate reader (ThermoFisher). Two technical replicates were performed for each standard and sample. Cell line supernatants were analysed using three biological replicates, each of which derived from a separate revival.

2.4. Immunofluorescence

2.4.1. Preparation of tissue section slides

5µm frozen glioblastoma (GBM) tissue sections affixed to glass slides were obtained from 50 GBM patients; either five or eight tissue sections were obtained per patient. Slides were provided by the Brain Tumour North West Tissue Bank in Preston, Lancashire, United Kingdom. Tissue sections were stored at -80°C.

2.4.2. Preparation of cell culture slides

Slides were pre-sterilised in ethanol and air-dried. 50-100µL of cell suspension (U251, U373, Monomac-6, THP1, human umbilical endothelial vein (HUVEC) or normal human astrocyte (NHA)) was added and slides were placed in a humidified tray within a 37°C 5% CO₂ incubator. For monocyte cell lines, media containing 20ng/mL PMA was used to ensure adherence. After 24 hours, slides were inspected under a light microscope to confirm cell adherence and fixed in ice-cold methanol for ten minutes. Slides were then rinsed three times in PBS, air-dried and stored at -20°C until required.

2.4.3. Deparaffinisation and antigen retrieval

Formalin-fixed paraffin-embedded human tonsils (Novus Biologicals) were dewaxed using three five-minute washes in 100% xylene. Sections were rehydrated in three five-minute washes in 100% ethanol, followed by one five-minute wash in each of 95%, 70% and 50% ethanol, then two five-minute washes in PBS. Antigen retrieval was performed by boiling sections five times in Vector Labs citrate retrieval buffer in a microwave, followed by cooling under running water. Sections were stored at 5°C before or -80°C after use.

2.4.4. Staining procedure

Slides were acclimatised to room temperature for a minimum of 30 minutes. For intracellular staining, sections were permeabilised with a 10-minute incubation in PBS 0.05% Tween-20 (v/v). Sections were blocked for 45 minutes using PBS 1% BSA (w/v). Sections were then incubated in 100 μ L of primary antibody for 60 minutes, followed by incubation in the dark for minimum 30 minutes with 100 μ L of secondary antibody, where relevant. Sections were counterstained with 1 μ g/mL 4',6-diamidino-2-phenylindole (DAPI) for 7-10 minutes. Coverslips were mounted using Prolong Gold Antifade Reagent (Invitrogen). After overnight curing, coverslips were sealed with clear nail varnish. All incubations were carried out in a humidified chamber at ambient temperature; antibodies were diluted in accordance with the manufacturer's recommendations. Sections were washed thrice with PBS 0.05% Tween-20 (v/v) between steps, or twice following DAPI staining.

2.4.5. Sample acquisition

Imaging was performed on a Zeiss LSM 880 laser scanning inverted confocal microscope, and Zen Black software. The microscope was equipped with laser lines at 405, 458, 488, 514, 561 and 633nm. The lenses used were Plan-Apochromat 10X/0.45 M27, EC Plan-Neofluar 20X/0.50 M27, EC Plan-Neofluar 40X/1.30 Oil DIC M27 and Plan-Apochromat 63X/1.40 Oil DIC M27. Zeiss Immersol 518F was used with the 40 and 63x oil immersion lenses.

Averaging was manually set to 16; images were recorded with a bit depth of 16. For each individual experiment, the Smart Setup function was used to set the optimum parameters for the fluorophores used. Images were saved as both .czi and TIF files, and a split-channel TIF was saved for each image. Image processing was performed using ImageJ (NIH, Maryland, USA) and Zen Blue where necessary. Cells were counted manually using the ImageJ Cell Counter plugin.

2.4.6 Antibodies used in immunofluorescence

Antibodies used in immunofluorescence experiments are listed in Table 2.04. All antibodies were stored according to the manufacturer's instructions at either 5-8°C or at -20°C in 50% glycerol.

Table 2.04. Antibodies used in immunofluorescence.

Specificity	Conjugate	Host	Clone	Isotype	Supplier	Catalog number
CD3	AlexaFluor 647	Mouse	UCHT8	IgG1 κ	BioLegend	300416
CD19	FITC	Mouse	4G7	IgG1 κ	BioLegend	392507
CD31	FITC	Mouse	WM59	IgG1 κ	BioLegend	303103
CD56	N/A	Rabbit	JF1021	IgG	Novus Biologicals	NBP2-66968
CD68	N/A	Rabbit	Poly-clonal		Invitrogen	PA5-80441
CTGF	N/A	Goat	Poly-clonal	IgG	R&D Systems	AF660-SP
Galectin-1	N/A	Goat	Poly-clonal	IgG	R&D Systems	AF1152-SP
Galectin-3	N/A	Goat	Poly-clonal	IgG	R&D Systems	AF1197-SP
Galectin-4	N/A	Goat	Poly-clonal	IgG	R&D Systems	AF1227-SP

Galectin-7	N/A	Goat	Poly-clonal	IgG	R&D Systems	AF1339-SP
Galectin-9	AlexaFluor 594	Mouse	9M1-3	IgG1 κ	BioLegend	348921
GFAP	N/A	Goat	Poly-clonal	IgG	Novus Biologicals	NB100-53809
LYVE1	N/A	Rabbit	Poly-clonal	IgG	Novus Biologicals	NB100-725SS
PD-L1	AlexaFluor 594	Mouse	29E.2A3	IgG2b κ	BioLegend	329742
PDGFR β	N/A	Mouse		IgG1	Novus Biologicals	MAB1263-SP
Goat	FITC	Donkey	Poly-clonal	IgG	Jackson Immuno Research	705-097-003
Mouse	AlexaFluor 594	Donkey	Poly-clonal		Jackson Immuno Research	705-587-003
Rabbit	RRX	Donkey	Polyclonal	IgG	Jackson Immuno Research	711-297-003

Table 2.04. Antibodies used in immunofluorescence.

2.5. Bioinformatics

Mass spectrometry data of the secretome of 30 whole human GBM was obtained as an Excel spreadsheet. These data were a preliminary dataset from the work described in Gkanatsiou, 2022.

Pathway analysis was performed using Reactome (Gillespie et al., 2022). The entire dataset was uploaded to Reactome and a list of the top 25 overrepresented pathways was downloaded as a PDF. Additionally, immunity-related pathways with a false discovery rate <0.05 were noted.

Protein functions were identified using a manual search of the Uniprot (UniProt Consortium, 2023) and Genecards databases (Stelzer et al., 2016). Protein presence in extracellular vesicles was examined using a manual search of the VesiclePedia database (Pathan et al., 2019). Differentially-expressed genes and survival curves associated with genes of interest were generated from The Cancer Genome Atlas's firehose legacy GBM dataset (Brennan et al., 2013), comprising 136 patients, using the online tools in cBioPortal (Cerami et al., 2012; Gao et al., 2013). Differentially-expressed gene lists were uploaded to Reactome and overrepresented pathways were identified as described above.

2.6. Statistical analyses

Statistical analysis and data processing was performed using GraphPad PRISM version 8 and above (GraphPad, Boston, Massachusetts, USA) and Microsoft Excel. Significance was set at $p=0.0050$.

To compare two means, t-test was used. If standard deviations were found to be significantly different according to the Brown-Forsythe test, t-test with Welch's correction was used. To compare three or more means, one-way analysis of variance (ANOVA) was used. If these data were found not to follow a normal distribution by D'Agostino-Pearson's normality test, the non-parametric Kruskal-Wallis test was used instead of ANOVA. If standard deviations were found to be significantly different according to the Brown-Forsythe test, ANOVA with Welch's correction was used, regardless of distribution. The recommended multiple comparisons test for each condition was used.

To correlate two outcomes, Fisher's exact test was used.

Bonferroni's correction for multiple comparisons was performed using the formula α/n to calculate a new significance level, where α =the previous significance level (0.05) and n =the number of tests. The Benjamini-Hochberg correction for multiple comparisons was performed by ranking p-values from smallest to largest. For each p-value, a critical value was calculated using the formula $(i/m)Q$, where i =rank, m =number of tests and Q =false discovery rate.

3: IDENTIFICATION OF PROTEINS OF INTEREST IN GBM MASS SPECTROMETRY DATA AND CULTURE SUPERNATANTS

3.1. Introduction

The first stages of this work analysed a preliminary database of proteins secreted by glioblastoma (GBM). These proteins were identified by Gkanatsiou (2022) using mass spectrometry analysis of transport media (TM) from 30 human whole GBM tumours. In this work, extracellular vesicles (EVs) were isolated using column filtration and analysed alongside directly secreted (DS) proteins.

As discussed above, this project aimed to identify secreted proteins that may be responsible for immunomodulatory effects in GBM. DS proteins would exert local effects, whereas proteins in EVs may also enter the circulation, from where they may be able to affect systemic immunity (Figueroa and Carter, 2018). Likely candidates for immunosuppression were then brought forward to a further series of experiments, examining their intratumoural distribution, cellular sources and effects on immune effector populations *in vitro*.

In this chapter, proteins of interest were identified from mass spectrometry data and their concentrations in liquid samples from glioma primary tumours and *in vitro* cultures were ascertained using enzyme-linked immunosorbent assays (ELISA). Concurrently, the mass spectrometry data and publicly available messenger RNA

(mRNA) sequencing data was used for bioinformatic analyses to examine the prevalence and potential differential regulation of immune pathways.

3.2. Results

3.2.1. Bioinformatics

3.2.1.1. Initial analysis of mass spectrometry data

3.2.1.1.1. Screening of mass spectrometry data

Initial screening of the mass spectrometry dataset was performed using inclusion and exclusion markers to remove irrelevant proteins while highlighting those that may be of interest (shown in Table 3.01). The EV dataset contained 1693 proteins, while the DS dataset comprised 1198 proteins. Exclusion criteria were primarily used to remove structural proteins, while inclusion criteria were designed to highlight immunity- and growth-related proteins. This filtering refined the datasets to 1436 and 1080 proteins for EV and DS fractions respectively.

Exclusion markers were primarily structural proteins and other families, such as histones, that were irrelevant to the work. Inclusion terms included those associated with immune function, such as immunoglobulin; those with potential to identify immune effector populations, such as CD; and aberrant growth-related proteins such as Ras.

Table 3.01. Inclusion and exclusion criteria for mass spectrometry dataset filtering

Inclusion	Exclusion
CD	Actin
CDK	Albumin
Complement	Apolipo
Growth factor	Collagen
HLA	Dynein
Immunoglobulin	Fibrinogen
Ras	Fibronectin
	Hemoglobin
	Histone
	Keratin
	Kinesin
	Laminin
	Macroglobulin
	Microtubul
	Myosin
	Proteasome
	Ribosomal
	Spectrin
	Tubulin
	Vimentin

Table 3.01. Inclusion and exclusion criteria for mass spectrometry dataset filtering. The Americanised spelling of haemoglobin was used as a search term in accordance with the spelling in the mass spectrometry dataset.

3.2.1.1.2. *Identification of protein families*

Refined datasets were examined for proteins of interest, both individually and by family. Protein functions were identified using the UniProt (UniProt Consortium, 2023) and Genecards (Stelzer et al., 2016) databases; metabolism-related families were omitted as they are far beyond the scope of this work. Members of the cellular communication network (CCN), carcinoembryonic cell adhesion molecule (CEACAM), galectin, serpin and high mobility group (HMG) proteins were all present in the secretome, as well as immunoglobulin (Ig) fragments and multiple interleukin (IL) receptors. Protein families with more than one member present are shown in Table 3.02.

Multiple conclusions can be drawn from these data. First, several proteins and protein families were directly secreted but not exported in EVs, showing that secretion may be tightly regulated. This is reminiscent of results observed by de Vrij et al. in their 2015 paper, which described limited overlap between EV-secreted and intracellular proteins from the U87 glioma cell line. The presence of class-switched Ig fragments was also of interest, as this may indicate B-cell activity within GBM; however, ascertaining whether these antibodies are directed against tumour antigens was beyond the scope of this project. Human leukocyte antigen (HLA) molecules, also known as major histocompatibility complex (MHC), and various scavenger receptors were also present in the EV but not the DS proteins tab, suggesting the presence of professional antigen-presenting cells (APCs). There were also several proteins associated with innate immune effector presence, such as neutrophil elastase and the natural killer cell-associated molecule CD244, implying that these cells may also be present.

Table 3.02. Protein families identified in the GBM secretome by mass spectrometry

Family	EV fraction	DS fraction
14-3-3 proteins	ϵ , ζ/δ , θ , σ	α/β , ζ/δ , η , θ , σ
Annexins	A1, A2, A3, A4, A5, A6, A11	A1, A2, A3, A5, A6
Aquaporins	1, 4	4
CCN	1, 2	1, 2
CEACAM	1, 6	6
Complement system	C1qA, C1qB, C1qC, C1s, C1r, C3, C4a, C5, C6, C7, C8, C9, B, I	C1qC, C2, C3, C4a, C4b, B, I
Galectins	1, 3, 4, 7	1, 3
High mobility group	A1, B1, B2	B1, B2
HLA	A and B DR, DP, DQ	N/A
Immunoglobulins	κ , λ , α , μ and γ variable and constant regions	κ and λ variable and constant regions α , γ and μ constant regions
Integrins	α 2, 3, 7, V and M β 1 and 2	α 1
Interleukin receptors	IL-5R α , 11R α	N/A
NACHT, LRR and PYD containing proteins	2, 3, 10	N/A

Olfactory receptors	1F12, 1M1, 5L1, 8H2, 10X1, 56B1	1M1, 1P1, 4K13, 13C4
S100 proteins	A6, A7, A8, A9, A10, A11, A13, A16, B	A2, A4, A6, A8, A9, A11, B, P
Scavenger receptors	FCGR1, FCGRN	FCGRB, FCG3A, FCGBP
Serpins	A1, A3, A5, A7, B3, B6, C1, D1, E1, F1, F2, G1, H1	A1, A3, A6, A7, B1, B6, B9, C1, D1, E1, F1, G1
Sushi repeat proteins	SRPX, SRPX2, SUSP2	N/A
XRCC	5, 6	N/A

Table 3.02. Protein families identified in the GBM secretome by mass spectrometry.

3.2.1.1.3. *Pathway analysis of mass spectrometry data*

Reactome (Gillespie et al., 2022) was used to identify upregulated pathways in mass spectrometry data. The top 25 upregulated pathways for each dataset are shown in Table 3.03. A variety of cellular processes were altered. Additionally, seven immunity-related processes were upregulated in the DS proteins dataset, and three in the EV dataset.

In the EV dataset, the immunity-related pathways were neutrophil degranulation, platelet degranulation and viral mRNA translation. In the DS dataset, the same degranulation processes were observed as well as innate immunity, IL-12 signalling, JAK-STAT signalling following IL-12 signalling, antigen cross-presentation and virally-mediated CD4 degradation. Platelet activation, signalling and aggregation was also noted in this dataset, and may represent immune-related platelet activation or a response to tissue injury. It is worth noting that Reactome, like other similar tools, does not definitively state that these pathways are relevant, but rather indicates that they are likely to be based on the presence of multiple functionally-related proteins.

These data have several interesting implications. First, both innate and adaptive immunity were clearly represented, but adaptive immunity is most prevalent in the DS dataset, suggesting that activity and modulation of adaptive immunity takes place on a local scale in GBM. However, systemic T-cell lymphopaenia has also been observed in these patients: this appears to be modulated through downregulation of the S1P1 receptor, resulting in sequestration, especially of naïve T-cells, in the bone marrow in response to intracranial tumours regardless of type

(Chongsathidkiet et al., 2018). It is currently unclear if this downregulation is related to the tumour secretome.

Second, both datasets contained an upregulated pathway related to viral infection. Specifically, Vpu-mediated CD4 degradation is related to human immunodeficiency virus (HIV) (Andrew and Strebel, 2010), but no HIV+ patients were present in the mass spectrometry dataset. As GBM patients often suffer from systemic immunosuppression, it is not impossible that viral infection is common in these patients; however, it is also likely that CD4 is affected by non-viral GBM-related immunosuppression.

Third, IL-12, a pro-inflammatory cytokine that promotes Th1 cell function and is produced by APCs, was represented in the DS fraction, and the presence of APCs was also supported by upregulated cross-presentation. These data support the idea that modulation of T-cell function is common within GBM.

Table 3.03. Top 25 upregulated pathways in mass spectrometry data as identified by Reactome

EV fraction	DS fraction
SRP-dependent co-translational protein targeting to membrane	Neutrophil degranulation
Neutrophil degranulation	Platelet degranulation
Regulation of expression of SLITs and ROBOs	Response to elevated platelet cytosolic Ca ²⁺
Axon guidance	Innate immune system
Nervous system development	Platelet activation, signalling and aggregation
Eukaryotic translation elongation	AUF1 (hnRNP D0) binds and destabilises mRNA
Peptide chain elongation	Regulation of mRNA stability by proteins that bind AU-rich elements
Signalling by ROBO receptors	The role of GTSB1 in G2/M progression after G2 checkpoint
Eukaryotic translation termination	Negative regulation of NOTCH4 signalling
Response to elevated platelet cytosolic Ca ²⁺	Apoptosis
Nonsense mediated decay (NMD) independent of the exon junction complex (EJC)	Gene and protein expression by JAK-STAT signalling after interleukin-12 stimulation
Platelet degranulation	Interleukin-12 signalling

LI3a-mediated translational silencing of ceruloplasmin expression	FBXL7 down-regulates AURKA during mitotic entry and in early mitosis
Response of EIF2AK4 (GCN2) to amino acid deficiency	SCF-beta-TrCP mediated degradation of Emi1
Selenocysteine synthesis	Regulation of ornithine decarboxylase (ODC)
GTP hydrolysis and joining of the 60S ribosomal subunit	SCF(Skp2)-mediated degradation of p27/p21
Eukaryotic translation initiation	Ubiquitin-dependent degradation of cyclin D
Cap-dependent translation initiation	Programmed cell death
Formation of a pool of free 40S subunits	Degradation of DVL
Viral mRNA translation	Vpu mediated degradation of CD4
Extracellular matrix organisation	Regulation of apoptosis
Selenoamino acid metabolism	Metabolism of polyamines
ECM proteoglycans	NIK > noncanonical NF-kB signalling
Nonsense-mediated decay (NMD)	Cross presentation of soluble exogenous antigens (endosomes)
Nonsense-mediated decay (NMD) enhanced by the exon junction complex (EJC)	Regulation of activation PAK-2p34 by proteasome mediated degradation

Table 3.03. Top 25 upregulated pathways in mass spectrometry data as identified by Reactome.

In accordance with accepted measures of statistical significance, all pathways identified by Reactome with a false discovery rate (FDR) less than 0.05 were examined.

Reactome identified 1629 upregulated pathways for DS proteins, of which 188 met this significance threshold; and for EV proteins 1796 pathways were upregulated, of which 242 met the threshold. 27 and 23 immunity-related pathways, respectively, were isolated from these datasets (shown in Table 3.04). In this analysis, scavenging by class A receptors and binding and uptake of ligands by scavenger receptors, which were identified by Reactome as vesicle-mediated transport pathways, were considered to be immune-related functions.

Both datasets showed similar representation of immune functions. Adaptive immunity processes such as T-cell and B-cell receptor (TCR and BCR) signalling, scavenger receptor function and antigen presentation-associated functions were present in both, providing further evidence that T-cells, B-cells and macrophages are present and active within the tumour. In addition, IL-1 and IL-12 signalling was noted in both datasets, both of which are pro-inflammatory cytokines. Thus, it appeared that in terms of both pathway and protein family analysis, immune processes relating to B-cells, T-cells and mononuclear phagocytes were clearly represented.

Table 3.04. Upregulated immunity pathways in mass spectrometry data

EV fraction	DS fraction
Neutrophil degranulation	Neutrophil degranulation
ER-Phagosome pathway	Innate immune system
Antigen processing-Cross presentation	Gene and protein expression by JAK- STAT signaling after Interleukin-12 stimulation
Innate Immune System	Interleukin-12 signaling
NIK-->noncanonical NFkB signaling	Vpu mediated degradation of CD4
Dectin-1 mediated noncanonical NFkB signaling	NIK-->noncanonical NFkB signaling
Cross-presentation of soluble exogenous antigens (endosomes)	Cross-presentation of soluble exogenous antigens (endosomes)
MHC class II antigen presentation	Interleukin-12 family signaling
Scavenging by Class A Receptors	Downstream signaling events of B Cell Receptor (BCR)
Activation of NFkB in B cells	Activation of NFkB in B cells
Downstream signaling events of B Cell Receptor (BCR)	Interleukin-1 signaling
Terminal pathway of complement	Signaling by Interleukins
CLEC7A (Dectin-1) signaling	CLEC7A (Dectin-1) signaling
Gene and protein expression by JAK- STAT signaling after Interleukin-12 stimulation	Signaling by the B Cell Receptor (BCR)
Interleukin-12 signaling	Activation of C3 and C5

Downstream TCR signaling	Scavenging by Class A Receptors
Interleukin-1 signaling	Interleukin-1 family signaling
Interleukin-12 family signaling	Downstream TCR signaling
Complement cascade	Binding and Uptake of Ligands by Scavenger Receptors
TNFR2 non-canonical NFκB pathway	Advanced glycosylation endproduct receptor signaling
Binding and Uptake of Ligands by Scavenger Receptors	FCERI mediated NFκB activation
TCR signaling	TNFR2 non-canonical NFκB pathway
Immune System	Regulation of actin dynamics for phagocytic cup formation
	Regulation of TLR by endogenous ligand
	Metal sequestration by antimicrobial proteins
	TCR signaling
	Complement cascade

Table 3.04. Upregulated immunity pathways in mass spectrometry data.

3.2.1.2. Identification and analysis of proteins of interest

3.2.1.2.1. *Identification of proteins of interest*

After identifying that multiple protein families were represented in the mass spectrometry data, and that immune pathways appear to be differentially regulated, proteins that may have an immunomodulatory function were considered.

Galectins – small carbohydrate-binding proteins – were considered to be of particular interest, and several members of this family were identified in 3.2.1.1.2. Firstly, the presence of galectin-1 (gal-1) and galectin-3 (gal-3) in both fractions was of interest as both these proteins are widely held to be immunosuppressive in various solid tumours (for review of gal-1 biology see Camby et al., 2006; for gal-3 in cancer, see Song et al., 2014). Furthermore, human galectin-4 (gal-4) and galectin-7 (gal-7) are known to be restricted under normal conditions to the gastrointestinal tract and stratified epithelial cells, respectively (Huflejt and Leffler, 2004; Magnaldo, Fowles and Darmon, 1998), rendering their presence in GBM of interest. Finally, galectins bind to the extracellular matrix (ECM) as well as cell-surface glycoproteins and can thus modulate cell behaviour in a variety of ways; due to differences in the carbohydrate recognition domain (CRD) of each protein, they can synergise or antagonise each other (Weinmann et al., 2018). As multiple galectins were identified, this synergy may occur within GBM to suppress immune effector function. Thus, all four members of this family were valid targets for further study.

Alongside the galectins, three further proteins of interest were selected for study: CTGF/CCN2 (connective tissue growth factor, or cellular communication network

family member 2); CEACAM1 (carcinoembryonic adhesion molecule family member 1); and S100A7, a small calcium-binding protein.

Aberrant expression of CTGF has been implicated in a variety of human cancers (for review see Wells et al., 2014). It is associated with resistance to temozolomide *in vitro* and *in vivo*, correlates with increased tumour growth in mouse models and worse prognosis in patients (Xie et al., 2004; Yin et al., 2010; Zeng et al., 2017). It has also been suggested to be an immunomodulatory protein in its own right and may promote tumour-infiltrating lymphocyte (TIL) adhesion and extravasation, and can upregulate CCL2 in some cell types (for review see Kular et al., 2011). There is almost no available data on S100A7 in glioma. It may be associated, in breast cancer, with influx of TAMs and expression of both CTGF and CCL2 (Mandal et al., 2007; Nasser et al., 2011) and bioinformatic analyses suggest its overexpression to be associated with differential regulation of several immune-related processes in breast cancer (Mandal et al., 2007); thus, analysis of S100A7 may elucidate novel facets of the GBM microenvironment. CEACAM1 is best-known as an adhesion molecule, but is also associated with glioma stemness (Kaneko et al., 2015); combined blockade of CEACAM1 and the immune checkpoint (IC) TIM-3, one of its receptors, facilitates the development of long-term immunological memory and recovery from glioma in mice (Li et al., 2017a). CEACAM1 expression is increased on peripheral blood mononuclear cells (PBMCs) and particularly TILs from glioma patients, and is associated with worse prognosis and decreased T-cell function (Li et al., 2018). Thus, each of these proteins has been linked with immunomodulatory function but their role in GBM is not yet particularly clear. The proteins selected for further study are summarised in Table 3.05.

Table 3.05. Target proteins shortlisted from mass spectrometry data

Protein	Family	Fraction
CEACAM1	Carcinoembryonic adhesion molecule	EV
CTGF	Cellular communication network	Both
Galectin-1	Galectin	Both
Galectin-3	Galectin	Both
Galectin-4	Galectin	EV
Galectin-7	Galectin	EV
S100A7	S100 calcium binding	EV

Table 3.05. Target proteins shortlisted from mass spectrometry data.

3.2.1.2.2. *Presence of target proteins in VesiclePedia*

Next, VesiclePedia was searched (Pathan et al., 2019) to ascertain if these proteins had been previously documented in EVs and if so, from which tissues. The results are displayed in Table 3.06.

All proteins of interest had been previously identified in brain cancer EVs. Gal-1, -3 and -7 and CTGF had been specifically documented in GBM; the rest had not. Furthermore, gal-1, -3 and -7 and CTGF were all identified in one paper studying EVs derived from primary GBM (Skog et al., 2008); of these four, only galectin-1 had been confirmed to be present in brain tumour EVs by subsequent researchers (de Vrij et al., 2015). Of the other three proteins – galectin-4, CEACAM1 and S100A7 – indications of their presence in brain tumour EVs were based on work performed in cell lines. Thus, for gal-1, -3 and CTGF, the mass spectrometry analysis herein confirmed the results of Skog et al.; for the other proteins, this was the first determination that they exist in primary GBM-derived EVs.

The target proteins were also expressed in various other solid cancers as well as leukaemia. Tissue- and cell-type-specific data from VesiclePedia revealed multiple potential sources of most target proteins besides cancer cells. Because the mass spectrometry data in Gkanatsiou (2022) was derived from bulk tumour secretome and not a purified population, further work was necessary to elucidate the likely cellular sources of these proteins.

Table 3.06. Presence of target proteins in VesiclePedia

Source	Gal-1	Gal-3	Gal-4	Gal-7	CTGF	CEACAM1	S100A7
Brain cancer	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Bladder cancer	Yes	Yes	No	No	No	No	No
Breast cancer	Yes	Yes	Yes	No	No	No	Yes
Colorectal cancer	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Duodenal cancer	No	No	No	No	Yes	No	No
Kidney cancer	Yes	No	Yes	Yes	Yes	Yes	Yes
Leukaemia	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Lung cancer	Yes	Yes	Yes	No	Yes	Yes	Yes
Melanoma	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ovarian cancer	Yes	Yes	Yes	Yes	Yes	Yes	No
Prostate cancer	Yes	Yes	Yes	Yes	No	No	Yes
Squamous cancer	Yes	No	No	No	No	No	No
B-cells	Yes	No	No	No	No	No	No
Dendritic cells	Yes	Yes	No	No	No	No	Yes

Epithelial cells	Yes	Yes	No	No	No	No	Yes
Endothelial cells	No	No	No	No	Yes	Yes	Yes
Erythrocytes	No	Yes	No	No	No	No	Yes
Keratinocytes	No	No	No	Yes	No	No	No
Macrophages	No	Yes	No	No	No	No	No
Monocytes	No	No	No	Yes	No	No	No
Osteoblasts	Yes	No	No	No	No	No	No
T-cells	No	No	No	Yes	No	No	Yes

Table 3.06. Presence of target proteins in VesiclePedia.

3.2.1.2.3. Pathway analysis of differentially expressed genes associated with target proteins

Next, changes in gene expression associated with target protein expression were examined. This analysis would reveal if particular behaviours, particularly those associated with immunity, were altered in correlation with target protein expression.

cBioPortal (Cerami et al., 2012; Gao et al., 2013) was used to examine mRNA sequencing data from 136 GBM included in The Cancer Genome Atlas (TCGA)'s firehose legacy GBM dataset (Brennan et al., 2013). This analysis revealed almost 11,000 differentially expressed genes (DEGs) associated with four of the seven proteins; no data was available for galectin-4, galectin-7 or CEACAM1-associated changes in expression. Details are given in Table 3.07.

Table 3.07. Differentially expressed genes in mRNA sequencing data associated with target protein mRNA

Target	Differentially expressed genes
Galectin-1	10975
Galectin-3	10975
Galectin-4	No data
Galectin-7	No data
CTGF	10975
CEACAM1	No data
S100A7	10958

Table 3.07. Differentially expressed genes in mRNA sequencing data associated with target protein mRNA. mRNA sequencing data from 136 patients was assessed for genes differentially expressed in relation to expression of target protein mRNA.

An initial comparison revealed that the total list of DEGs was identical for each protein, barring the 17 genes absent from the S100A7 dataset. These four datasets were then analysed in Reactome to identify differentially-regulated pathways. As with the mass spectrometry data, pathways with a false discovery rate (FDR) less than 0.05 were considered. A summary is available in Table 3.08.

Table 3.08. Summary of pathway numbers associated with target protein mRNA expression

Target	Total upregulated pathways	Upregulated pathways with FDR <0.05	Total downregulated pathways	Downregulated pathways with FDR <0.05
Galectin-1	2222	65	2117	94
Galectin-3	2212	170	2174	12
CTGF	2237	48	2145	99
S100A7	2260	7	2143	133

Table 3.08. Summary of pathway numbers associated with target protein mRNA expression as identified by Reactome.

Next, immunity-related pathways were isolated from both upregulated and downregulated datasets from the pathways with FDR less than 0.05 (shown in Table 3.09). 20 pathways were represented, of which 19 were upregulated. Most of these pathways were also differentially regulated in the broader mass spectrometry dataset: the only exceptions were the general cytokine signalling pathway, non-canonical NF κ B pathway, MHC class I mediated antigen processing and presentation (all upregulated) and antiviral mechanism by IFN γ -stimulated genes (downregulated), which were not found to be differentially regulated in the mass spectrometry dataset.

It can be concluded that the proteins in the mass spectrometry data and mRNA sequencing data associated with proteins of interest may have a high level of concordance, and both datasets showed differential regulation of immune pathways, which were also highly concordant. Immunoregulation was represented in multiple types of data from different GBM patients, and intriguingly, innate and adaptive immunity were both upregulated. Neutrophil degranulation was also highly represented in both datasets, as were processes showing signalling through the BCR and TCR. Clearly, multiple immune effectors were present and retained at least some activity within the tumour microenvironment (TME). However, it remained to be seen whether the proteins of interest identified in 3.2.1.2.1. were responsible for suppressing their activity.

Table 3.09. Differentially regulated immune pathways associated with target protein mRNA expression

Pathway	Associated with expression of	Regulation
Neutrophil degranulation	Galectin-1, galectin-3, CTGF, S100A7	Up
Innate immunity	Galectin-1, CTGF	Up
MHC class II antigen presentation	CTGF	Up
Immune system	Galectin-1	Up
Signaling by interleukins	Galectin-1	Up
Cytokine signaling in immune system	Galectin-1	Up
Interleukin-1 family signaling	Galectin-1, galectin-3	Up
TCR signaling	Galectin-1, galectin-3	Up
Interleukin-1 signaling	Galectin-1, galectin-3	Up
Activation of NFkB in B-cells	Galectin-1, galectin-3	Up
Downstream TCR signaling	Galectin-1, galectin-3	Up
Vpu-mediated degradation of CD4	Galectin-1, galectin-3	Up
Cross-presentation of soluble exogenous antigens	Galectin-1, galectin-3	Up
Antigen processing cross-presentation	Galectin-3	Up
ER-phagosome pathway	Galectin-3	Up

NIK--> non-canonical NFκB signaling	Galectin-3	Up
Downstream signaling events of BCR	Galectin-3	Up
Non-canonical NFκB pathway	Galectin-3	Up
MHCI mediated antigen processing and presentation	Galectin-3	Up
Antiviral mechanism by IFNγ stimulated genes	CTGF	Down

Table 3.09. Differentially regulated immune pathways associated with target protein mRNA expression.

3.2.1.3. Effects of identified target proteins on patients

TCGA's firehose legacy GBM dataset was then analysed using cBioPortal to discern the effects of target protein mutation and copy number variation on patient survival.

Figure 3.01 shows overall survival (OS) for 269 patients for which these data were available, stratified by mutation status of genes encoding gal-1 (A), gal-3 (B), gal-4 (C), gal-7 (D), CTGF (E), CEACAM1 (F) and S100A7 (G). In line with the current understanding, OS was approximately 15 months. Target genes in these patients were generally not profiled for mutations, and in those that were, mutations were rare. Copy number alterations (CNAs) were more prevalent. LGALS1, encoding gal-1, was usually diploid or showed a shallow deletion; gain was detected in a minority of samples. The same pattern was seen in LGALS3, encoding gal-3, and CTGF. CEACAM1, LGALS4 and LGALS7, encoding gal-4 and -7 respectively, were primarily diploid or showed some gain, with shallow deletion being a sizeable minority. Two samples for each gene showed amplification. S100A7 was generally diploid, with both gain and shallow deletion a minority. In every case, CNA did not appear to influence patient survival. Amplification and deep deletions were rare, accounting for single-digit numbers of patients.

The Ensembl Genome Browser (Harrison et al., 2024) showed that gal-4 and -7 are both located at 19q13.2 (Figure 3.02) and thus could be expected to show a similar CNA profile; however, CEACAM1 is at the same locus and has a visibly different profile. This could be explained by greater distance between the genes; as shown in Figure 3.02, LGALS4 and LGALS7 are located extremely close together and thus could be affected by mutations at the 19q13.2 locus that do not affect CEACAM1.

This hypothesis was supported by the dataset: both samples with gain of LGALS4 and LGALS7 were from the same two patients, whereas both samples that gained CEACAM1 were from different patients. The other genes are at different loci: gal-1 at 22q13.1, gal-3 at 14q22.3, CTGF at 6q23.2 and S100A7 at 1q21.3. Therefore, the similarity in CNA between gal-1, gal-3 and CTGF could not be explained through the same mechanism. The most widely known chromosomal aberrations in GBM are chromosome 7 amplification and loss of heterozygosity at chromosome 10, which obviously do not account for the CNAs seen here; however, they could be explained by other, less extreme mutation events. It is uncertain whether the resulting CNAs promote tumour growth and survival, which was beyond the scope of this project.

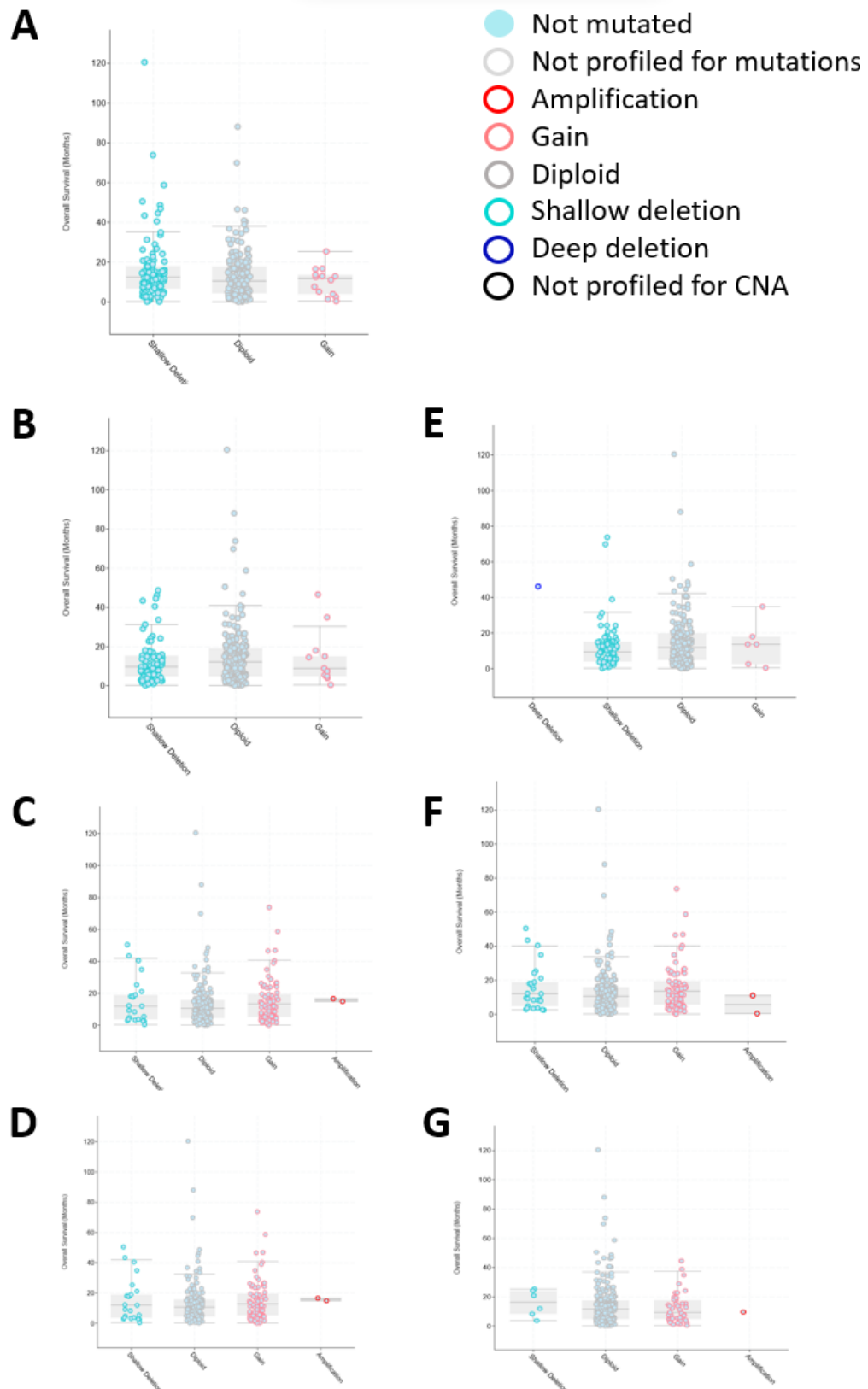


Figure 3.01. Overall survival and copy number in 269 patients. Data points are coloured to show mutation and CNA in genes encoding galectin-1 (A), galectin-3 (B), galectin-4 (C), galectin-7 (D), CTGF (E), CEACAM1 (F) and S100A7 (G).

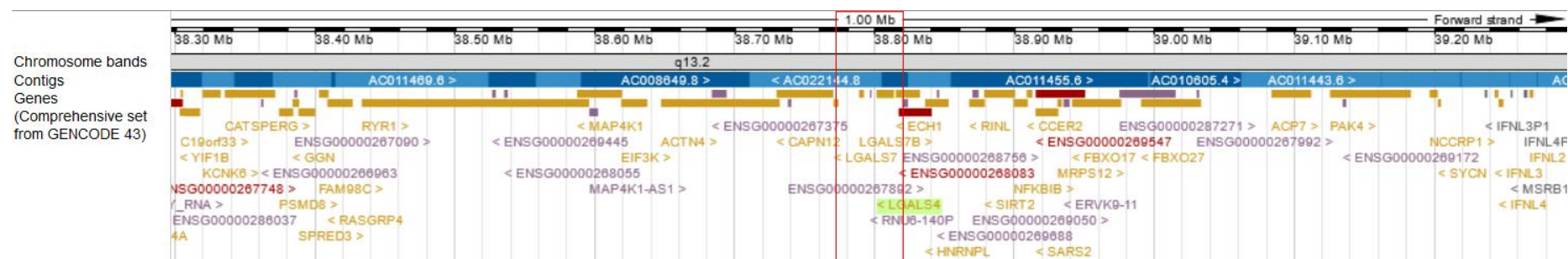


Figure 3.02. Chromosomal location of genes encoding galectin-4 (LGALS4) and galectin-7 (LGALS7) on chromosome 19. Both genes are close together; the CEACAM1 gene is more distant and not shown in this image. The window is centred on LGALS7; LGALS4 is highlighted below. Image captured from Ensembl genome browser, release 112.

3.2.1.4. Protein subsets of interest

It was also considered that analysis of the mass spectrometry dataset may answer specific questions about the behaviour of specific intratumoural populations. To this end, a list of markers indicative of macrophage polarisation was compiled, to ascertain if tumour-associated macrophages (TAMs) appeared to be clearly polarised towards either an M1 or M2 phenotype.

A phenotypic analysis of T-cells could not be performed in the same way. The macrophage polarisation analysis was dependent on using a large number of molecules that, if heavily skewed in one direction, may indicate the presence of an M1- or M2-like phenotype amongst intratumoural macrophages. This sort of plastic cell surface marker expression does not apply well to T-cells, which are divided into helper or cytotoxic categories dependent on the presence of either CD4 or CD8, respectively. Any further phenotyping would be dependent on detection of lineage-specific transcription factors.

The presence of galectin binding partners was also examined, as the presence of many galectin ligands may indicate that these proteins were exerting widespread or cell-type-specific effects. As GBM is a highly immunosuppressive tumour, there is much interest in use of IC inhibitors in combination with the standard treatment regimen (reviewed in Huang et al., 2017; see also 1.4.6.); as this is an area of interest, IC ligands were also studied to examine which interactions may be represented in the dataset.

3.2.1.4.1. Macrophage polarisation markers

M1- and M2-associated markers were compiled using data from Chávez-Galán et al., 2015 and Krausgruber et al., 2011. Their presence in the mass spectrometry output is shown in Table 3.10.

Four of 30 M1-associated proteins and six of 22 M2-associated proteins were present in the dataset. While these data showed a skew in favour of M2 polarisation, it is worth noting that macrophage polarisation markers are often more strongly associated with, rather than exclusive to, one phenotype. Further, the M1/M2 dichotomy is an oversimplification that may not apply well to TAMs (see 1.4.5.). GBM TAMs may be more likely to express M2-like than M2-like characteristics, but further work would be necessary to define these in detail and examine how target proteins of interest may affect macrophage behaviour.

Table 3.10: Macrophage polarisation markers in mass spectrometry data

Phenotype	Marker	Fraction
M1	CCL4	None
M1	CCL5	None
M1	CCL22	None
M1	CCR7	None
M1	CD1a	None
M1	CD40	None
M1	CD80	None
M1	CD83	None
M1	CD86	None
M1	CXCL9	None
M1	FCER2	None
M1	HLA-A	EV
M1	HLA-DR	DS
M1	ICAM	EV, DS
M1	IL-1 β	None
M1	IL-2R α	None
M1	IL-6	None
M1	IL-12 α	None
M1	IL-12 β	None
M1	IL-12p70	None
M1	IL-18	None
M1	IL-18R	None
M1	IL-23 α	None

M1	IL-23p19	None
M1	iNOS/NOS2	DS
M1	IRF5	None
M1	SOCS3	None
M1	STAT1	None
M1	STAT2	None
M1	TNF	None
M2	CCL2	EV
M2	CCL13	None
M2	CD14	EV, DS
M2	CD163	None
M2	CD200R	None
M2	CD206	None
M2	CD209	None
M2	CLEC4A	None
M2	CLEC7A	None
M2	F13A1	EV, DS
M2	FCGR1A	EV
M2	FCGR1B	DS
M2	FCGR2B	None
M2	FCGR3A	DS
M2	FGL2	None
M2	IGF1	None
M2	IL-10	None
M2	IRF4	None

M2	MAF	None
M2	MGL1	None
M2	MGL2	None
M2	MRC1	None

Table 3.10. Macrophage polarisation markers in mass spectrometry data.

3.2.1.4.2. *Galectin binding partners*

Next, the mass spectrometry data was searched for galectin binding partners to identify if these proteins were likely to have local immunomodulatory effects on specific cellular populations. Results are shown in Table 3.11.

The list of binding partners was compiled for various human tissues from Camby et al., 2006 (gal-1); Feuk-Lagerstedt et al., 1999 (gal-3); Stillman et al., 2006 and references therein (gal-3); Mauris et al., 2011 (gal-3); Tan et al., 2021 and references therein (gal-3); and Nio-Kobayashi and Itabashi 2021 (gal-1, -3 and -4). Identification of binding partners for gal-4 and gal-7 was difficult as not only are these proteins less well characterised, but also much of the research into their ligands focuses on the specific saccharides their CRD domains bind, not the glycoproteins said saccharides are covalently attached to; thus, very few gal-4 and no gal-7 binding partners are shown in Table 3.11.

16 of 30 gal-1 and eight of 36 gal-3 binding partners were present, strongly indicating that intratumoural gal-1 and gal-3 were able to act on some cellular populations. Gal-4 ligands were not identified, although this does not mean that intratumoural gal-4 has no biological activity; the same was true of gal-7. Most of the ligands identified were not cell-type-specific, and many were ECM components. CD2, a T-cell marker and gal-1 binding protein, was present; CD45, a pan-leukocyte marker expressed on T-cells, was not identified, and therefore absence of a protein in this analysis could not be taken to mean it was absent within the tumour. In conclusion, it was likely that gal-1 and gal-3 were likely to be acting on varied intratumoural populations, and possible that gal-4 and gal-7 were.

Table 3.11. Galectin binding partners in mass spectrometry data

Galectin	Binding partner	Fraction
Galectin-1	$\alpha 1\beta 1$ integrin	DS ($\alpha 1$)
Galectin-3	$\alpha 4$ integrin	None
Galectin-1	$\alpha 5\beta 1$ integrin	None
Galectin-1	$\alpha 7\beta 1$ integrin	EV ($\alpha 7$)
Galectin-1	$\alpha M\beta 2$ integrin	EV (both)
Galectin-1, 3	$\beta 1$ integrin	EV
Galectin-1	Actin	DS
Galectin-3	ALIX	None
Galectin-3	Annexin A7	EV
Galectin-3	Basigin-2	None
Galectin-3	BCL-2	None
Galectin-3	BMPRI α	None
Galectin-1	CA125	None
Galectin-1	CD2	DS
Galectin-1	CD3	None
Galectin-1	CD7	None
Galectin-1, 3	CD43	None
Galectin-1, 3	CD45	None
Galectin-3	CD47	None
Galectin-3	CD71	None
Galectin-3	CD95 (Fas)	None
Galectin-3	CD98	None
Galectin-3	CD231	None

Galectin-3	CD1a precursor	None
Galectin-4	CDKN1B	None
Galectin-3	CEACAM1	None
Galectin-1	CEACAM5	None
Galectin-3	CEACAM8	None
Galectin-3	CHMP4	Both
Galectin-3	Collagen IV	EV
Galectin-1	Cytochrome c subunit III	None
Galectin-3	Ephrin B1	None
Galectin-1, 3	Fibronectin	Both
Galectin-1, 3	Gemin-4	None
Galectin-1, 3	Galectin-3 binding protein	Both
Galectin-1	HBGp82	None
Galectin-1	H-Ras	None
Galectin-3	HSPC108	None
Galectin-3	IgE	None
Galectin-3	IGF2R	None
Galectin-3	Junctional adhesion molecule A	None
Galectin-1	Laminin	EV (α 2, 4, 5, β 1 and γ 1)
Galectin-1	LAMP-1	EV
Galectin-1	LAMP-2	EV
Galectin-3	Mannose-6-phosphate receptor	None
Galectin-3	MHCI	None
Galectin-3	MST1R	None
Galectin-1, 3	Mucins	Both (Mucin-16)

Galectin-1, 4	NCAML1	None
Galectin-1, 3	Neuropilin-1	None
Galectin-1	Osteopontin	DS
Galectin-1	Pre-BCR	None
Galectin-3	Sodium/potassium-transporting ATPase β unit	None
Galectin-1	SUMO-3	None
Galectin-1	Thrombospondin	Both
Galectin-1	Thy1	Both
Galectin-3	TLR4	None
Galectin-4	TSFP1	None
Galectin-3	TTF1	None
Galectin-3	Uveal autoantigen with coiled-coil domains and ankyrin domains	Both
Galectin-1	Vitronectin	Both

Table 3.11. Galectin binding partners in mass spectrometry data.

3.2.1.4.3. Immune checkpoint molecules

To conclude these analyses, mass spectrometry data was searched for ligands for the ICs outlined in 1.3.2.1.4., in order to identify any obvious mediators of GBM immunosuppression. However, as the ligands for CTLA-4 are the costimulatory molecules expressed on APCs, the search included CTLA-4 itself. Additionally, the TIM-3 ligand phosphatidylserine is not a protein and thus would not be identified in the mass spectrometry dataset, and therefore was omitted from this analysis. Results are shown in Table 3.12.

CTLA-4 was not identified in mass spectrometry data; neither were the PD-1 ligands PD-L1 and PD-L2. However, two of the three protein ligands for TIM-3 were present. TIM-3 may be a likely receptor through which T-cell function is suppressed in GBM. Additionally, both ligands were present in the EV fraction, suggesting they may enter the circulation and contribute to systemic immunosuppression.

Table 3.12. Immune checkpoint molecules in mass spectrometry data

Checkpoint	Ligand	Fraction
CD80/86	CTLA-4	None
PD-1	PD-L1	None
PD-1	PD-L2	None
TIM-3	CEACAM1	EV
TIM-3	HMGB1	Both
TIM-3	Galectin-9	None

Table 3.12. Immune checkpoint molecules in mass spectrometry data.

3.2.2. Presence of target proteins in supernatants

In the above section, proteins of interest were identified in the GBM secretome using mass spectrometry. Pathway analysis showed upregulated immunity-related pathways in the dataset, lending weight to the idea that secretion of immunomodulatory proteins is a means through which GBM may facilitate immune escape. The same and similar pathways were upregulated in mRNA sequencing data associated with expression of the target proteins. Their presence was then examined in supernatants from glioma cell lines as well as primary cultures.

This work used seven low-grade glioma (LGG) and 23 GBM TM: serum-free media in which tumour samples were placed following surgical excision (see 2.1.3). For 16 GBM tumours, culture supernatant (CS) taken 24 hours after primary cultures were established was also available. This sample set allowed comparisons between LGG and GBM secretomes, and the GBM secretome before and after establishment of an *in vitro* culture. GBM TM was the closest approximation of the *in vivo* secretome, and so these samples were of particular interest. Details of experimental groups are given in Table 3.13. BCA (bicinchoninic acid) assays were used to determine total protein concentration and sandwich ELISA to determine the concentration of specific proteins of interest.

Table 3.13. Experimental groups used in BCA and ELISA experiments

Group	Timepoints (hours)	Media and blank	0.25µm filtered
U251 CS	24, 48, 72, 96	RPMI-1640, 10% FBS	Yes
U373 CS	24, 48, 72, 96	RPMI-1640, 10% FBS	Yes
GBM TM	24	Ham's F10, 0% FBS	No
LGG TM	24	Ham's F10, 0% FBS	No
GBM CS	24	Ham's F10, 0% FBS	No

Table 3.13. Experimental groups used in BCA and ELISA experiments.

3.2.2.1. BCA results

Initial experimental work used BCA assays to determine the mean average total protein in glioma cell line supernatants. These experiments required protein to be precipitated using trichloroacetic acid (TCA) and resuspended in phosphate-buffered saline (PBS), as phenol red found in culture media interferes with the BCA assay. A BCA assay standard curve was performed using known concentrations of bovine serum albumin (BSA), and is shown in Figure 3.03. This result demonstrates that the assay worked as expected.

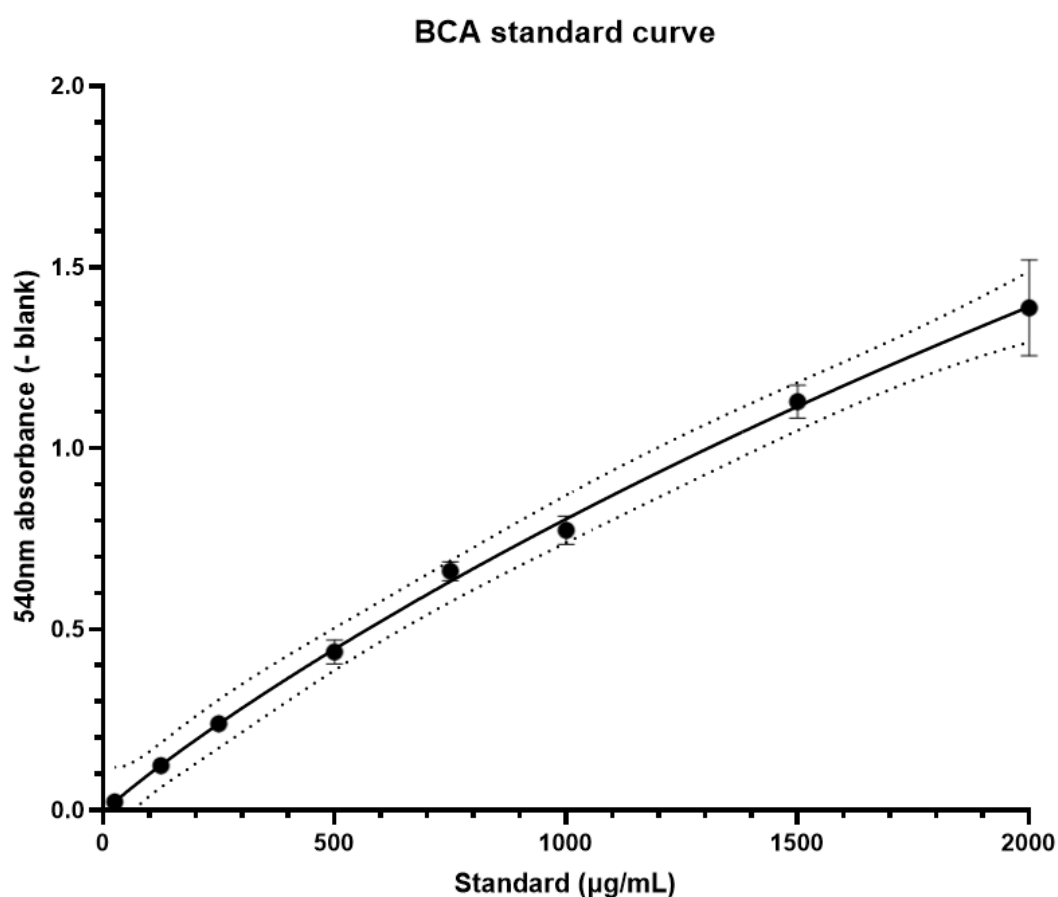


Figure 3.03. BCA standard curve. Standards were diluted and 25µL of each was incubated with 200µL BCA working reagent in a 96-well plate at 37°C. After 30 minutes, plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 540nm. The blank reading was subtracted from experimental readings. Data were analysed in Microsoft Excel and Graphpad PRISM. Error bars=standard error of the mean (SEM). Solid line=line of best fit. Dotted lines=95% CI (confidence interval). N=1. All standards were run in duplicate.

Total protein in supernatants from the U251 and U373 cell lines was examined. Protein concentration in U251 supernatant was consistent at the 24- and 48-hour timepoints, at a mean average of 631 and 622µg/mL respectively. Protein concentration then dropped to 375µg/mL at the 72-hour mark and increased to 824µg/mL by 96 hours (Figure 3.04A). Up to 48 hours post-seeding, cells were in the exponential growth phase; protein concentration may be relatively static at this time as growth factors are secreted and taken up. By 72 hours, flasks were confluent and this decrease in protein concentration may reflect the transition to the plateau phase. At 96 hours, the increased levels of total protein were likely caused by high levels of cell death.

In U373 cells, mean total protein rose from 634 to 744µg/mL between the 24- and 48-hour timepoints, then declined to 599 and 450µg/mL at 72 and 96 hours post-seeding, respectively (Figure 3.04B). These results imply that U373 cells secreted protein faster than it is taken up in the exponential phase, and there was no increase in protein secretion by the 96-hour mark; it is possible that these cells took longer to enter the death phase, or that the protein released by dying cells was efficiently taken up by other cells. These data demonstrated different patterns of growth and protein secretion in two GBM cell lines, reflective of the heterogeneity of GBM *in vivo*.

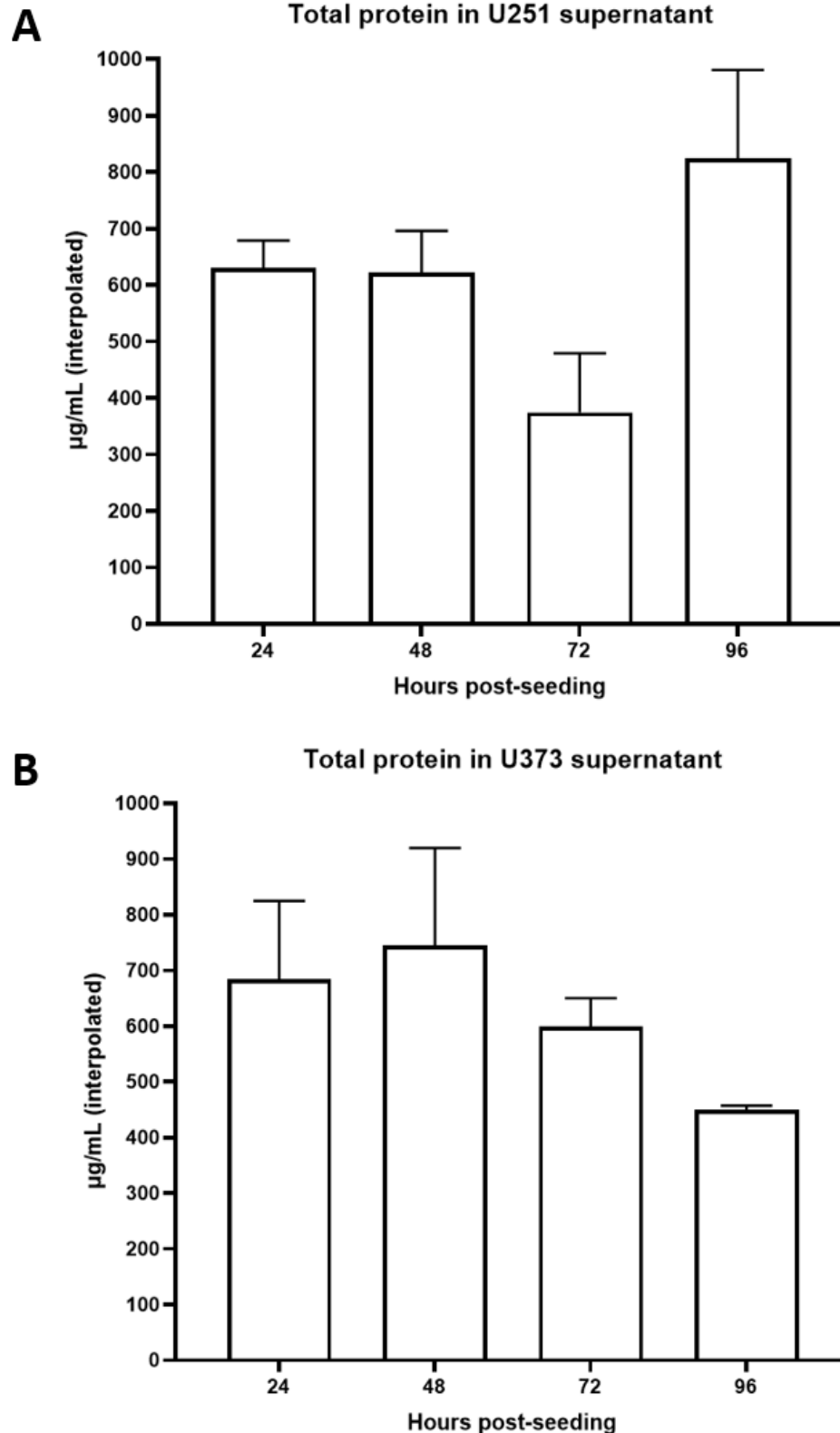


Figure 3.04. Total protein in U251 (A) and U373 (B) supernatant. Cells were seeded at $1.8 \times 10^5/\text{mL}$ and supernatants were collected after 24, 48, 72 and 96 hours' growth. Supernatants were passed through a $0.25\mu\text{m}$ filter to remove remaining cells. Protein was precipitated and resuspended in PBS. $25\mu\text{L}$ of each standard or sample was incubated with $200\mu\text{L}$ BCA working reagent in a 96-well plate at 37°C . After 30 minutes, plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 540nm . The blank reading was subtracted from experimental readings. Data were analysed in Microsoft Excel and Graphpad PRISM. Error bars = mean and standard error of the mean (SEM). $N=3$ for each timepoint. Samples and standards were run in duplicate.

Next, total protein in transport media from GBM and LGG was assayed.

Across 23 GBM TM samples, protein concentration could be interpolated in 22. The remaining sample had a concentration too high to be determined even after fourfold dilution in PBS. Across the 22 remaining samples, most GBM TM samples had a protein concentration below 350 μ g/mL, with a mean average of 146 μ g/mL. In the seven LGG TM, there was also one sample for which a concentration could not be interpolated. Across the remaining six, the mean average was 458 μ g/mL, which was entirely driven by one outlier; all other samples had a concentration below 100 μ g/mL. When the two diagnoses were compared, the higher mean average in LGG TM was not statistically significant, likely because standard error of the mean in this group was broadened to a great degree by the one outlier (Figure 3.05).

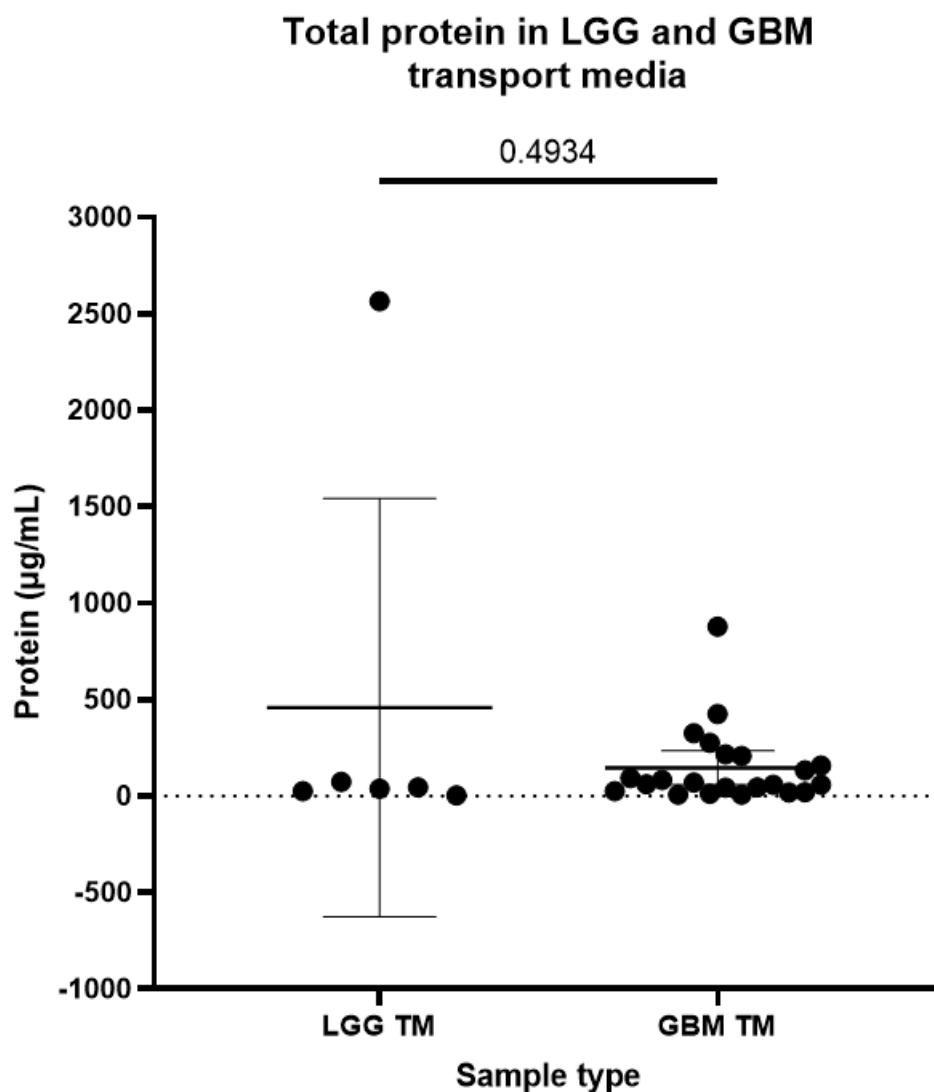


Figure 3.05. Comparison of total protein in LGG and GBM transport media. Protein concentrations were determined by BCA after precipitation with TCA. Ham's F10 media with 0% FBS was used as a blank; blank value was subtracted from experimental values. Data were analysed by unpaired two-tailed t-test. Error bars=mean + SEM. N=6 for LGG and 22 for GBM groups. Samples and standards were run in duplicate.

For 16 of the 22 GBM tumours CS taken 24 hours after tumours were plated *in vitro* was also available. A BCA assay was performed on these CS samples and the change in protein secretion for these 16 GBM was compared. As these tumours were collected and grown in serum-free media, there is no interference from foetal bovine serum (FBS) in these results. However, it is worth noting that cell numbers during the transition to primary culture could not be controlled for.

Results are shown in Figure 3.06. As stated above, mean protein concentration in GBM TM was 146µg/mL; in CS, mean concentration was determined to be 1570µg/mL. This upregulation of protein secretion upon establishment of *in vitro* cultures was found to be statistically significant by unpaired two-tailed t-test ($p < 0.0001$). This result was potentially reflected in the increased protein secretion by U251 and U373 glioma cell lines during exponential growth. It was also likely that a large amount of this protein originated from dead and dying cells that did not survive the transition to *in vitro* growth.

Total protein in paired GBM transport media and culture supernatant

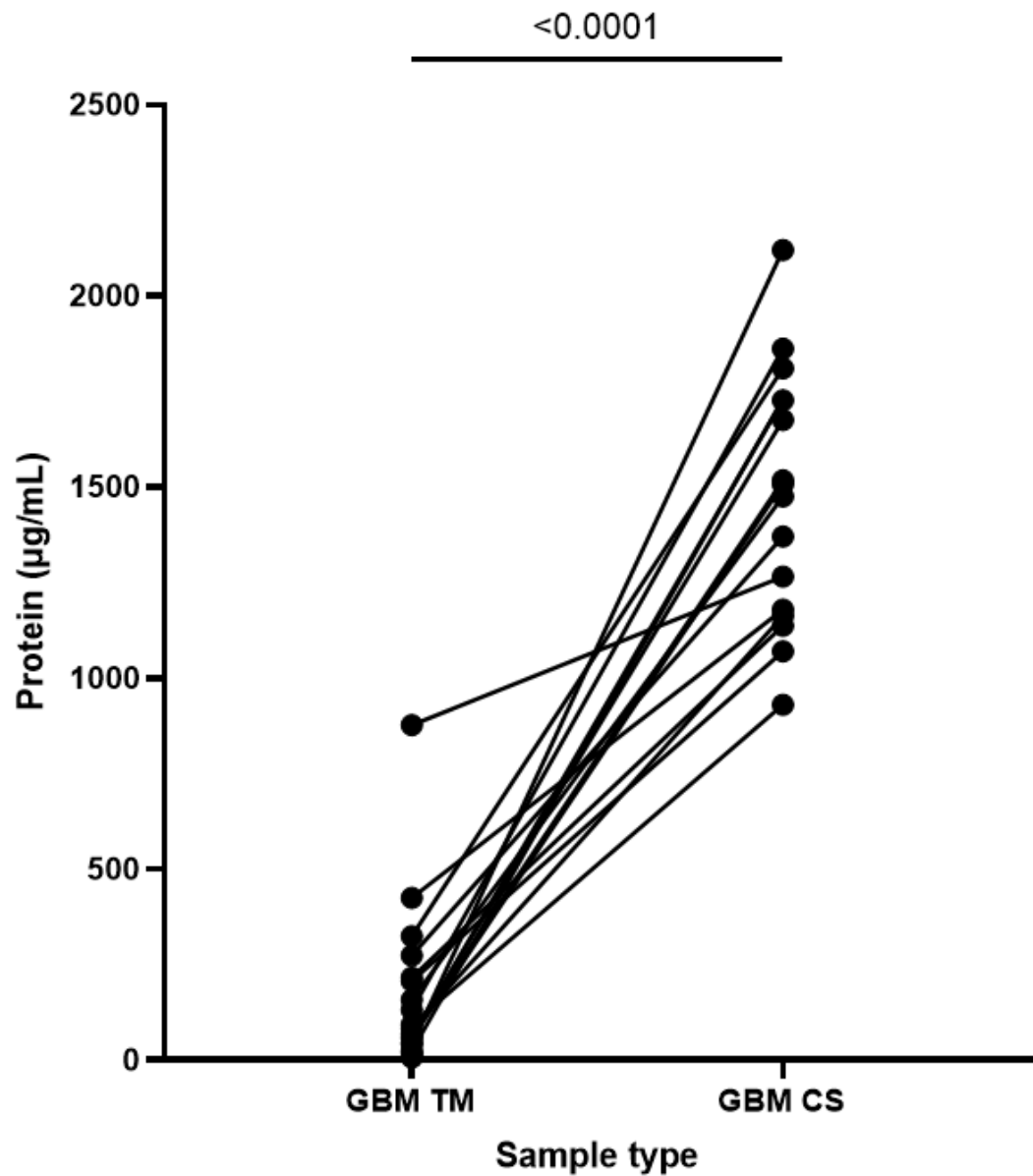


Figure 3.06. Total protein in paired GBM transport media and culture supernatant. Protein concentrations were determined by BCA after precipitation with TCA. Ham's F10 media with 0% FBS was used as a blank; blank value was subtracted from experimental values. Data were analysed by unpaired two-tailed t-test. $N=15$ for each sample type. Samples and standards were run in duplicate.

3.2.2.2. ELISA results

In the above section, total protein secretion from the U251 and U373 cell lines, and primary GBM and LGG tumours post-surgery, was characterised. Total protein secreted by GBM tumours before and after the establishment of *in vitro* cultures was also examined. Next, the presence of proteins of interest in the same samples was assayed using sandwich ELISAs.

3.2.2.2.1. *MIF*

Initial assays examined macrophage migration inhibitory factor (MIF), which is known to be expressed in GBM at high levels (Bacher et al., 2003) and was also found in both fractions of the mass spectrometry database. Thus, MIF was determined to be an appropriate positive control. A representative standard curve is shown in Figure 3.07.

MIF was present in U373, but not U251, supernatants (Figure 3.08). Mean MIF concentration was highest 24 hours post-seeding, at 929.0pg/mL; it then decreased to 395.5, 363.4 and 0pg/mL at 48, 72 and 96 hours post-seeding respectively. This decline is the opposite of what is seen in terms of total protein secretion; MIF may have been secreted and taken up by actively growing cells.

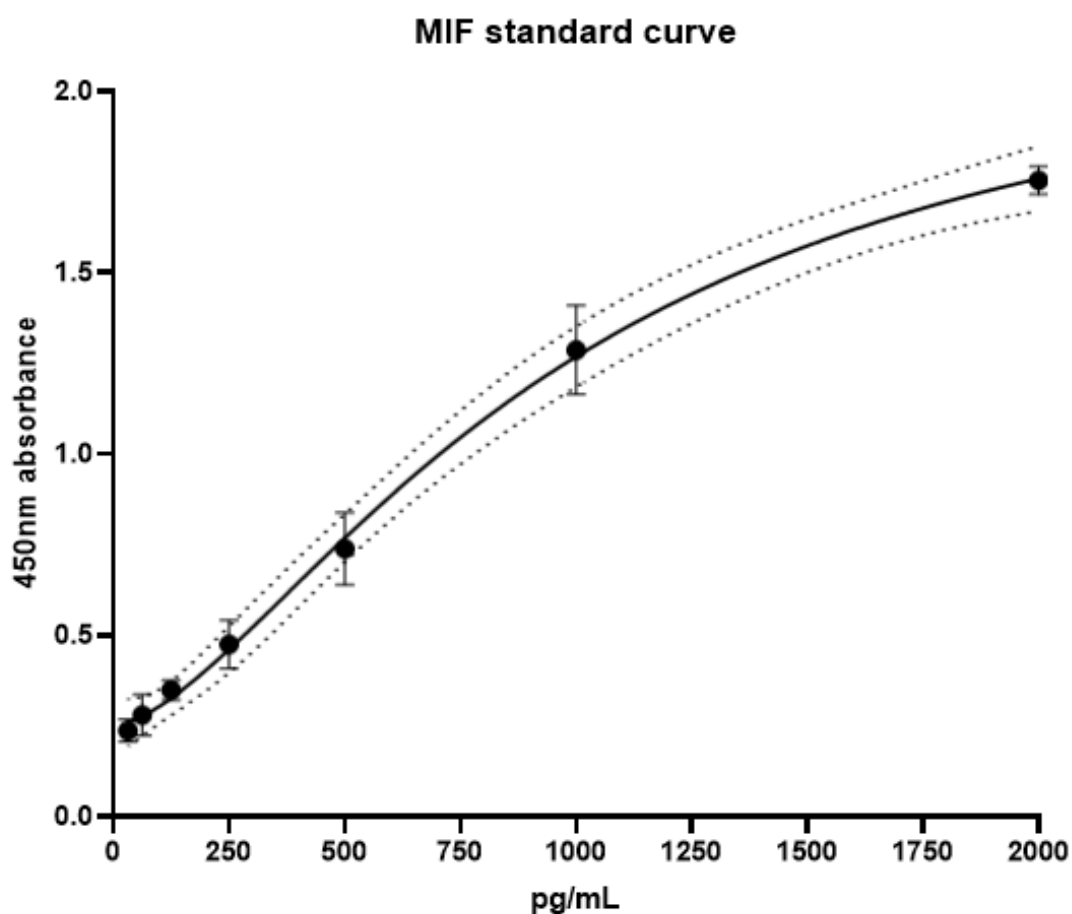


Figure 3.07. MIF standard curve. Standards were diluted and 100 μ L of each was incubated in a 96-well plate pre-coated with capture antibody. After two hours, plates were washed and incubated for a further two hours with detection antibody. Plates were washed again and incubated for 20 minutes each with streptavidin-HRP (horseradish peroxidase) and ELISA substrate. The reaction was stopped with acid and plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Solid line=line of best fit. Dotted lines=95% CI. Error bars=SEM. N=1. All standards were run in duplicate.

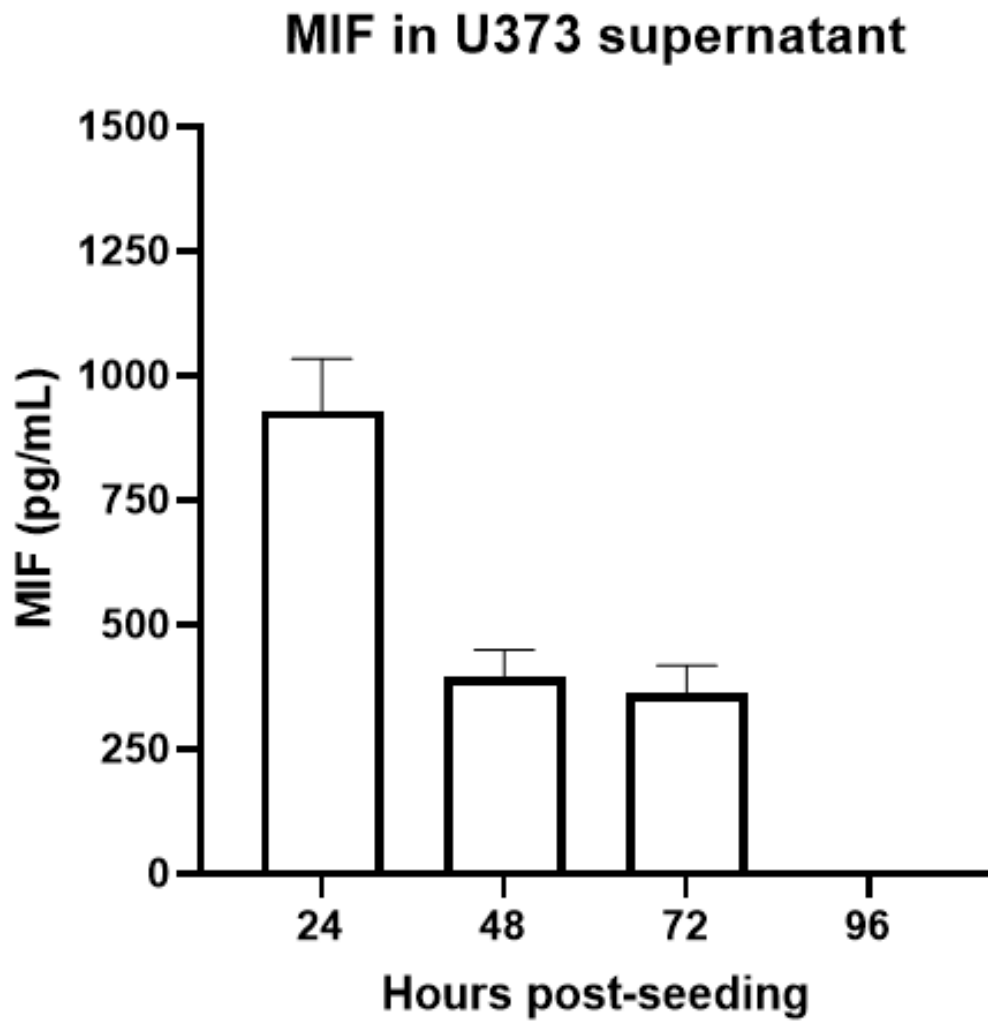


Figure 3.08. MIF in U373 supernatant. Cells were seeded at $1.8 \times 10^5/\text{mL}$ and supernatants were collected after 24, 48, 72 and 96 hours' growth. Supernatants were passed through a $0.25\mu\text{m}$ filter to remove remaining cells. MIF ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Error bars=SEM. N=3 for each timepoint. Samples and standards were run in duplicate.

17 of 23 GBM TM samples and six of seven LGG TM samples were found to contain MIF. In GBM samples, MIF was in the thousands of picograms range, up to 83988.1pg/mL or 83.9ng/mL in one sample; LGG samples showed a similar range, peaking at 63475.0pg/mL or 63.5ng/mL. Mean averages were also similar, at 23247.9pg/mL in GBM TM and 27468.0pg/mL in LGG TM. These means were not found to be significantly different (Figure 3.09).

Finally, MIF expression in the 16 pairs of GBM TM and GBM CS was assayed. These data implied an overall reduction in MIF secretion following the transition to *in vitro* growth, although a subset of tumours did increase MIF secretion. This decrease was found to reach significance ($p=0.0233$) (Figure 3.10).

Overall, MIF was widely expressed in both GBM and LGG, although it was absent in the U251 cell line. MIF expression was lowered in CS as compared to TM, and thus it is possible that U251 cells were derived from a rare MIF-null tumour.

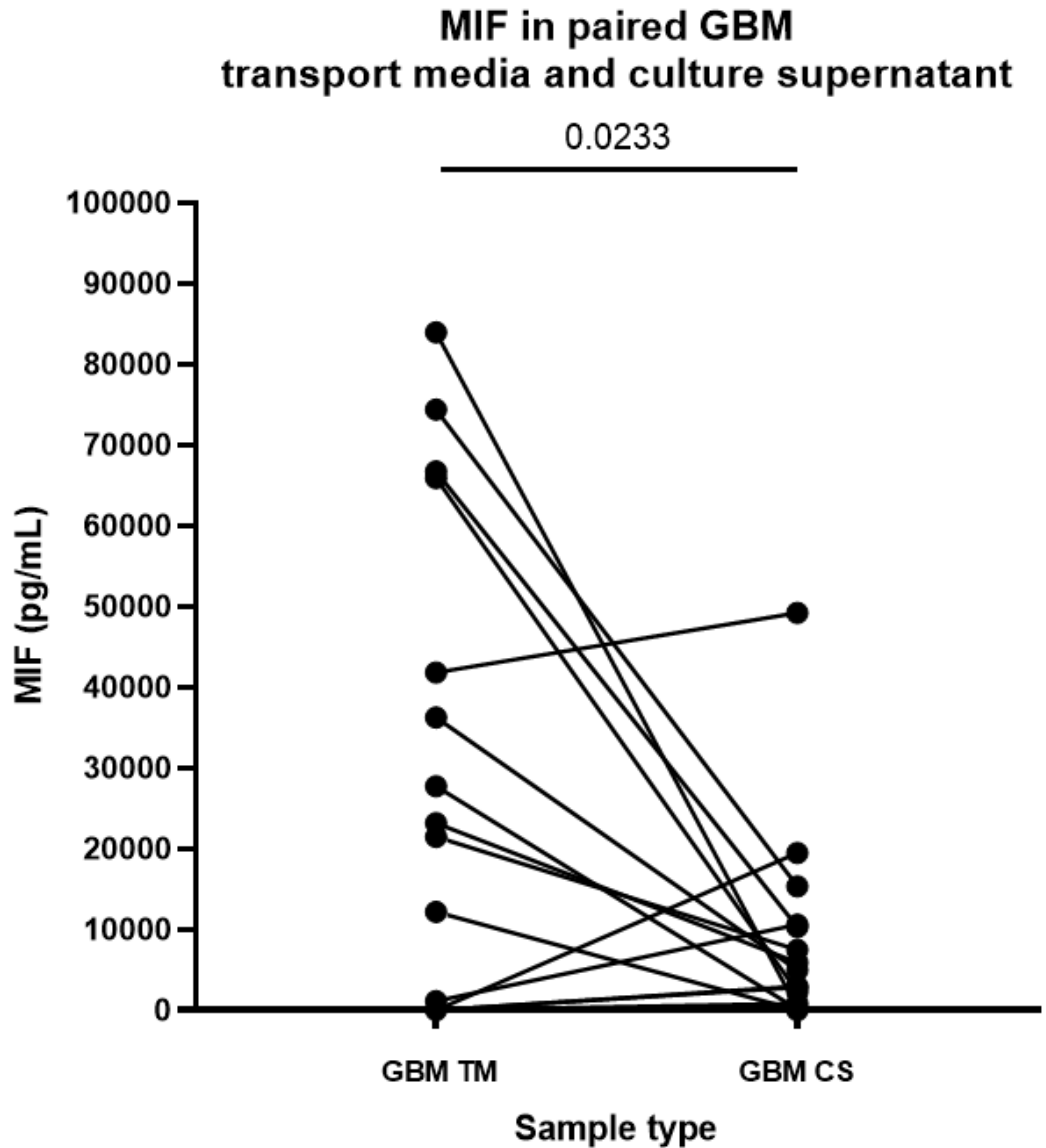


Figure 3.10. MIF in GBM transport media and culture supernatant. MIF ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed t-test with Welch's correction. Error bars=mean + SEM. N=16. Samples and standards were run in duplicate.

3.2.2.2.2. *Galectin-1*

Next, the concentration of galectin-1 in glioma cell line supernatant was assessed. A representative standard curve is shown in Figure 3.11. In both U251 (Figure 3.12A) and U373 (Figure 3.12B), concentrations of galectin-1 rose steadily over the four-day timecourse, suggesting that galectin-1 was secreted but not taken up by these cells. In the U251 samples, mean gal-1 concentration rose from 3.8ng/mL to 6.3, 8.2 and 12.9ng/mL at 48, 72 and 96 hours respectively. These were overall very similar to the levels seen in U373 cells, which were 3.5, 5.9, 7.9 and 8.6ng/mL at 24, 48, 72 and 96 hours post-seeding respectively. Increased gal-1 secretion in U251 cells at 96 hours post-seeding might have been caused by increased cell death and was in line with the BCA results, where increased levels of total protein were observed at this timepoint compared to U373 cells.

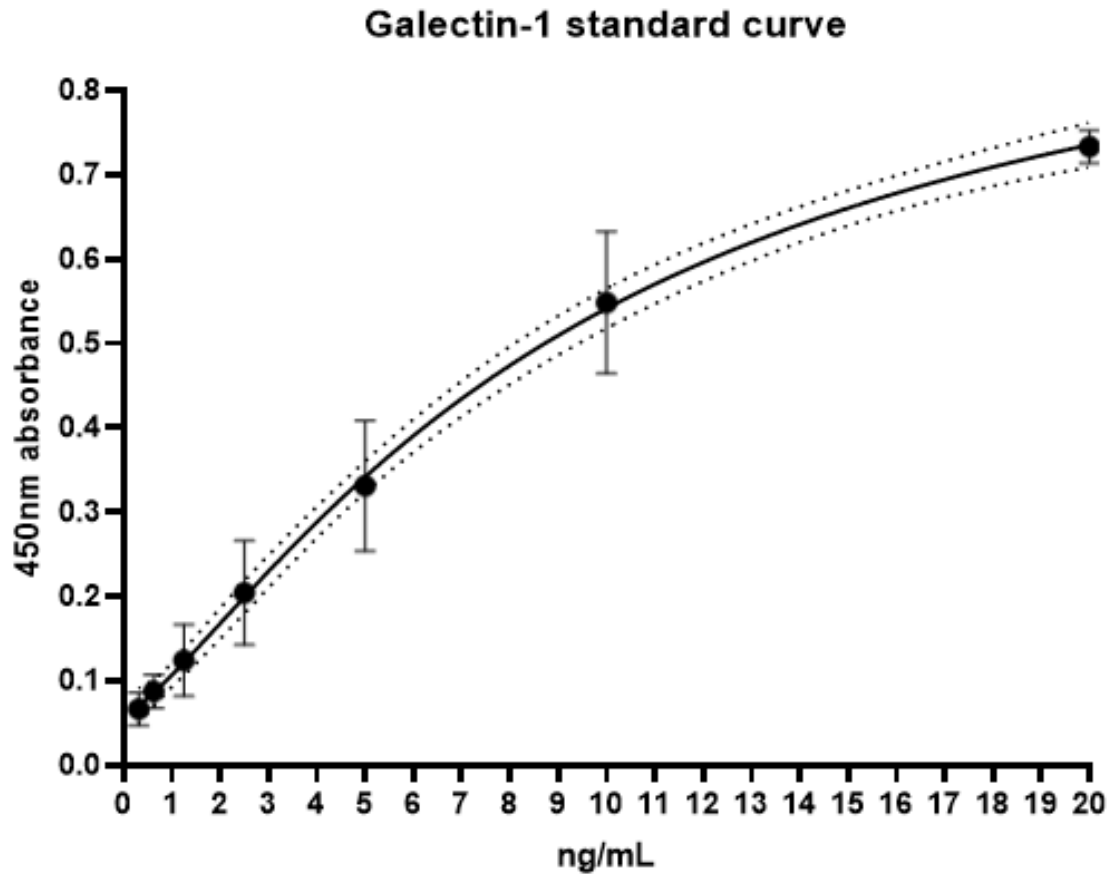
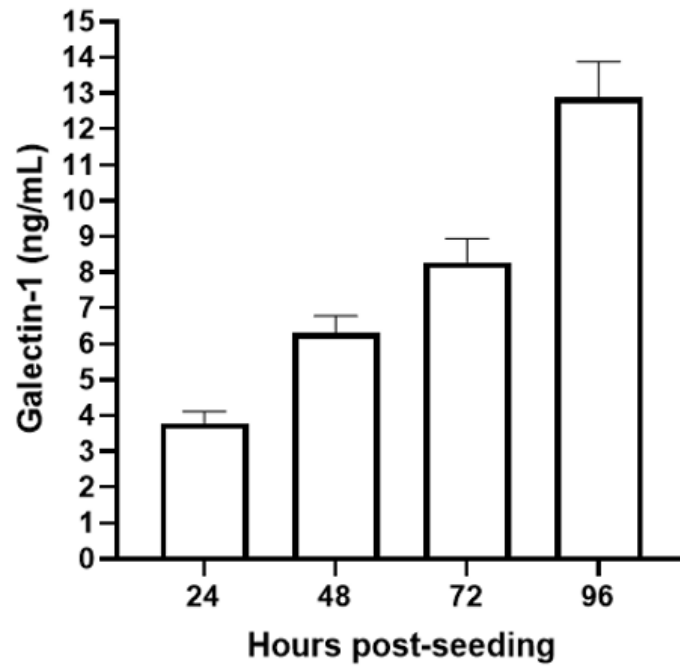


Figure 3.11. Galectin-1 standard curve. Standards were diluted and 100 μ L of each was incubated in a 96-well plate pre-coated with capture antibody. After two hours, plates were washed and incubated for a further two hours with detection antibody. Plates were washed again and incubated for 20 minutes each with streptavidin-HRP (horseradish peroxidase) and ELISA substrate. The reaction was stopped with acid and plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Solid line=line of best fit. Dotted lines=95% CI. Error bars=SEM. N=1. All standards were run in duplicate.

A Galectin-1 in U251 supernatant



B Galectin-1 in U373 supernatant

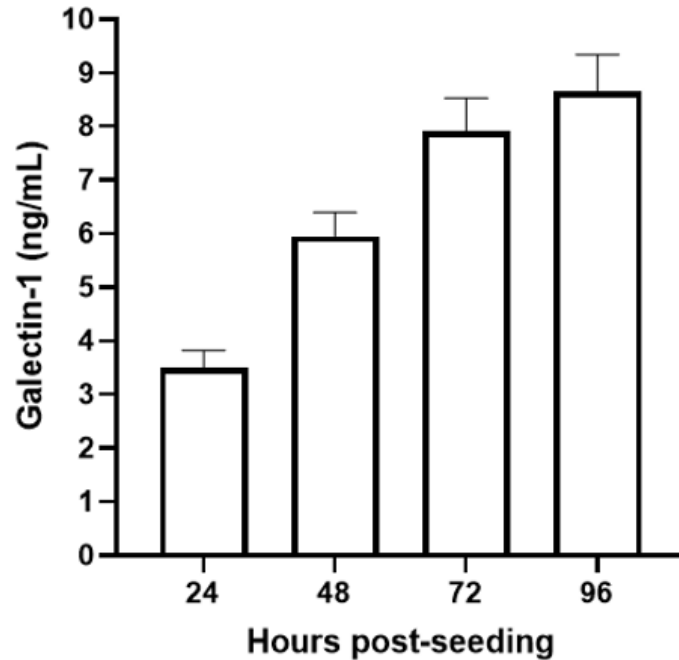


Figure 3.12. Galectin-1 in U251 (A) and U373 (B) supernatant. Cells were seeded at $1.8 \times 10^5/\text{mL}$ and supernatants were collected after 24, 48, 72 and 96 hours' growth. Supernatants were passed through a $0.25 \mu\text{m}$ filter to remove remaining cells. Galectin-1 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Error bars=SEM. N=3 for each timepoint. Samples and standards were run in duplicate.

Next, galectin-1 in transport media from GBM and LGG tumours was examined (Figure 3.13). Gal-1 was found to be present in eight of 23 GBM TM and two of seven LGG TM samples. The mean concentration was 11.2ng/mL in GBM TM, which showed a widespread range from 0 to 118.1ng/mL. Gal-1 in LGG TM was found at 53.1 and 4.1ng/mL for a mean average of 8.2ng/mL. This difference was not found to be significant by t-test. Thus, gal-1 secretion may not differ by glioma grade.

To conclude galectin-1 experiments, the change in gal-1 secretion upon establishment of an *in vitro* culture was studied. The results are shown in Figure 3.14. Gal-1 secretion decreased from a mean average of 13.7ng/mL in the 16 TM samples to 1.6ng/mL in corresponding CS samples, which approached but did not reach significance ($p=0.0972$). A subset of tumours did increase gal-1 secretion.

To conclude, gal-1 was overall expressed at high levels by a subset of gliomas and was downregulated on *in vitro* growth, although a subset of tumours began to secrete gal-1 after primary cultures were established. Given gal-1's presence in both cell lines, also in the ng/mL range, it was possible that either these cell lines originated from gal-1+ tumours or gal-1 expression was important for immortalisation; the data were not sufficient to draw any conclusion on this matter. However, the apparent lack of uptake by cell lines favoured the former explanation, although low-level uptake would still be masked by high-level secretion.

Galectin-1 in paired GBM transport media and culture supernatant

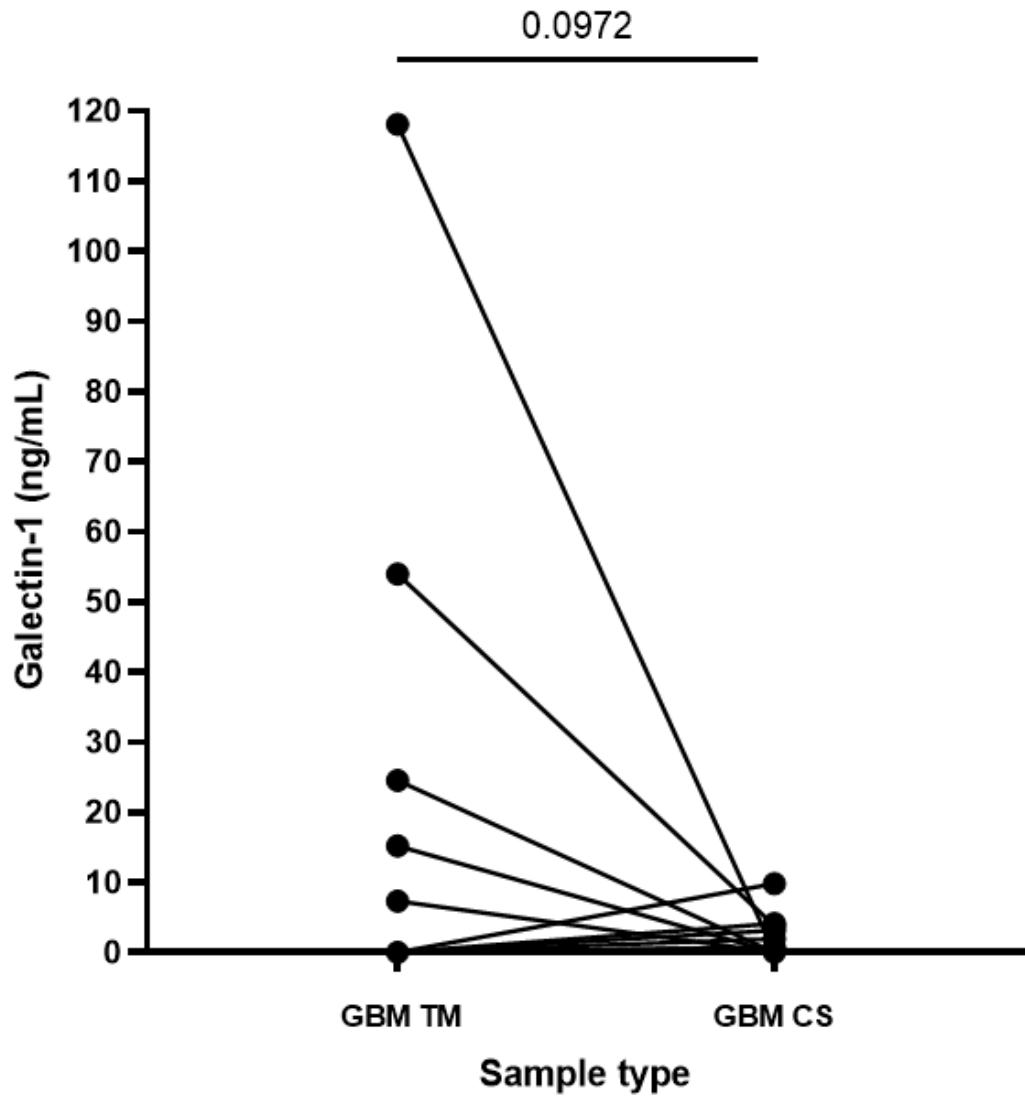


Figure 3.14. Galectin-1 in paired GBM transport media and culture supernatant. Galectin-1 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed t-test with Welch's correction. N=16. Samples and standards were run in duplicate.

3.2.2.2.3. *Galectin-3*

Next, samples were assayed for galectin-3. A representative standard curve is shown in Figure 3.15. Gal-3 was not detectable in U251 supernatants at any timepoint. However, it was present in U373 supernatants at mean concentrations of 170.0, 157.2, 236.2 and 308.4pg/mL at 24, 48, 72 and 96 hours post-seeding respectively (Figure 3.16). Thus, gal-3 showed a similar pattern of secretion as gal-1 at a lower overall concentration, and moreover was differentially regulated between the U373 and U251 glioma cell lines.

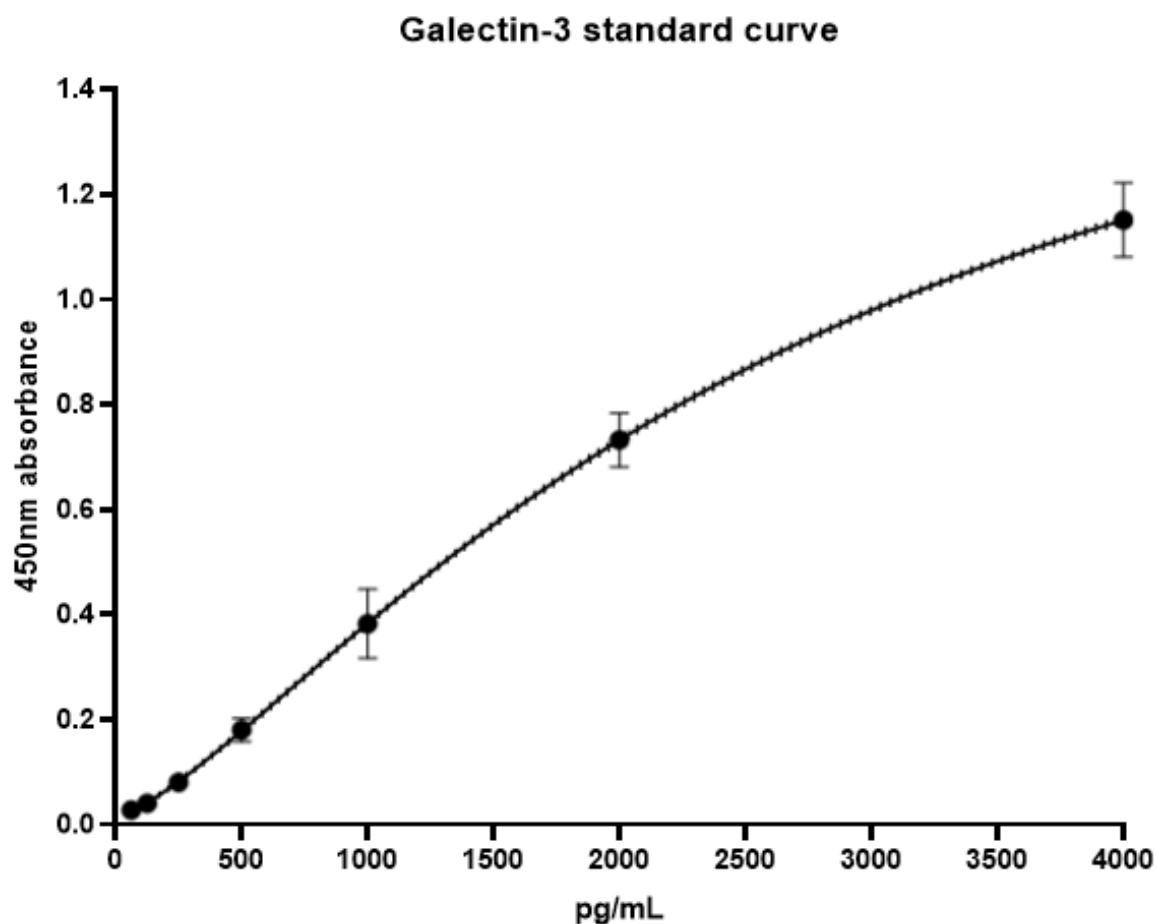


Figure 3.15. Galectin-3 standard curve. Standards were diluted and 100 μ L of each was incubated in a 96-well plate pre-coated with capture antibody. After two hours, plates were washed and incubated for a further two hours with detection antibody. Plates were washed again and incubated for 20 minutes each with streptavidin-HRP (horseradish peroxidase) and ELISA substrate. The reaction was stopped with acid and plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Solid line=line of best fit. Dotted lines=95% CI. Error bars=SEM. N=1. All standards were run in duplicate.

Galectin-3 in U373 supernatant

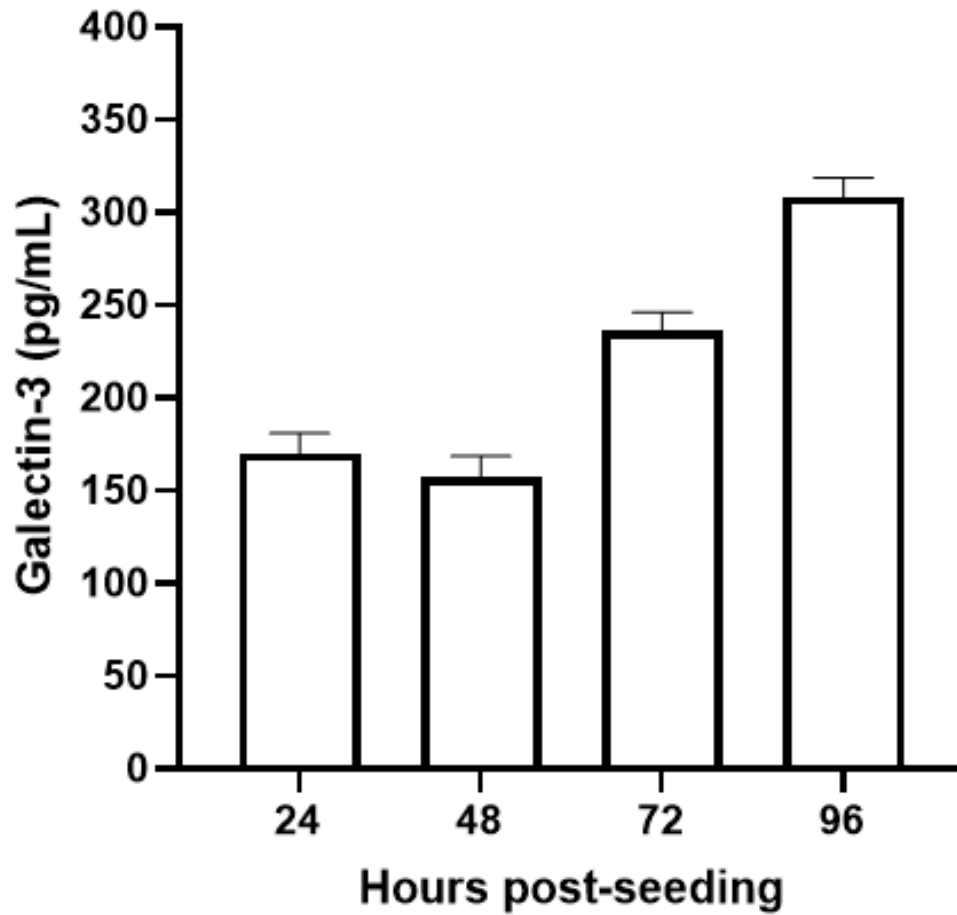


Figure 3.16. Galectin-3 in U373 supernatant. Cells were seeded at $1.8 \times 10^5/\text{mL}$ and supernatants were collected after 24, 48, 72 and 96 hours' growth. Supernatants were passed through a $0.25\mu\text{m}$ filter to remove remaining cells. Galectin-3 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Error bars=SEM. N=3 for each timepoint. Samples and standards were run in duplicate.

The concentration of gal-3 in transport media from GBM and LGG tumours was then studied. Results are shown in Figure 3.17. Gal-3 was present in 17 of 23 GBM TM and six of seven LGG TM, with mean average concentrations of 1800.3 and 595.4pg/mL respectively. This difference reached significance ($p=0.0089$). It appeared that gal-3 was widely expressed by gliomas, with potential differential regulation between high- and low-grade glioma.

Finally, gal-3 concentration was compared across the 16 paired GBM CS and TM samples. The data is shown in Figure 3.18. Gal-3 – or gal-3-producing cells – was clearly and significantly lost ($p < 0.0001$) following the transition to *in vitro* growth. Mean average gal-3 concentration was 1558.5pg/mL in the 16 TM samples, compared to 7.4pg/mL in CS samples.

These data demonstrated that gal-3 was widely expressed *in vivo* but quickly lost *in vitro*, and thus likely plays a role in supporting tumour growth *in vivo*. In addition, gal-3 was differentially expressed in U251 and U373 cell lines, suggesting that the U373 secretome may be more reflective of the *in vivo* environment.

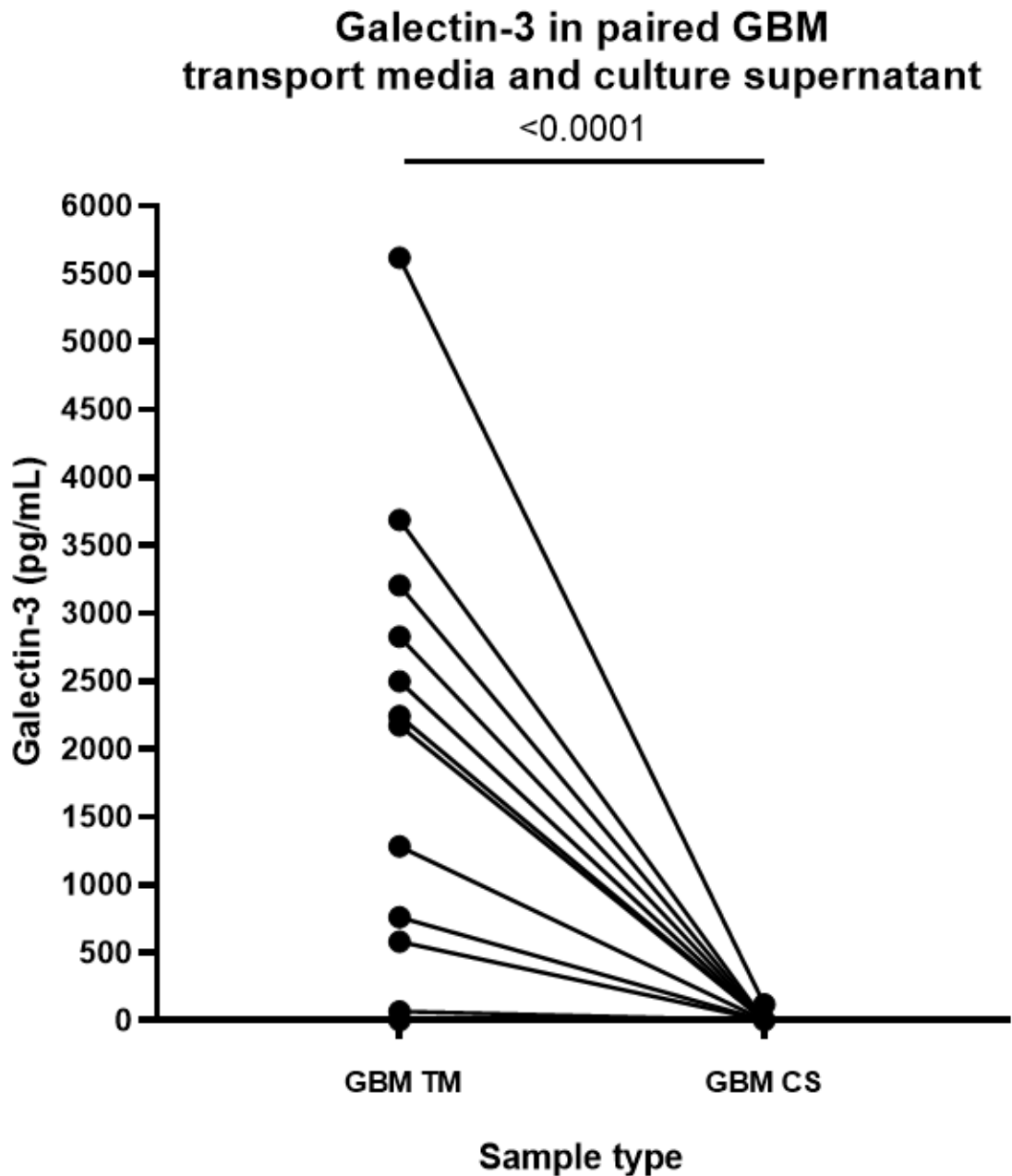


Figure 3.18. Galectin-3 in GBM transport media and culture supernatant. Galectin-3 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed t-test with Welch's correction. N=16. Samples and standards were run in duplicate.

3.2.2.2.4. *Galectin-4*

Galectin-4 was studied next. This protein was absent in both cell lines and LGG TM (data not shown); a representative standard curve is shown in Figure 3.19. Gal-4 was present in two of 23 GBM TM samples, at concentrations of 765.3 and 10.0pg/mL, giving a mean average of 33.7pg/mL (Figure 3.20). It was concluded that gal-4 is expressed at variable levels in a minority of GBM samples.

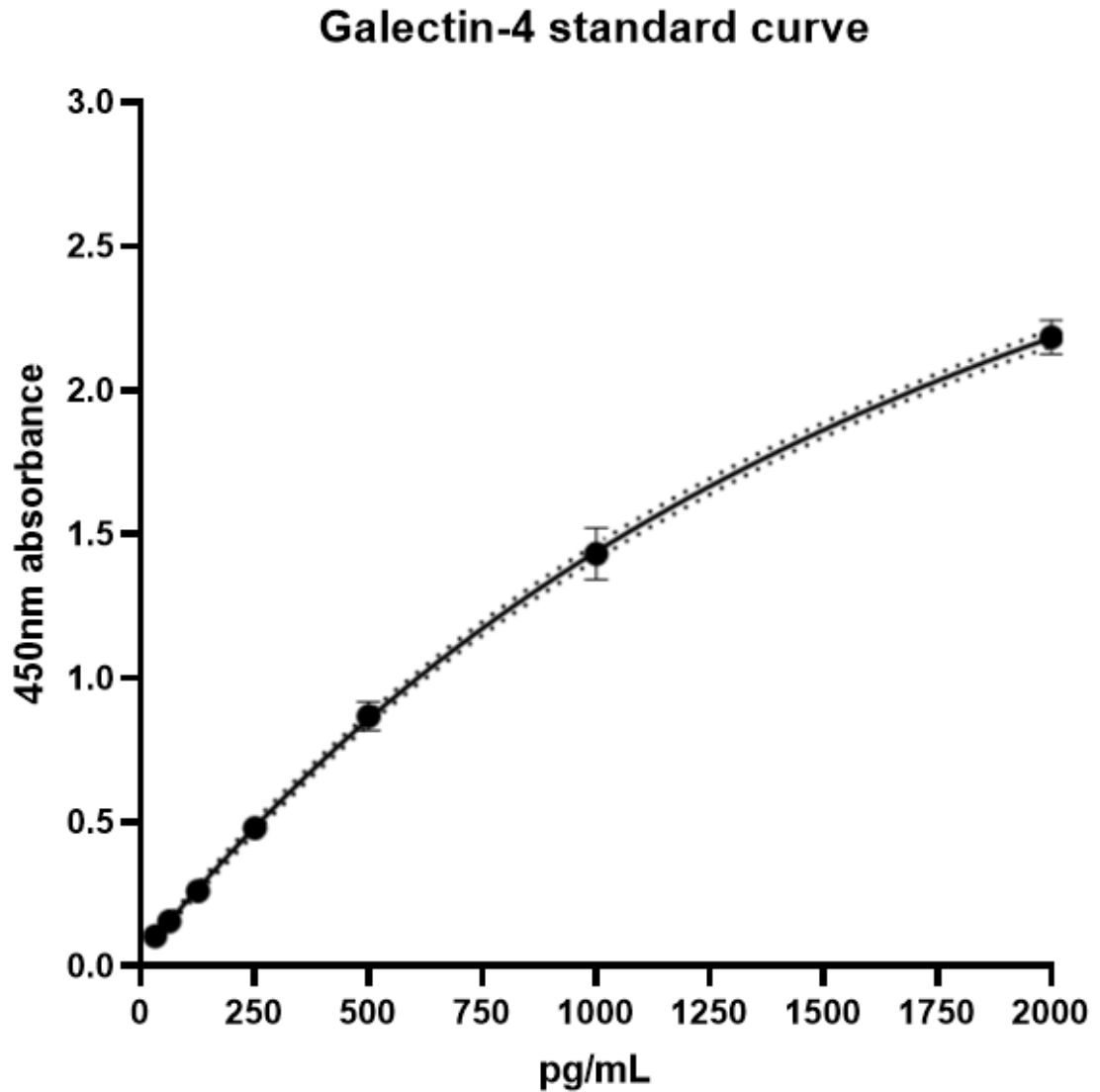
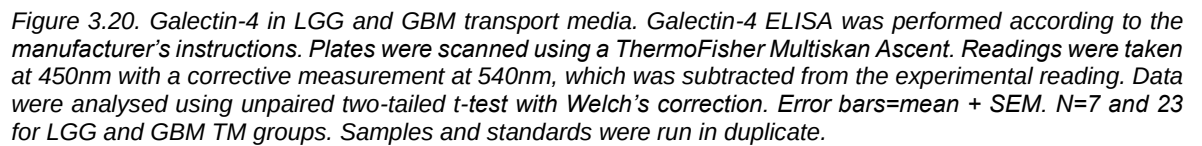


Figure 3.19. Galectin-4 standard curve. Standards were diluted and 100 μ L of each was incubated in a 96-well plate pre-coated with capture antibody. After two hours, plates were washed and incubated for a further two hours with detection antibody. Plates were washed again and incubated for 20 minutes each with streptavidin-HRP (horseradish peroxidase) and ELISA substrate. The reaction was stopped with acid and plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Solid line=line of best fit. Dotted lines=95% CI. Error bars=SEM. N=1. All standards were run in duplicate.

0.3218



When comparing the 16 GBM TM to their CS counterparts, it was found that gal-4 disappeared after primary cultures were established. The mean average concentration of gal-4 in the 16 TM samples was 48.5pg/mL. However, this downregulation did not approach significance (Figure 3.21).

In conclusion, gal-4 expression may be limited to GBM, and its absence in glioma cell lines correlated with the observed low occurrence of gal-4 in primary culture supernatant.

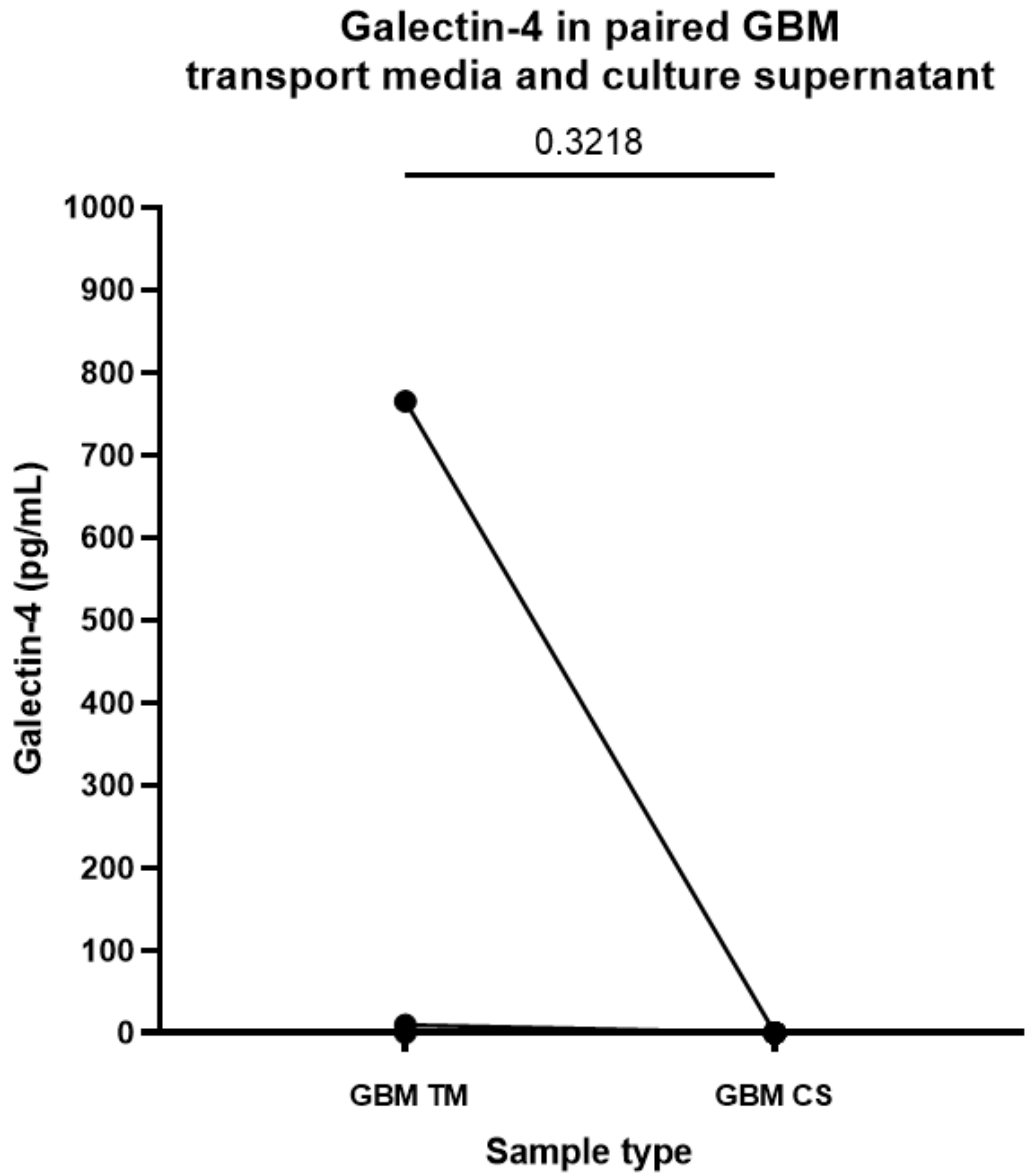


Figure 3.21. Galectin-4 in GBM transport media and culture supernatant. Galectin-4 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed t-test with Welch's correction. Error bars=mean + SEM. N=16. Samples and standards were run in duplicate.

3.2.2.2.5. *Galectin-7*

A representative standard curve for gal-7, the last member of the galectin family identified in mass spectrometry data, is shown in Figure 3.22. Like gal-4, this protein was also absent in both cell lines. Also like gal-4, gal-7 was found in a minority of samples: one of 23 GBM at 848.2pg/mL giving a mean average of 36.9pg/mL (Figure 3.23), and no LGG TM.

Galectin-7 standard curve

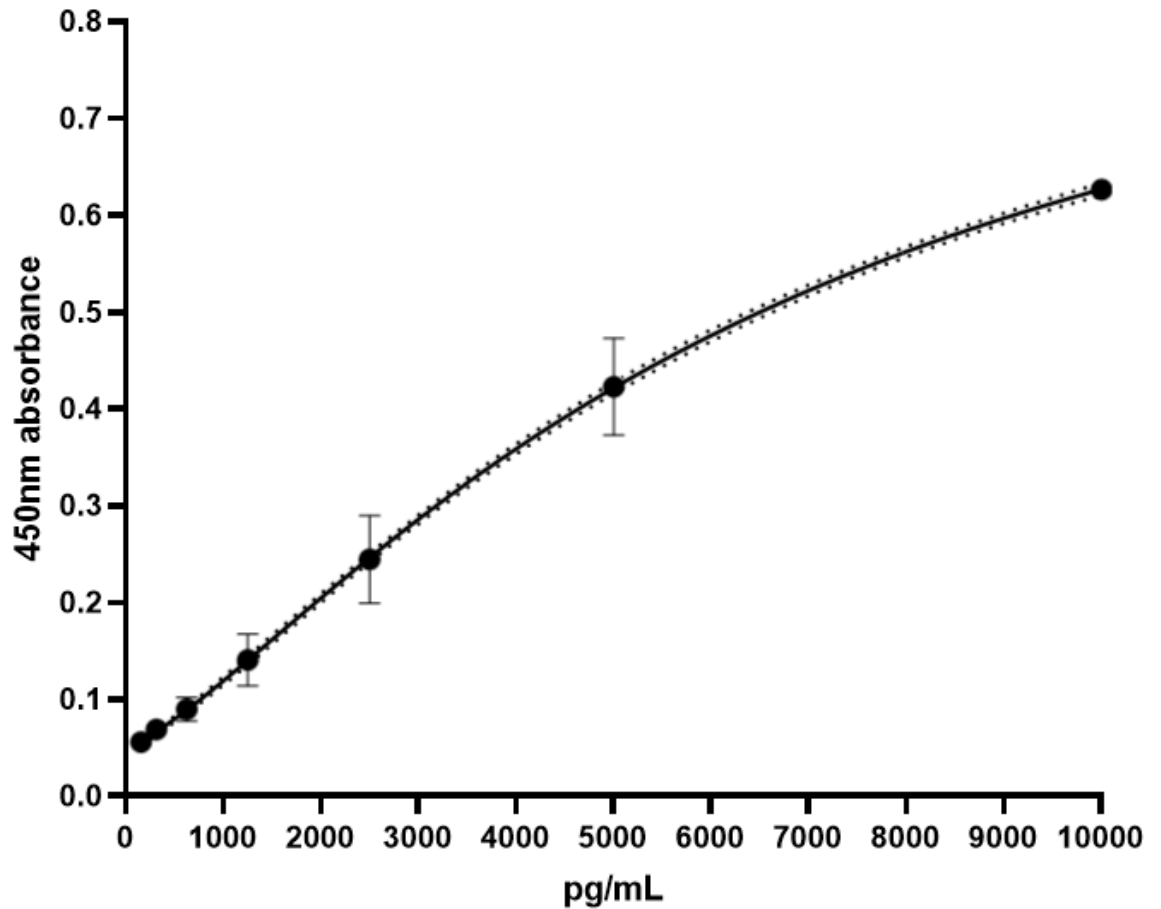


Figure 3.22. Galectin-7 standard curve. Standards were diluted and 100 μ L of each was incubated in a 96-well plate pre-coated with capture antibody. After two hours, plates were washed and incubated for a further two hours with detection antibody. Plates were washed again and incubated for 20 minutes each with streptavidin-HRP (horseradish peroxidase) and ELISA substrate. The reaction was stopped with acid and plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Solid line=line of best fit. Dotted lines=95% CI. Error bars=SEM. N=1. All standards were run in duplicate.

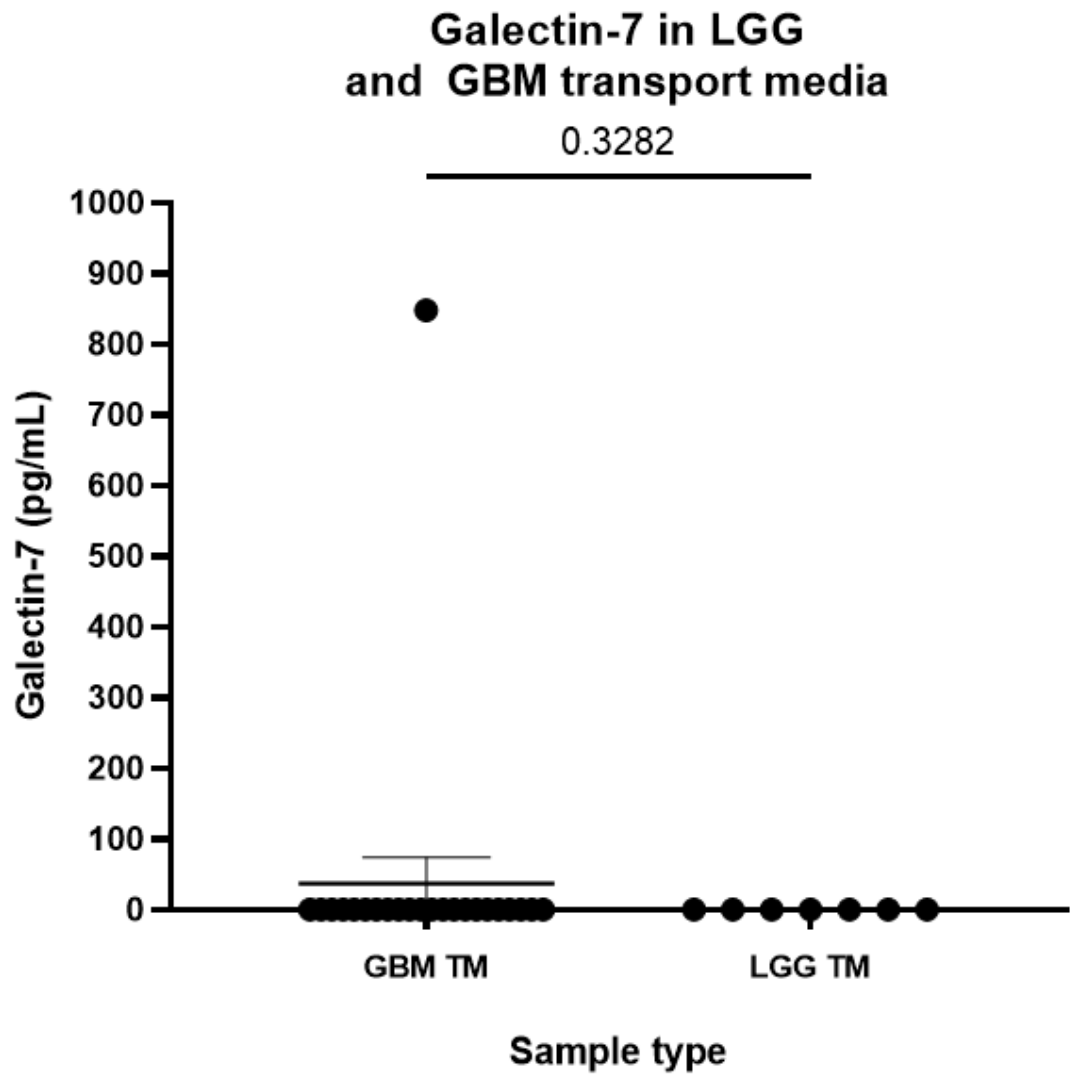


Figure 3.23. Galectin-7 in LGG and GBM transport media. Galectin-7 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed t-test with Welch's correction. Error bars=mean + SEM. N=7 and 23 for LGG and GBM TM groups. Samples and standards were run in duplicate.

Of the 16 TM samples for which paired CS was available, the mean average gal-7 concentration was 0pg/mL, whereas the protein was more highly expressed in CS samples. Gal-7 was found in three of 16 CS samples at 6193.2, 1795.0 and 359.9pg/mL for a mean average of 566.8pg/mL; however, this increase did not approach significance (Figure 3.24).

These data suggested that gal-7 was present in a minority of GBM samples, but was in some cases gained upon the transition to *in vitro* growth. Regardless, its absence in both cell lines suggested gal-7 is not necessary for either *in vivo* or *in vitro* growth.

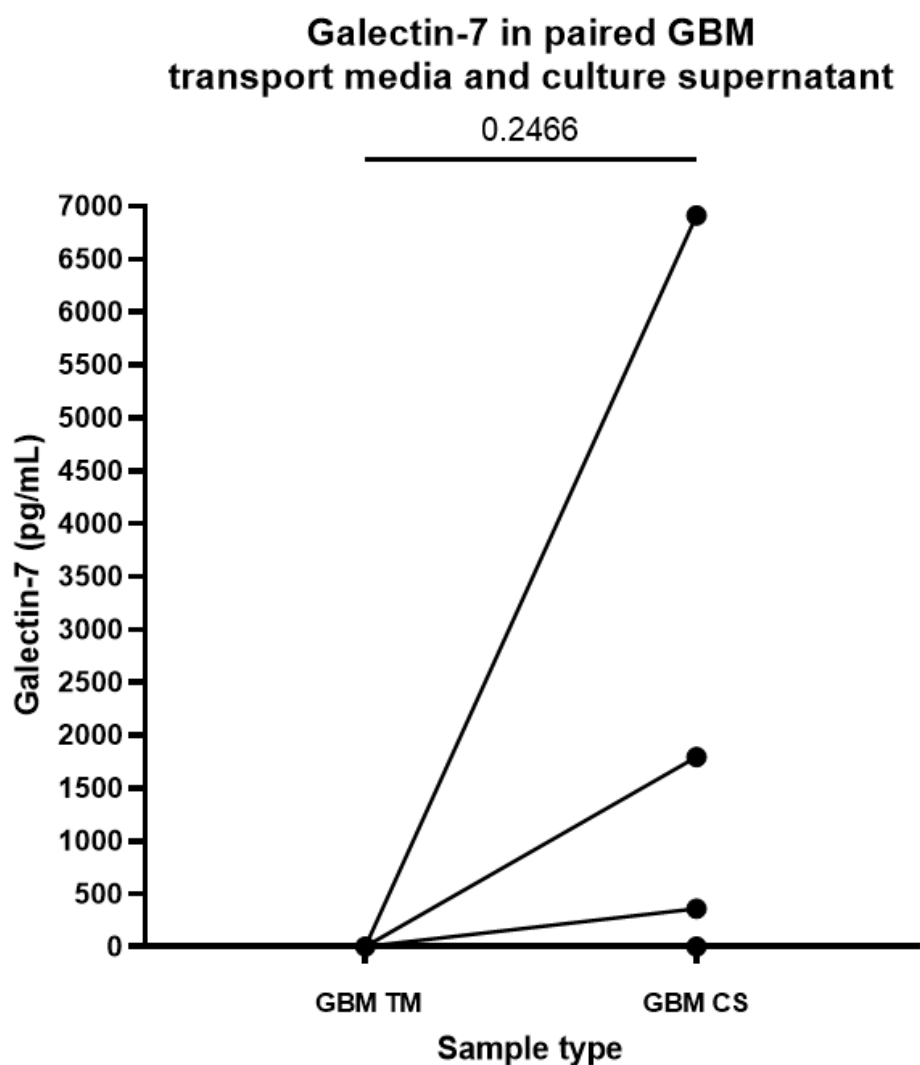


Figure 3.24. Galectin-7 in GBM transport media and culture supernatant. Galectin-7 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed t-test with Welch's correction. N=16. Samples and standards were run in duplicate.

3.2.2.2.6. CTGF

A representative standard curve for CTGF is shown in Figure 3.25. CTGF was detectable in both cell lines at relatively high concentrations (Figure 3.26). The mean concentration in U251 supernatants taken at 24, 48, 72 and 96 hours post-seeding was 1146.2, 1855.7, 2060.2 and 1946.1pg/mL respectively. U373 supernatants showed a similar pattern, with concentrations of 1767.2, 2240.3, 2513.5 and 2021.3pg/mL over the same timecourse. The pattern of secretion was different from that of either gal-1, gal-3 or total protein: instead, CTGF concentration increased up to 72h and then declined at 96h, although concentrations in U373 supernatants were a little higher. It is possible that CTGF was secreted and rapidly taken up by growing cells, which decreased at the 96-hour mark either due to cell death or confluence.

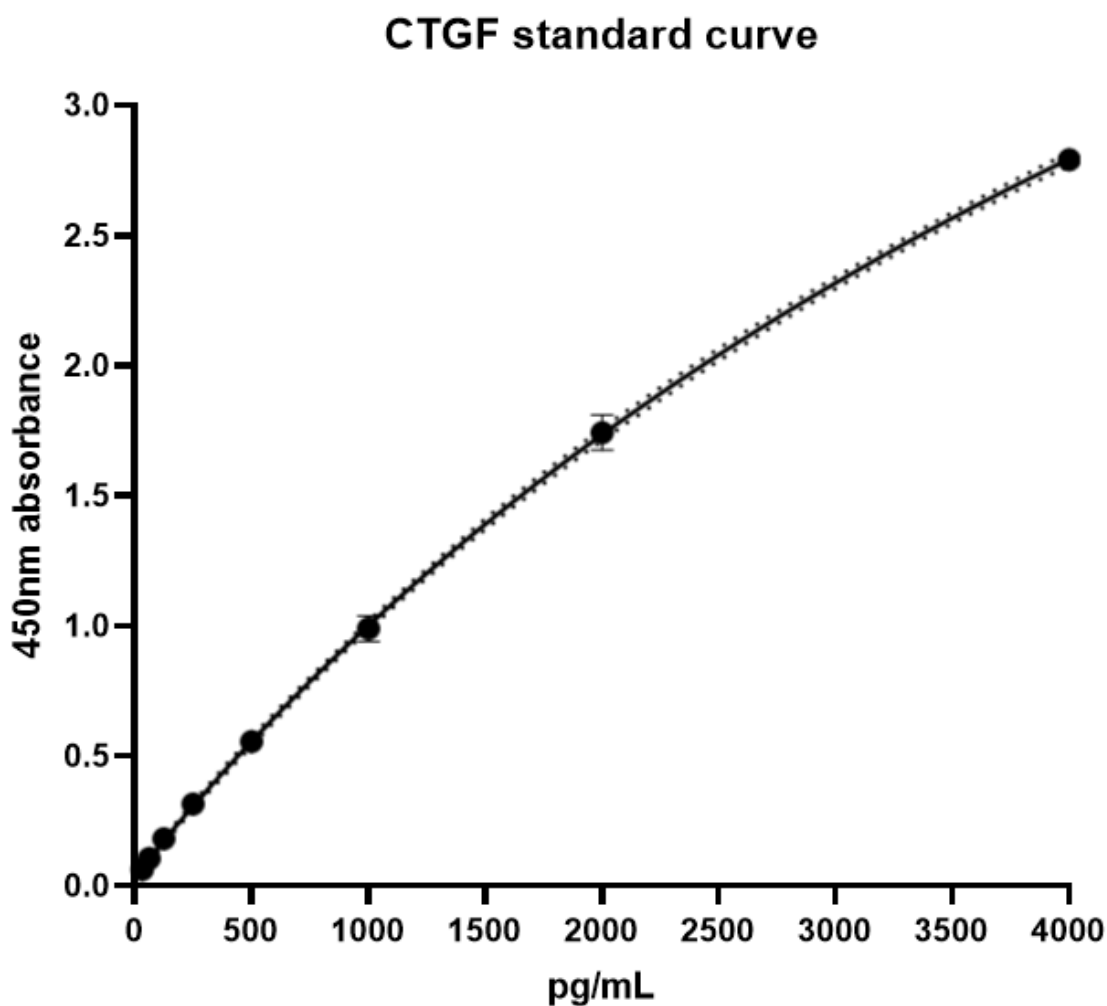


Figure 3.25. CTGF standard curve. Standards were diluted and 100 μ L of each was incubated in a 96-well plate pre-coated with capture antibody. After two hours, plates were washed and incubated for a further two hours with detection antibody. Plates were washed again and incubated for 20 minutes each with streptavidin-HRP (horseradish peroxidase) and ELISA substrate. The reaction was stopped with acid and plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Solid line=line of best fit. Dotted lines=95% CI. Error bars=SEM. N=1. All standards were run in duplicate.

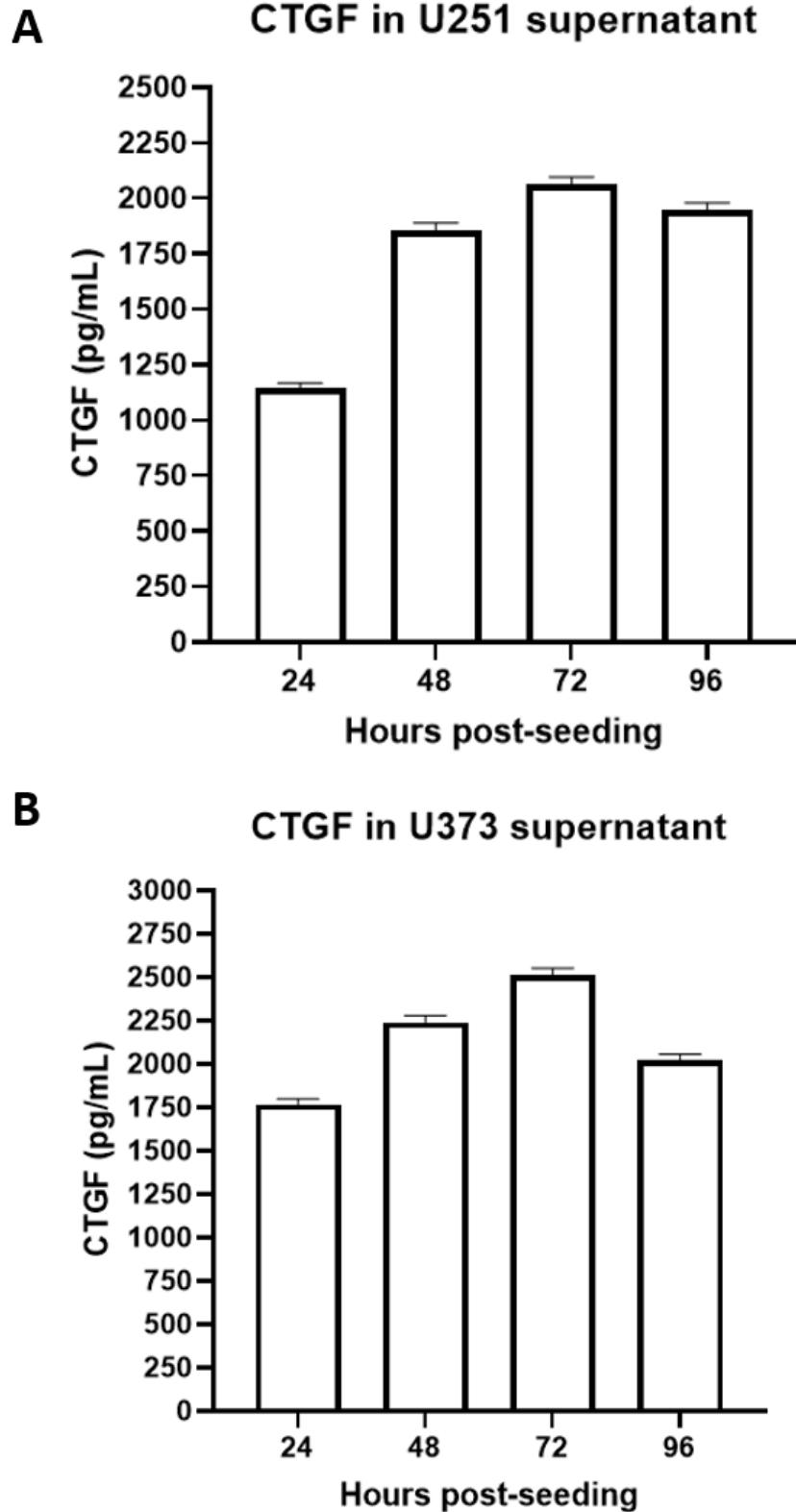
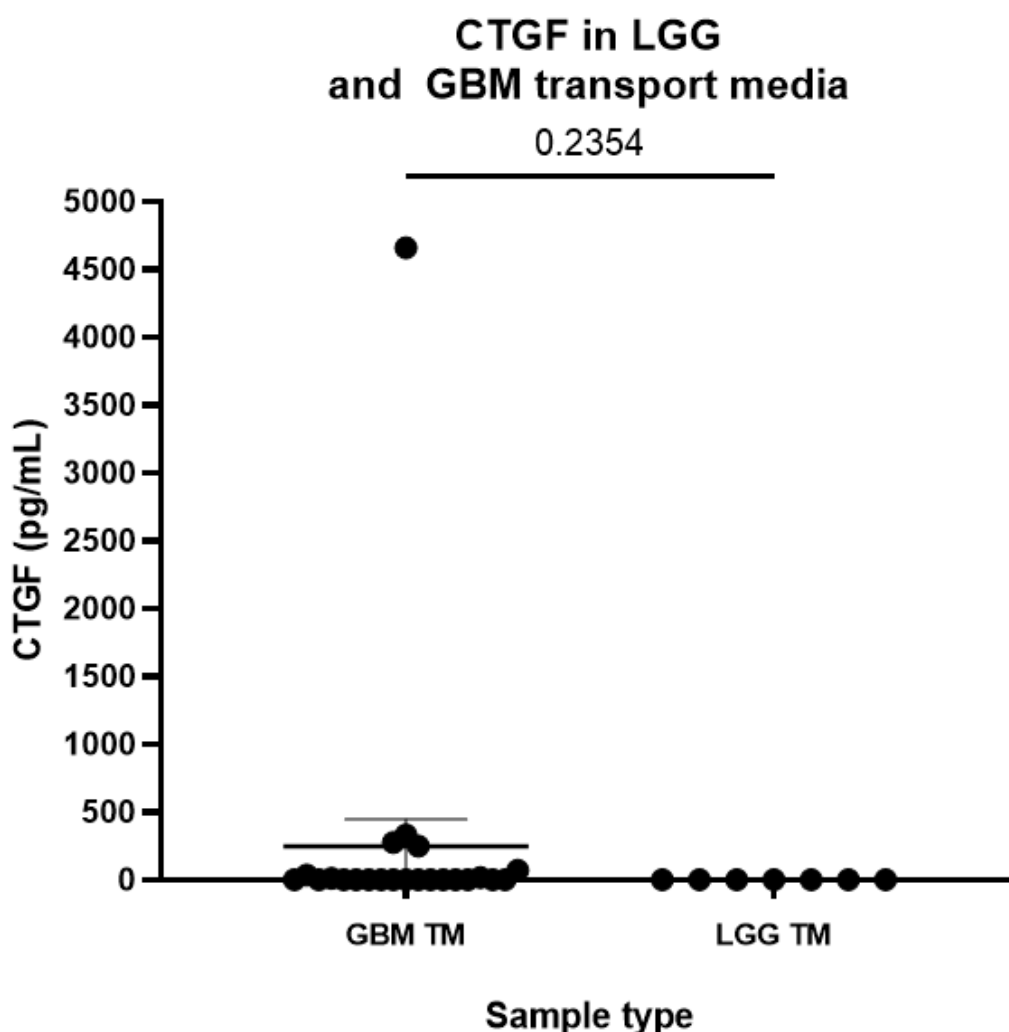


Figure 3.26. CTGF in U251 (A) and U373 (B) supernatant. Cells were seeded at $1.8 \times 10^5/\text{mL}$ and supernatants were collected after 24, 48, 72 and 96 hours' growth. Supernatants were passed through a $0.25\mu\text{m}$ filter to remove remaining cells. CTGF ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Error bars=SEM. N=3 for each timepoint. Samples and standards were run in duplicate.

CTGF was completely absent in LGG TM, but was found in eight of 23 GBM TM (Figure 3.27). CTGF concentrations ranged from 11.9 to 4659.0pg/mL, but were generally in the lower end of the range, with a mean concentration of 246.0pg/mL. These data suggested that CTGF was expressed in a subset of GBM and no LGG.



Next, CTGF expression before and after establishment of *in vitro* culture was studied (Figure 3.28). The mean concentration in the 16 TM in this sample set was 54.5pg/mL, and mean concentration in the paired CS samples was 0pg/mL. This did not approach significance.

Based on these data, it appeared that CTGF was expressed in a subset of GBM and lost upon the transition to *in vitro* growth. As CTGF was present in both cell lines, its role as a growth factor may support growth of immortalised cells; the absence in CS samples may have been a temporary downregulation. Alternately, CTGF may have been compensating for a loss of supportive intratumoural factors that do not play a role *in vitro*.

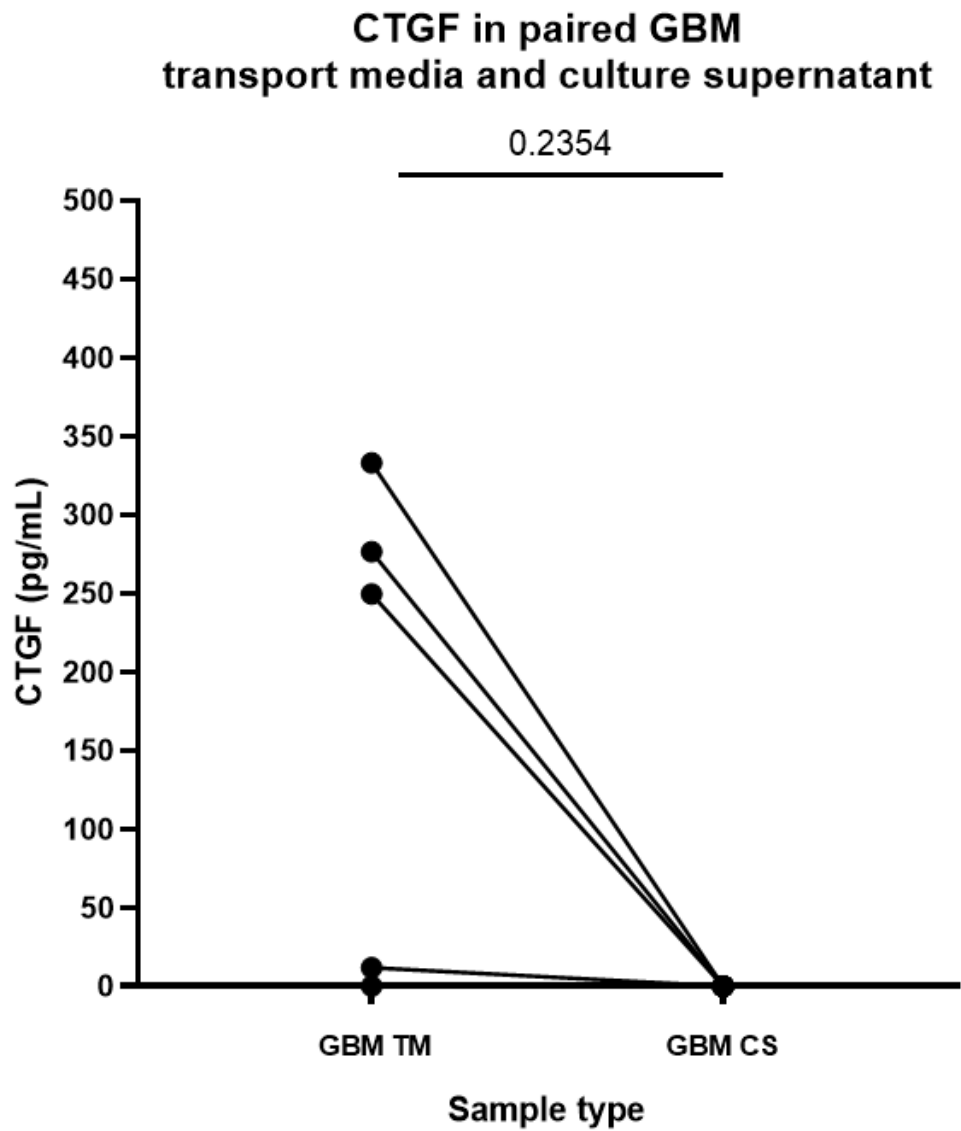


Figure 3.28. CTGF in GBM transport media and culture supernatant. CTGF ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed *t*-test with Welch's correction. *N*=16. Samples and standards were run in duplicate.

3.2.2.2.7. CEACAM1

CEACAM1 in supernatant samples was then analysed; a representative standard curve is shown in Figure 3.29. As with galectins 4 and 7, this protein was undetectable in cell line supernatant and LGG TM, and was found to be present in a minority of GBM TM: three of 23 samples. CEACAM1 concentration in these samples was varied, from 102.1 to 5854.6pg/mL, with a mean average of 278.7pg/mL (Figure 3.30).

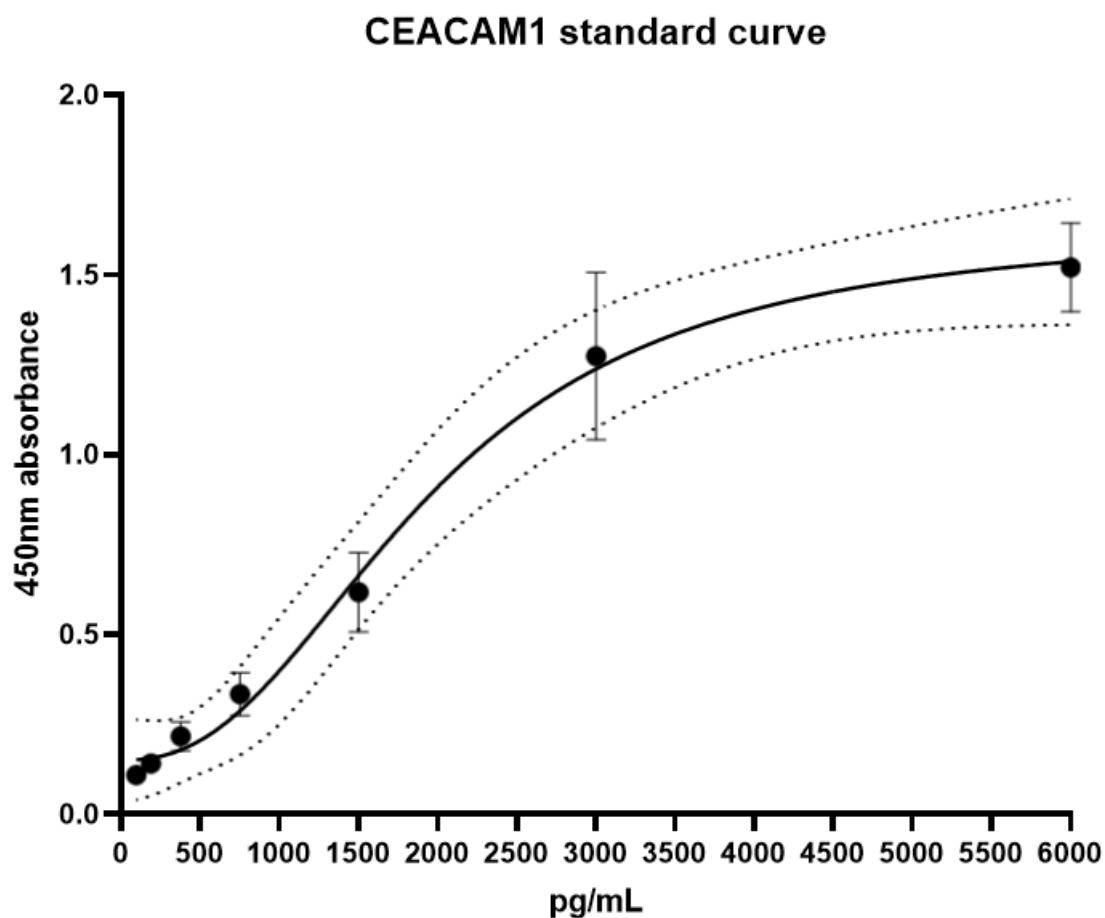


Figure 3.29. CEACAM1 standard curve. Standards were diluted and 100 μ L of each was incubated in a 96-well plate pre-coated with capture antibody. After two hours, plates were washed and incubated for a further two hours with detection antibody. Plates were washed again and incubated for 20 minutes each with streptavidin-HRP (horseradish peroxidase) and ELISA substrate. The reaction was stopped with acid and plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Solid line=line of best fit. Dotted lines=95% CI. Error bars=SEM. N=1. All standards were run in duplicate.

Figure 3.31 shows a comparison of CEACAM1 secretion across paired GBM TM and CS samples. CEACAM1 was undetectable in CS, although the downregulation was not found to be significant.

From these data, it appeared that CEACAM1 was expressed in a minority of GBM and no LGG samples; its disappearance *in vitro* implied it plays no role in supporting *in vitro* growth.

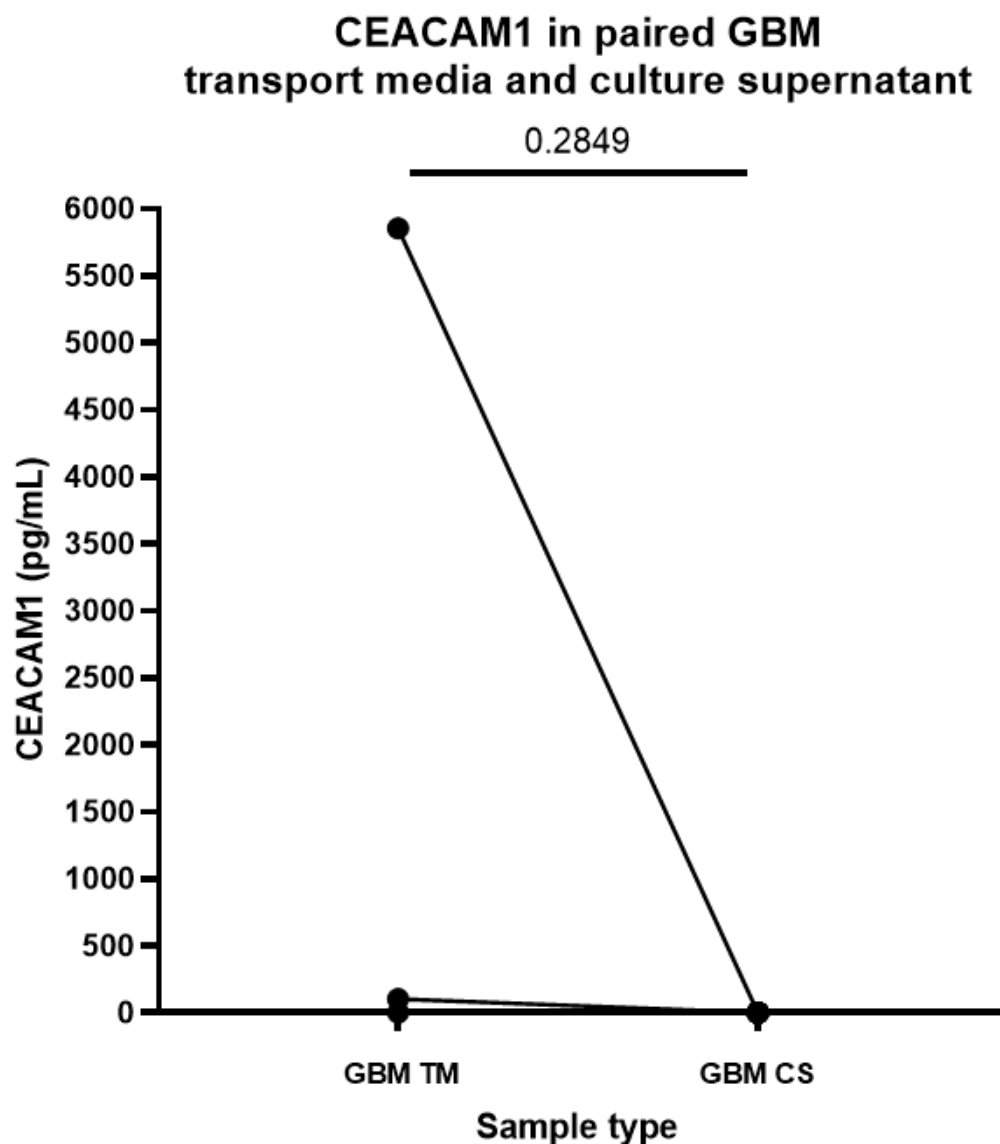


Figure 3.31. CEACAM1 in GBM transport media and culture supernatant. CEACAM1 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed t-test with Welch's correction. N=16. Samples and standards were run in duplicate.

3.2.2.2.8. S100A7

ELISA assays were concluded with an assay for S100A7. Due to limited kit size, only the 16 paired CS and TM samples were included in this experiment. An example standard curve is shown in Figure 3.32.

All 16 TM samples contained S100A7 in a narrow range of concentration, from 22.1 to 55.8pg/mL (Figure 3.33). S100A7 expression was lost in all but 4 tumours on the transition to *in vitro* growth; the range in these samples was comparable to that in TM. However, the difference in mean averages – 33.7 and 7.2pg/mL – was found to be statistically significant ($p < 0.0001$). In conclusion, S100A7 likely conferred a benefit to GBM growth *in vivo* that is lost *in vitro*; however, unlike the other proteins in these assays, these results could not be compared to LGG or cell line data.

S100A7 standard curve

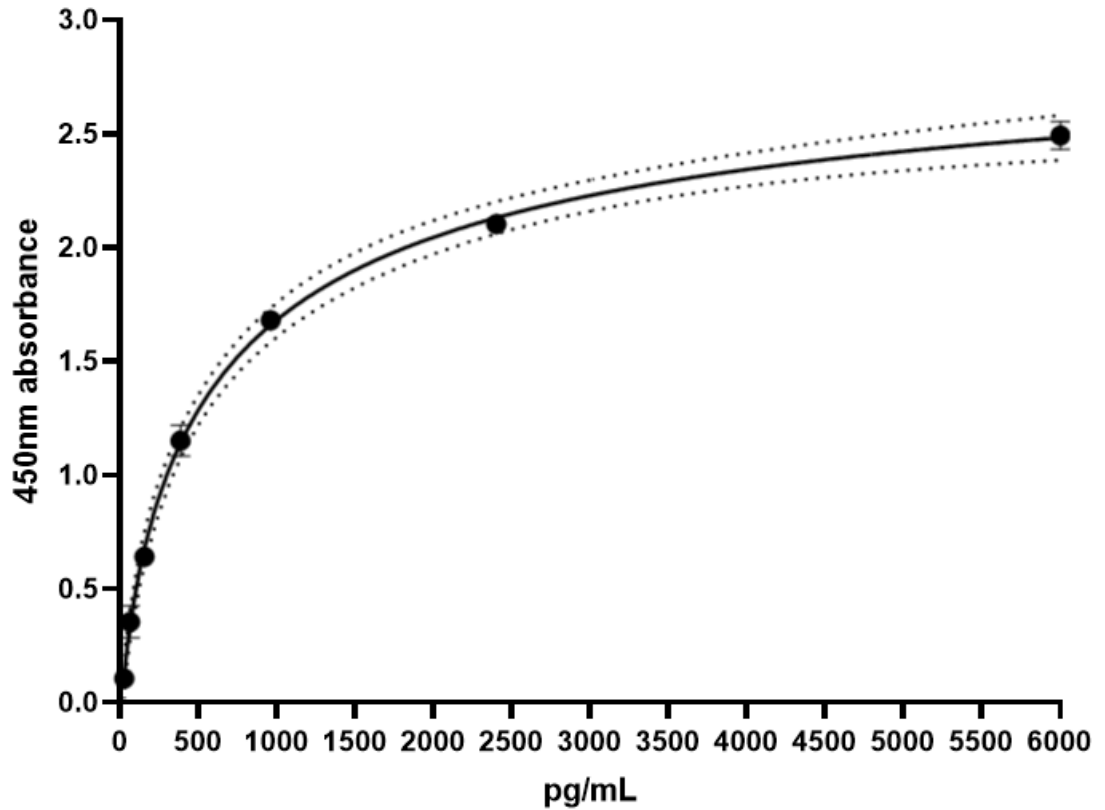


Figure 3.32. S100A7 standard curve. Standards were diluted and 100 μ L of each was incubated in a 96-well plate pre-coated with capture antibody. After two hours, plates were washed and incubated for a further two hours with detection antibody. Plates were washed again and incubated for 20 minutes each with streptavidin-HRP (horseradish peroxidase) and ELISA substrate. The reaction was stopped with acid and plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, was subtracted from the experimental reading. Solid line=line of best fit. Dotted lines=95% CI. Error bars=SEM. N=1. All standards were run in duplicate.

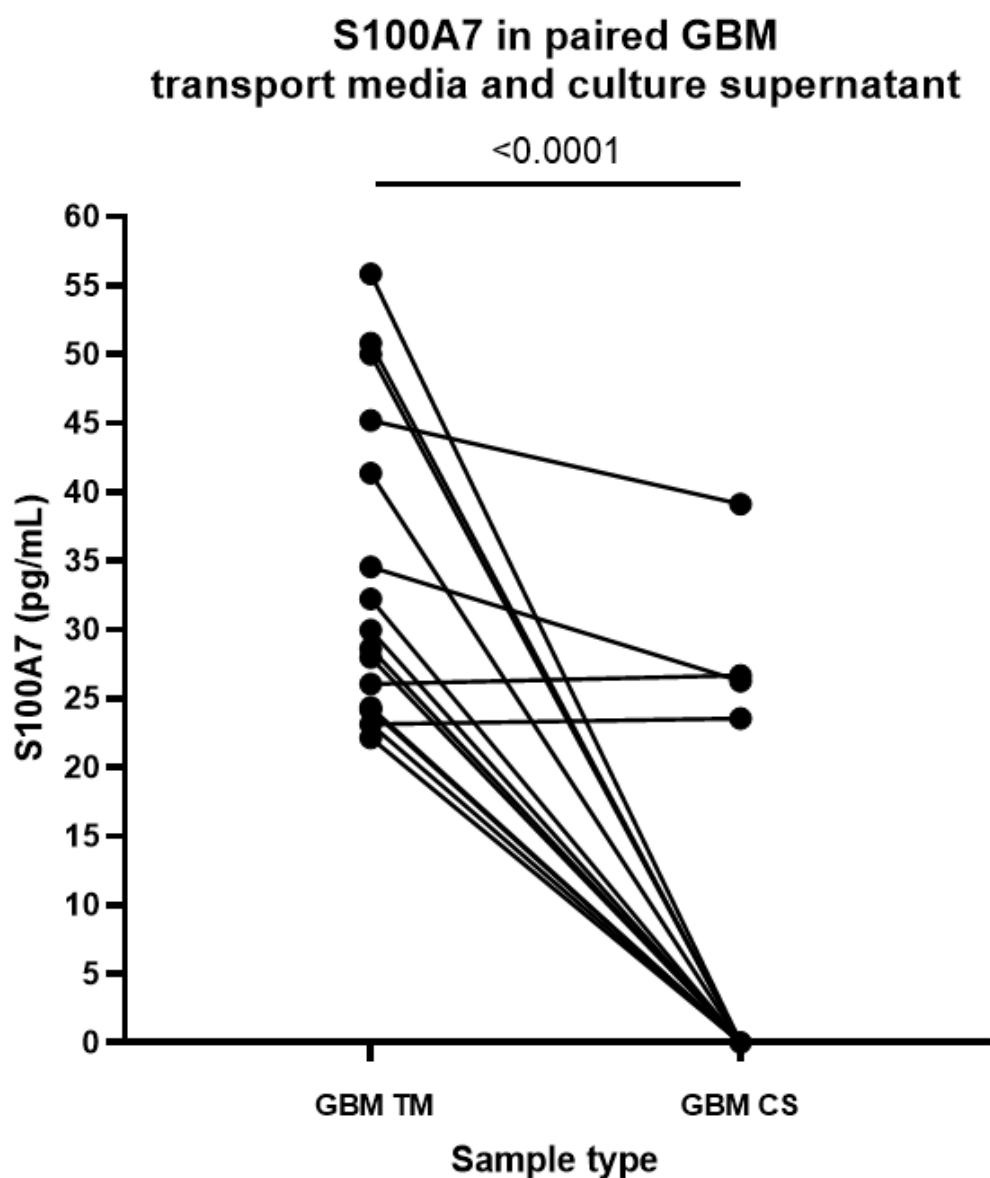


Figure 3.33. S100A7 in GBM transport media and culture supernatant. S100A7 ELISA was performed according to the manufacturer's instructions. Plates were scanned using a ThermoFisher Multiskan Ascent. Readings were taken at 450nm with a corrective measurement at 540nm, which was subtracted from the experimental reading. Data were analysed using unpaired two-tailed t-test. N=16. Samples and standards were run in duplicate.

3.2.3. Secretory profiles

The above section described the presence of a variety of proteins of interest in CS from glioma cell lines, GBM and LGG TM and GBM primary CS. Each protein of interest was detectable in some samples, and some were either present in the majority of samples, e.g. gal-3, or at very high concentration, such as MIF and gal-1. Next, relationships between the secreted proteins were considered using the 23 GBM TM samples, which were most reflective of the *in vivo* environment.

Each tumour was scored for the presence or absence of each protein. This compiled data, shown in Figure 3.34, did not reveal any obvious secretory patterns. Presence of each protein was then compared to presence of every other protein; this analysis used Fisher's exact test instead of chi-squared due to small sample size. A significant correlation ($p=0.0119$) was noted between presence of gal-4 and CEACAM1 in GBM TM, and the correlation between galectin-1 and MIF secretion also approached significance ($p=0.0582$) (Table 3.14.).

Because 28 comparisons were performed in this analysis, two different post-hoc tests were performed to correct for multiple comparisons. Running 28 tests at the 0.05 significance level means 1.4 tests would be expected to return a positive result even if all null hypotheses – that presence of two proteins in GBM TM is not correlated – were true. Bonferroni's test was used to control for family-wise error rate (FWER): the probability of making at least one false positive in a family of tests. This method is known to be conservative, and indeed returned an adjusted significance level of 0.0018, which no correlations reached (Table 3.14).

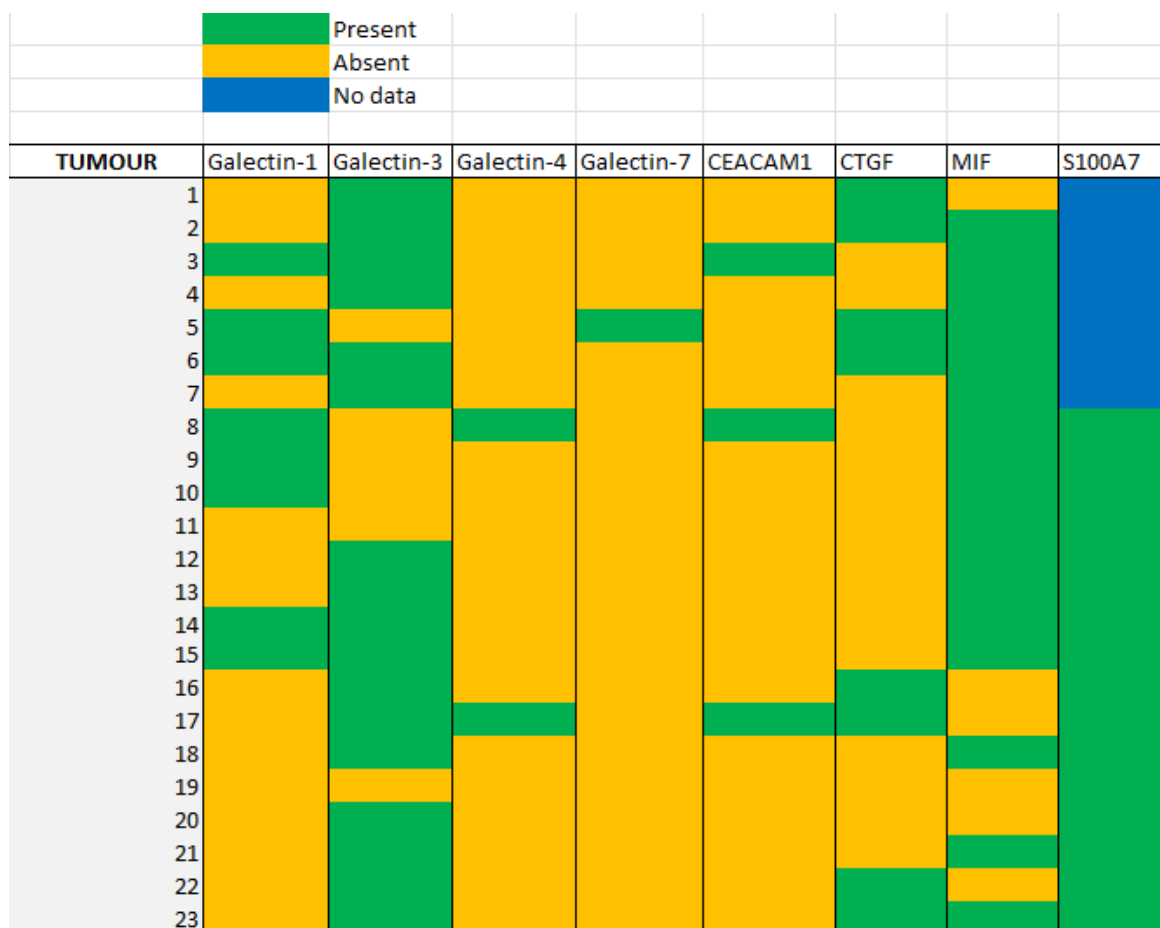


Figure 3.34. Secretion patterns of target proteins in 23 GBM transport media based on protein presence and absence.

Table 3.14. Secreted protein comparisons with Bonferroni's correction

Comparison	P-value (Fisher's exact test)	Significance
Galectin-1 and galectin-3	0.1310	No
Galectin-1 and galectin-4	>0.9999	No
Galectin-1 and galectin-7	0.3478	No
Galectin-1 and CTGF	>0.9999	No
Galectin-1 and CEACAM1	0.2688	No
Galectin-1 and MIF	0.0582	No
Galectin-1 and S100A7	>0.9999	No
Galectin-3 and galectin-4	0.4625	No
Galectin-3 and galectin-7	0.2609	No
Galectin-3 and CTGF	0.3690	No
Galectin-3 and CEACAM1	>0.9999	No
Galectin-3 and MIF	>0.9999	No
Galectin-3 and S100A7	>0.9999	No
Galectin-4 and galectin-7	>0.9999	No
Galectin-4 and CTGF	>0.9999	No
Galectin-4 and CEACAM1	0.0119	No
Galectin-4 and MIF	0.4625	No
Galectin-4 and S100A7	>0.9999	No
Galectin-7 and CTGF	0.3478	No
Galectin-7 and CEACAM1	>0.9999	No
Galectin-7 and MIF	>0.9999	No
Galectin-7 and S100A7	>0.9999	No
CTGF and CEACAM1	>0.9999	No

CTGF and MIF	0.1310	No
CTGF and S100A7	>0.9999	No
MIF and CEACAM1	>0.9999	No
MIF and S100A7	>0.9999	No
CEACAM1 and S100A7	>0.9999	No

Table 3.14. Secreted protein comparisons with Bonferroni's correction. GBM TM samples were scored for presence or absence of each target protein and correlations between pairs were assessed using Fisher's exact test. Bonferroni's correction was performed to give an adjusted significance level of 0.0018.

The same data were then analysed using the Benjamini-Hochberg correction, which is less conservative than the Bonferroni method. This method controls the FDR, which is the percentage of values that may be false positives. The false discovery rate was set at 0.05 for this analysis. This correction also indicated that no correlations were significant (Table 3.15).

In conclusion, correlations between secreted proteins were likely not significant, and no secretory profiles were clearly identifiable in this dataset.

Table 3.15. Secreted protein comparisons with Benjamini-Hochberg correction

Correlation	p-value (Fisher's exact test)	Rank	Critical value	Significance
Galectin-4 and CEACAM1	0.0119	1	0.001785714	No
Galectin-1 and MIF	0.0582	2	0.003571429	No
Galectin-1 and galectin-3	0.1310	3	0.005357143	No
CTGF and MIF	0.1310	4	0.007142857	No
Galectin-3 and galectin-7	0.2609	5	0.008928571	No
Galectin-1 and CEACAM1	0.2688	6	0.010714286	No
Galectin-1 and galectin-7	0.3478	7	0.0125	No
Galectin-7 and CTGF	0.3478	8	0.014285714	No
Galectin-3 and CTGF	0.3690	9	0.016071429	No
Galectin-3 and galectin-4	0.4625	10	0.017857143	No
Galectin-4 and MIF	0.4625	11	0.019642857	No
Galectin-1 and galectin-4	>0.9999	12	0.021428571	No
Galectin-1 and CTGF	>0.9999	13	0.023214286	No
Galectin-1 and S100A7	>0.9999	14	0.025	No
Galectin-3 and CEACAM1	>0.9999	15	0.026785714	No
Galectin-3 and MIF	>0.9999	16	0.028571429	No
Galectin-3 and S100A7	>0.9999	17	0.030357143	No
Galectin-4 and galectin-7	>0.9999	18	0.032142857	No
Galectin-4 and CTGF	>0.9999	19	0.033928571	No

Galectin-4 and S100A7	>0.9999	20	0.035714286	No
Galectin-7 and CEACAM1	>0.9999	21	0.0375	No
Galectin-7 and MIF	>0.9999	22	0.039285714	No
Galectin-7 and S100A7	>0.9999	23	0.041071429	No
CTGF and CEACAM1	>0.9999	24	0.042857143	No
CTGF and S100A7	>0.9999	25	0.044642857	No
MIF and CEACAM1	>0.9999	26	0.046428571	No
MIF and S100A7	>0.9999	27	0.048214286	No
CEACAM1 and S100A7	>0.9999	28	0.05	No

Table 3.15. Secreted protein comparisons with Benjamini-Hochberg correction. GBM TM samples were scored for presence or absence of each target protein and correlations between pairs were assessed using Fisher's exact test with the Benjamini-Hochberg correction.

It was then hypothesised that changes in protein secretion upon the transition to *in vitro* growth may reveal GBM profiles that cannot be distinguished based on TM profiles alone; these data are summarised in Table 3.16. This analysis was limited to the 16 tumours for which paired TM and CS samples were available. For this analysis, tumours that lost or gained expression were included under the downregulated and upregulated groups, respectively.

No secretory profiles were obvious from this dataset either. However, all tumours that gained galectin-7 expression after *in vitro* growth also upregulated galectin-1. Furthermore, the four tumours that maintained S100A7 expression *in vitro* downregulated galectin-3 and did not express galectin-7 at any stage (Figure 3.35).

While these analyses did not elucidate the presence of any secretory profiles, it cannot be concluded that they are absent; however, given the very heterogenous nature of GBM, it is entirely possible that such profiles simply do not exist.

Table 3.16. Changes in protein secretion upon transition to *in vitro* growth

MIF	Either up- or downregulated
Galectin-1	Either up- or downregulated
Galectin-3	Either downregulated or absent
Galectin-4	Either downregulated or absent
Galectin-7	Either upregulated or absent
CTGF	Either downregulated or absent
CEACAM1	Either downregulated or absent
S100A7	Either downregulated or no change

Table 3.16. Changes in protein secretion upon transition to *in vitro* growth.

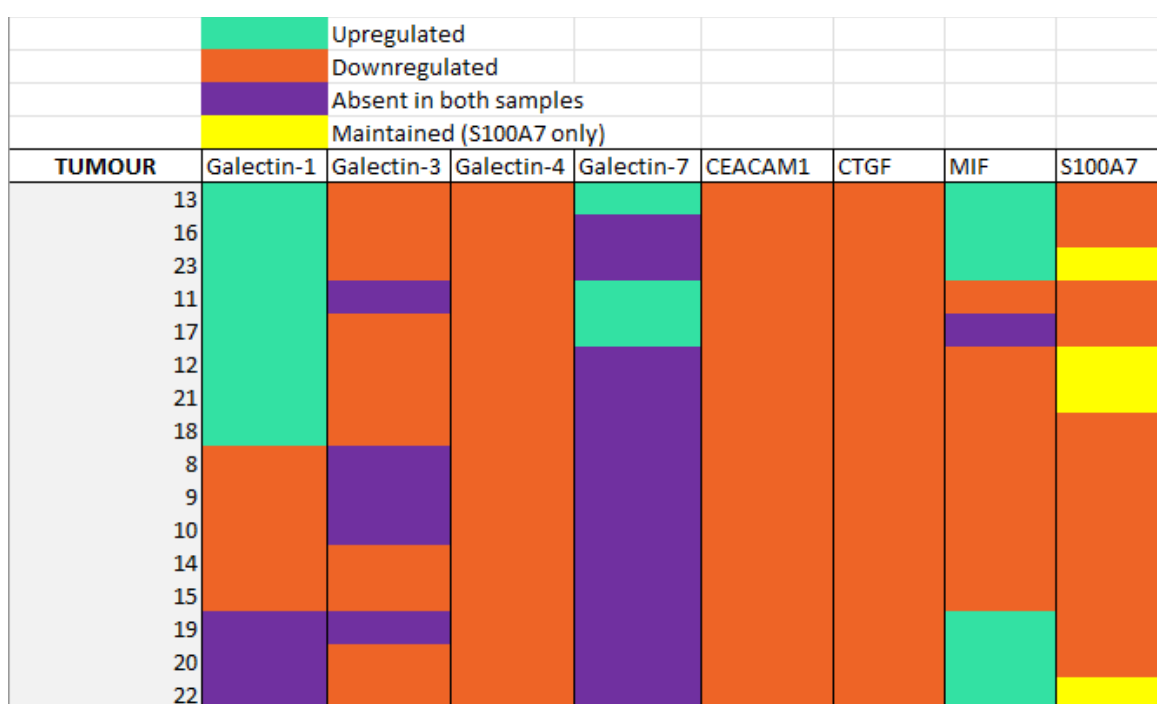


Figure 3.35. Secretion patterns of target proteins in 23 paired GBM transport media and culture supernatant. Paired TM and CS from 16 GBM were analysed by ELISA for the presence of target proteins. Tumours were categorised as showing either upregulation, downregulation, maintenance or absence of each protein. Secretory patterns were not readily apparent.

3.3. Discussion and conclusions

3.3.1. Immune effector presence and activity

This series of experiments aimed to identify potential immunosuppressive proteins and differential regulation of immunity pathways in mass spectrometry data of molecules secreted by 30 GBM.

Pathway analysis revealed a high presence of immune regulation pathways within the tumour. Both adaptive and innate immunity were clearly represented in the data, and while innate immunity is outside the scope of this project, it may be of interest to future researchers. In terms of adaptive immunity, processes related to T-cells, B-cells and APCs were observed, providing circumstantial evidence of anti-tumour activity. The presence of class-switched antibodies was of particular interest, as B-cells are rarely studied in GBM; furthermore, the presence of IL-12-related pathways suggested some Th1-mediated immunity. While antigen presentation-related pathways were also apparent, examination of polarisation markers revealed that TAMs may adopt a more M2-like phenotype. Overall, analysis of Gkanatsiou's preliminary mass spectrometry data implied some level of basal anti-tumoural immunity was present within GBM. Highly-concordant upregulation of multiple immunity-related pathways was observed in both mass spectrometry data and publicly available lists of DEGs associated with target protein mRNA, showing that these processes were consistent across different cohorts using different analyses.

3.3.2. Immune checkpoints

Mass spectrometry data was examined for the presence of ligands for ICs TIM-3, PD-1 and CTLA-4. PD-1 and its ligands PD-L1 and PD-L2 were not observed; however, it is widely understood that PD-1/PD-L1 signalling is an important immune checkpoint molecule in GBM (Liu et al., 2013; Berghoff et al., 2015; Nduom et al., 2016); so is TIM-3, which correlates with poor prognosis and may be widely expressed, as well as more highly expressed than PD-1 (Zhang et al., 2020; Ni et al., 2022; Guo Q et al., 2022).

TIM-3 has been found to be upregulated on TILs compared to PBMCs from glioma patients, and correlated with higher grade (Liu et al., 2016). Later authors found that TIM-3 presence correlates with the mesenchymal subtype of GBM, reduced survival and reduced T-cell anti-tumoural immunity; they noted a seeming redundancy between TIM-3- and PD-1-mediated immunosuppression (Li et al., 2017b). While much attention has been given to the primary TIM-3 ligand galectin-9, which was absent in Gkanatsiou's preliminary data, the ligands CEACAM1 and HMG1 were present. GBM may exploit the multiple ligands of this IC to suppress T-cell-mediated immunity.

Overall, an initial analysis of molecules secreted by 30 bulk GBM suggested that, while subject to high levels of suppression, TILs may still retain some basal function: a finding which offers hope for the potential of improved prognosis if intratumoural suppression could be alleviated. Additionally, these data suggested that the TIM-3 immune checkpoint may be a key player in GBM immunosuppression.

3.3.3. Relevance of galectins to GBM

3.3.3.1. Galectins – an overview

Once the likely presence and function of TILs was identified, proteins that may suppress their intratumoural activity were considered. Galectins – of which four were present – were considered to be valid candidates for further study, as these small soluble carbohydrate-binding proteins are known to have varied roles in tumour growth and immunosuppression. Additionally, CEACAM1, S100A7 and CTGF – three further proteins with poorly-characterised roles in GBM immunomodulation – were selected for further study.

All galectins have a carbohydrate recognition domain (CRD) and can be further divided into prototypic (one CRD), tandem (two CRDs connected by a linker) or chimeric members. Galectins lack a conventional export sequence, but can be found in various intracellular compartments as well as the cell surface and extracellular space. Gal-1, a prototype galectin that dimerises at concentrations over 10 μ M (Perillo et al., 1995), was the first to be discovered. Galectin-3 is the only chimeric galectin, having a single CRD and a long N-terminal tail. It appears to exist primarily as a monomer up to 45 μ M, with a small fraction of pentamers, the formation of which is mediated through the tail (Ahmad et al., 2004). Gal-4 is a tandem galectin and is readily able to crosslink glycosylated molecules; gal-7 is a prototypic member which can dimerise. For review of galectins in glioma biology, see Le Mercier et al., 2010; for review of galectin biology, see Thiemann and Baum, 2016.

3.3.3.2. Galectin-1 in GBM

Gal-1 has been widely studied in immunology and various solid tumours, including glioma. Gal-1 is generally regarded as a poor prognostic indicator in GBM patients (Camby et al., 2001). Intratumorally, it is present in both the perivascular region and the tumour bulk (Camby et al., 2001; Chou et al., 2018); it is also elevated in patient sera, which has been proposed as a biomarker (Verschuere et al., 2013). Glioma-derived gal-1 has tumourigenic effects and its involvement in chemotherapy resistance has been documented for over a decade, as has its upregulation by temozolomide – the standard chemotherapy for GBM patients (Le Mercier et al., 2008). Murine experiments suggest that gal-1 promotes glioma cell invasiveness (Toussaint et al., 2012) and its knockdown (KD) improves survival (Verschuere et al., 2014).

However, a lot of the interest around gal-1 in GBM, especially for this project, pertains more specifically to its immunosuppressive effects. It was documented as early as 1995 that dimeric gal-1 can induce activated T-cell apoptosis (Perillo et al., 1995), a notion that has persisted since – with the notable caveat that gal-1 dimerises at 10 μ M and serum or secreted concentrations of the protein are often lower. In the ELISA assays, gal-1 was secreted at higher concentrations than every other protein assayed and yet did not reach this threshold (see 3.2.2.2.2.). Thus, apoptosis-inducing concentrations of gal-1, if they existed intratumorally, would likely be small pockets of locally increased concentration within the GBM TME.

Later work identified that Th2 cells appeared uniquely resistant to gal-1-mediated apoptosis as a result of differential cell surface glycosylation. In these experiments,

endogenous gal-1 also reduced Th1 function, showing that both exo- and endogenous gal-1 can exert suppressive functions. *In vivo*, this was found to be important for limiting the severity of inflammation in an EAE (experimental autoimmune encephalitis) model (Toscano et al., 2007). In the same year, gal-1 was found to be upregulated in human T-regulatory cells (Tregs), whose suppressive behaviours could be countered with an anti-galectin-1 antibody *in vitro* and *in vivo* (Garín et al., 2007). Activated T-cell apoptosis was inhibited with an anti-Fas antibody, suggesting that the Fas signalling pathway may be a major means through which gal-1 promotes T-cell apoptosis (Hahn et al., 2004).

Gal-1 also mediates intratumoural immunosuppression through other means. Transcriptional analysis has shown gal-1 expression in GBM to correlate with presence of multiple immune effector phenotypes, including M2 macrophages, and LGALS3, the gene encoding gal-3. Gal-1 KD or knockout (KO) resulted in reduced macrophage infiltration and fewer M2 macrophages (Verschuere et al., 2014; Chen et al., 2019), potentially by suppressing secretion of the chemoattractant CCL2 (Verschuere et al., 2014). It appears from these results that gal-1 exerts effects on a variety of immune effectors and may be important for anti-inflammatory macrophage presence.

Conversely, abrogation of gal-1 function provides benefits beyond the above-mentioned reduction of M2 TAMs. Some authors have noted improved cytotoxic T-cell function and better efficacy of dendritic cell (DC) vaccine therapy following gal-1 KD (Verschuere et al., 2014). Treatment with small interfering RNA (siRNA) against gal-1 improved survival in a murine model of glioma, and may alter the

M1/M2 polarisation ratio in favour of pro-inflammatory macrophages; however, these results were based off the presence of one marker for each phenotype and should be regarded as preliminary. These authors also noted increased presence of effector T-cells, which could also synergise with anti-PD-1 therapy to increased effect (Van Woensel et al., 2017).

Clearly, there are multiple lines along which gal-1 is biologically relevant to GBM immunosuppression. Its KD in various mouse models has shown benefit through various means, and it has been suggested as a target in human studies (for review see Astorgues-Xerri et al., 2014). Gal-1 was selected as an intriguing target for further study in this project.

3.3.3.3. Galectin-3 in GBM

Like gal-1, gal-3 has been quite extensively studied in GBM. Changes in GBM gal-3 expression relative to LGG or normal brain tissue are varied. Some authors have found it to be less widely expressed (Gordower et al., 1999; Camby et al., 2001), whereas others have found it to increase by either protein or mRNA analysis (Bresalier et al., 1997; Neder et al., 2004; Binh et al., 2013; He et al., 2019). It has also been suggested to be a marker of early neoplasms in a rat model (Binh et al., 2013). Regardless, papers on both sides of the aisle, so to speak, have noted a correlation between gal-3 presence and worse prognosis (Camby et al., 2001; He et al., 2019). It is clear that, from the early days of gal-3 research, its relationship to tumour grade has been unclear.

Its intratumoural source is also subject to some debate. Two studies have found gal-3 to be present in a minority of cells in healthy brain tissue, the second of which noted its presence in vascular cells (Gordower et al., 1999; Neder et al., 2004). Camby et al. in their 2001 paper also noted vascular gal-3 as well as a presence in the tumour bulk; other authors described its presence in HGG macrophages and astrocytes (Binh et al., 2013). Yet others have found its expression to be heterogenous: absent in areas of microvascular proliferation but present surrounding pseudopalisading necrosis (Neder et al., 2004). Analysis of a limited number of patients has also suggested that gal-3 is mainly produced by tumour and infiltrating monocytic cells (Zhang et al., 2023). It is possible that gal-3 is subject to high levels of intertumoural heterogeneity, resulting in the discordant results outlined here. It is worth noting that many of these studies used immunohistochemistry, and therefore relied on histological characteristics such as the presence of vasculature

and necrosis alongside cellular morphology to determine likely cellular sources. It appears that no large-scale analysis using multiple markers to identify intratumoural gal-3+ populations has been performed, but it appears likely that this protein has multiple cellular sources.

Gal-3 may play varied roles in tumourigenesis. At least two groups have documented SNPs (single nucleotide polymorphisms) in the gene LGALS3 to be associated with higher glioma grade and reduced survival, or to outright be risk factors for GBM development (Chen et al., 2012; Wang H et al., 2019), implying that gal-3 may be a contributory factor to glioma development or have large relevance to its clinical course. Furthermore, the protein has been associated with radio- and chemotherapy resistance *in vitro* (Wang H et al., 2019) and was upregulated via NFκB and HIF1α in response to hypoxia, which conferred a protective effect during hypoxia and nutrient depletion (Ikemori et al., 2014).

Like gal-1, gal-3 also has documented immunosuppressive effects. Toscano et al., 2007 described gal-3-induced T-cell apoptosis, which – unlike gal-1-mediated apoptosis – was not differential between Th1 and Th2 phenotypes and occurred at concentrations as low as 3μM. Glioma stem cells may use secretion of gal-3 among other means to suppress T-cell proliferation and induce apoptosis (Wei et al., 2010). Non-glioma CD8+ TILs showed impaired TCR-CD8 interaction due to the formation of a gal-3 lattice around the TCR, which inhibited function of tumour antigen-specific TILs (Demotte et al., 2008). Gal-3 also impaired the function of CD4+ T-cells: intracellular gal-3 resulted in inhibition of TCR signalling pathways and promoted TCR downregulation (Chen et al., 2009). It is also entirely possible that both

mechanisms operate in each T-cell subset: an extracellular lattice around the TCR that physically blocks CD8 but permits CD4 interaction appears unlikely, and the intracellular TCR recycling and signalling pathways are not phenotype-specific. Thus, both intra- and extracellular gal-3 may have negative effects on T-cell function.

Gal-3 promotes an M2 macrophage phenotype (Zhang et al., 2023). Gal-3 has been shown to be expressed in human monocytes and upregulated upon differentiation into macrophages; monocytes exposed to inflammatory agents reduced secretion of gal-3, suggesting an inverse relationship between gal-3 and inflammation (Liu et al., 1995). It may also function as a monocyte and macrophage chemoattractant (Sano et al., 2000). It begins to appear that, as well as directly inhibiting T-cell function, gal-3 may have further indirect effects through recruitment of anti-inflammatory macrophages.

And thus, as with gal-1, abrogation of gal-3 function may confer beneficial effects. Blockage of gal-3 has shown to be effective in recovering both CD4+ and CD8+ T-cell function in TILs isolated from various non-glioma tumours, and improved survival in tumour-bearing mice (Demotte et al., 2010). Gal-3-null glioma cells showed impaired growth when implanted into mice (Ikemori et al., 2014), although *in vitro* experiments suggested their invasiveness may not be impaired (Debray et al., 2004). For these reasons, gal-3 was also selected as a priority for further investigation in the course of this project.

3.3.3.4. Galectin-4 and -7 in GBM

Unlike their more well-known counterparts, galectin-4 and -7 have been studied very little in GBM. It appears there is currently no literature available about the role of either protein in GBM, or glioma more broadly. This may be partially due to their reputation as tissue-restricted proteins, and partially due to their rarity: gal-4 positivity was observed in two and gal-7 in one of 23 GBM TM samples. However, both proteins have been studied in the context of other solid tumours.

Gal-4 is primarily of interest in colorectal (CRC) and other gastrointestinal cancers; in many cases, its loss has been associated with disease progression and worsened prognosis – perhaps reflective of increasing deviation from the normal tissue environment. This led to the suggestion that gal-4 functioned as a CRC tumour suppressor by inhibiting the Wnt pathway (Satelli et al., 2011). However, in non-CRC cancers, gal-4 is generally – though not always – elevated, and this appears to be very dependent on cancer subtype rather than tissue of origin. For review of gal-4 in solid tumours, see Kaur, Singh and Kaur, 2023.

Serum gal-4 has been documented to be increased in CRC and breast cancer patients, and may assist metastasis through its promotion of both vascular endothelial cytokine secretion and tumour cell adhesion in *in vitro* assays (Barrow et al., 2011; Chen et al., 2014). Increased gal-4 expression in prostate cancer has also been associated with metastasis (Tsai et al., 2016); GBM rarely metastasises, and this may relate to the rarity of gal-4 in GBM TM.

In pancreatic cancer, gal-4 has been described to be immunomodulatory. It inhibits T-cell infiltration and correlates with exhaustion markers on the T-cell surface; gal-4 KO in a murine model resulted in greater T-cell and macrophage infiltration. Gal-4 interactions with CD3 can induce T-cell apoptosis (Paclik et al., 2008; Lidström et al., 2023). Thus, it is possible that gal-4 – in the minority of primary tumours in which it is present – may synergise with other galectins to further depress T-cell function.

Gal-7 is downstream of tumour suppressor p53, yet in breast, head and neck, some cervical and ovarian cancers, often correlates with metastasis and aggressive behaviour; this may be due to the activity of mutant p53 and the NFκB pathway that is also often active in transformed cells (Campion et al., 2013). Studies examining gal-7 in lymphoma cell lines have suggested another regulatory mechanism exists. In many tumour types, it appears to correlate more directly with the degree of tumoural differentiation than prognosis or grade (for review see Kaur et al. 2016 and references therein). Also like gal-4, gal-7 has been described as a tumour suppressor in gastric cancer, where it does not relate to differentiation (Kim et al., 2013), while correlating with aggressive behaviour in other tumours.

Gal-7 has also been described as immunosuppressive in *in vitro* assays, where it has been described to induce lymphocyte and monocyte apoptosis, and downregulates T-cell cytokine production (Yamaguchi et al., 2013; Labrie et al., 2014). It is possible that gal-7, like gal-4, may synergise with other proteins present in the GBM TME to suppress immune function.

3.3.4. The secretome in LGG and GBM

In a comparison of 23 GBM TM with seven LGG TM, the secretomes of these two clinical entities were not found to be particularly different. Gal-4, -7, CTGF and CEACAM1 were all absent from LGG TM; however, they were present in a minority of GBM TM samples assayed, and the differences in mean concentration did not reach significance. This was also the case for gal-1 and MIF. However, gal-3 – the most widely-expressed protein, present in 17 GBM and six LGG samples – did have a significantly higher concentration in GBM TM ($p=0.0089$). Gal-3 has been suggested as a biomarker in various clinical entities (Hara et al., 2020). If the difference observed here is replicable in a larger dataset, gal-3 could have use as a biomarker for distinguishing LGG from more aggressive gliomas, especially if the difference also extends to serum protein levels.

3.3.5. Conclusions and limitations

The early stages of this research project sought to identify immunomodulatory proteins for further study in TM from 30 GBM tumours. Analysis of mass spectrometry data revealed not only several proteins of interest, but also activity related to adaptive immune effector cells: T-cells, B-cells and macrophages. These data suggested that intratumoural immune effectors likely retain some basal level of activity. Yet this is clearly not sufficient to permit recovery, and thus alleviation of immunosuppression may be of significant benefit.

Mass spectrometry analysis revealed the presence of four members of the galectin family in GBM TM; these were detectable in GBM TM by ELISA, as were additional proteins CTGF, CEACAM1, MIF and S100A7. Detection by ELISA indicated that tumours produced full-length and correctly folded proteins, as stable antibody binding is contingent on recognition of a specific epitope, and thus biological activity can be inferred. By contrast, mass spectrometry is highly sensitive but relies on tryptic digests, thus giving no indication of biological activity, and thus confirmation of this data by ELISA was valuable.

Furthermore, proteins were found to be present in the picogram to nanogram range: a level at which they may be able to exert biological effects on immune effectors. Gal-1 was present in a sizeable minority of GBM TM in the nanogram range, suggesting high levels of secretion *in vivo*. Gal-3 was present at lower concentrations but was found in the majority of TM samples. In combination with the many identifiable galectin binding partners in the mass spectrometry data, it appeared likely that gal-1 and gal-3 are able to exert biological effects within the

tumour. Conversely, gal-4 and -7 were present in a minority of samples, and no binding partners were identifiable. However, given documented synergistic behaviour between some galectins (Weinmann et al., 2018) and these proteins' own immunomodulatory functions, it can be concluded all four members of the family merit further study in terms of how they may contribute to GBM's uniquely suppressive TME. The combined effect of multiple immunosuppressive proteins may be of just as much clinical relevance as the effect of isolated proteins.

Additionally, some of the proteins may support *in vitro* growth. Gal-1 and CTGF were expressed by both GBM cell lines and thus may confer some benefit post-immortalisation; gal-3 was restricted to the U373 line, and gal-4, -7 and CEACAM1 were absent in both, and so these proteins likely did not play any major role in supporting *in vitro* growth. It was not possible to assay cell line supernatants for the presence of S100A7, and so inferences about this protein's role in supporting *in vitro* growth cannot be drawn. Clearly, all the proteins of interest were differentially regulated.

This experimental work had several limitations. Firstly, all samples were collected before 2021, and thus some samples included in the dataset are likely IDH-mutant tumours that today would be classified as HGG. In addition to small sample size, this may have masked secretory profiles that would be identifiable in a larger, more refined dataset. This criticism can also be applied to the mass spectrometry data, and thus any extrapolation from this data to recently-diagnosed GBM patients must be done cautiously or, preferentially, with additional validation in a post-2021 dataset. Additionally, prognostic data for patients from whom the TM samples were

taken was not available, and so patient survival could not be correlated to presence, absence or concentration of immunomodulatory proteins.

However, the analyses in this chapter were sufficient to reveal the presence of conformationally-correct immunomodulatory proteins. Further experiments were then performed to address the questions of their cellular sources, intratumoural distribution and biological effects.

4: INTRATUMOURAL LOCATION OF PROTEINS AND IMMUNE EFFECTOR POPULATIONS

4.1. Introduction

In the previous chapter, seven secreted proteins of interest were identified within preliminary mass spectrometry data from 30 whole GBM tumours (Gkanatsiou, 2022). Further analysis revealed that all proteins were detectable in GBM transport media (TM) by enzyme-linked immunosorbent assay (ELISA), suggesting that they may be able to exert biological functions within the tumour. These immunomodulatory proteins may play a role in GBM immunosuppression; in light of the multiple immune effector function-related pathways identified in mass spectrometry and messenger RNA sequencing data, this merited further investigation. Having determined their presence, further experiments aimed to examine the intratumoural location of these proteins and document their potential interactions with immune effectors.

To this end, frozen tissue sections from 50 GBM tumours were examined for the presence of target proteins and tumour-infiltrating leukocytes (TILs) using immunofluorescence (IF) staining and confocal microscopy. This dataset allowed identification of intratumoural location and cellular source of target proteins, as well as any interactions with TILs. In addition, it allowed for a large-scale overview of TIL distribution and cell-cell interactions. These results were intended to provide insight into immune effector interactions and behaviour in the GBM tumour microenvironment (TME). It was hoped that this dataset would not only elucidate

new avenues for alleviating GBM immunosuppression, but as some of the proteins of interest were also detectable in low-grade glioma (LGG) TM, that it would have broader clinical relevance.

4.2. Results

4.2.1. Characterisation of immune effectors and vasculature

4.2.1.1. Preliminary work

An antibody panel was designed to identify TIL populations. Anti-CD3 conjugated to AlexaFluor 647 (henceforth referred to as anti-CD3) and anti-CD19 conjugated to fluorescein isothiocyanate (FITC) (anti-CD19) were used for direct staining of these surface markers, which should be sufficient to identify T- and B-cells respectively. For macrophages, an unconjugated anti-CD68 antibody raised in rabbit and a rhodamine red-X-conjugated (RRX) anti-rabbit secondary antibody were used. 4'6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Details of these antibodies are given in Table 4.01.

Table 4.01. Antibody panel for detection of TIL populations

Specificity	Cell type	Fluorophore	Host	Dilution	Clone
CD3	T-cell	AlexaFluor 647	Mouse	1:20	UCHT8
CD19	B-cell	FITC	Mouse	1:20	4G7
CD68	Macrophage	N/A	Rabbit	1:1000	Polyclonal
Rabbit	N/A	RRX	Donkey	1:200	Polyclonal

Table 4.01. Antibody panel for detection of TIL populations.

Positive controls were performed using formalin-fixed paraffin-embedded (FFPE) human tonsils to detect macrophages, T-cells and B-cells. Negative controls were performed using frozen, methanol-fixed U251 cells, which do not express any of the above markers.

A tonsil section stained with anti-CD3, -CD19 and -CD68 is shown in Figure 4.01. CD3+ cells were clearly visible in this section, indicated by arrows. B-cells and macrophages were also identifiable in tonsil sections (Figure 4.02). CD19 staining was weak against comparatively high background but identifiable; arrows indicate a B-cell (yellow) and a macrophage (red).

Methanol-fixed U251 cells were used to establish negative controls for anti-CD3 - CD19 and -CD68, shown in Figure 4.03 panel A, B and C respectively. Finally, a secondary-only control for the anti-rabbit RRX-conjugated secondary is shown in Figure 4.04. As no positivity for any marker was visible, the protocol was considered sufficient to distinguish TILs from other populations and suitable for use in GBM tissue sections.

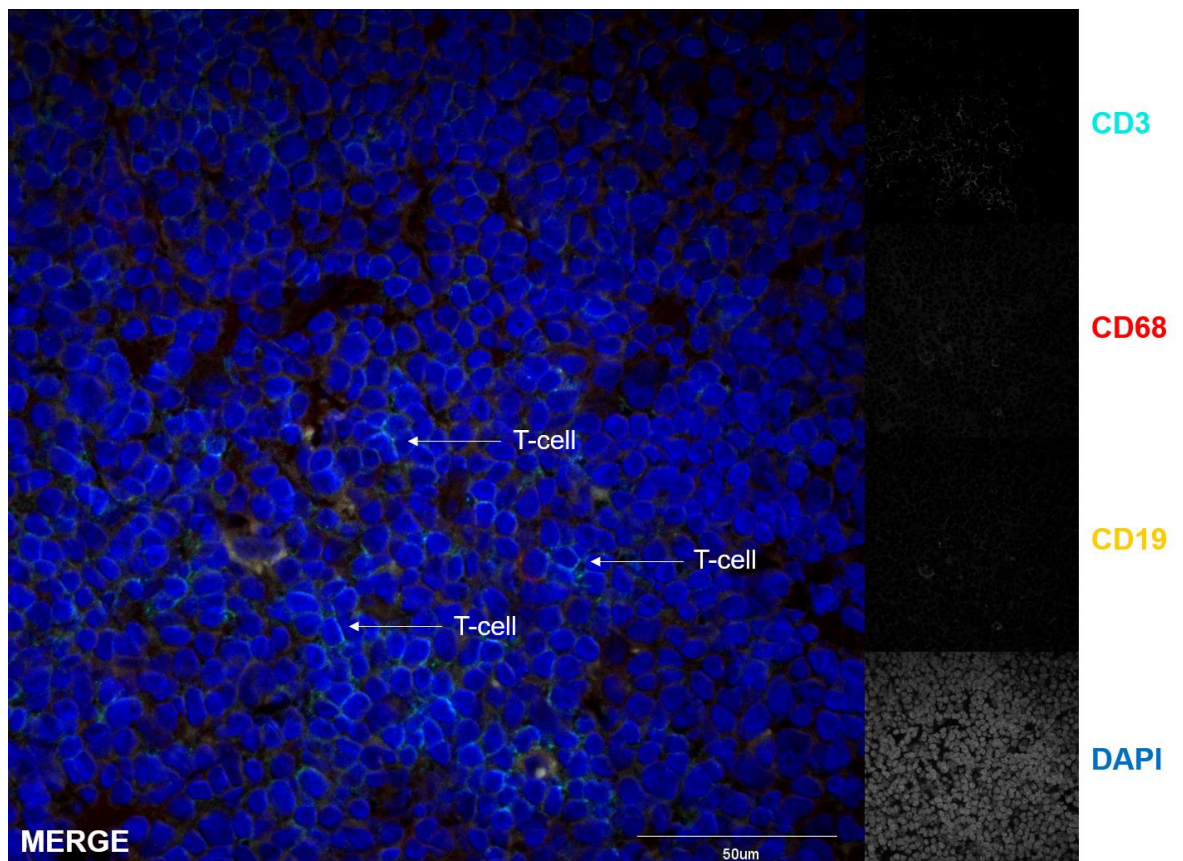


Figure 4.01. CD3 positive control. An FFPE human tonsil section was dewaxed in xylene and antigen retrieval was carried out using five rounds of heating in citrate buffer. Section was permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. The section was stained with anti-CD3, anti-CD19, anti-CD68, RRX-conjugated secondary antibody and DAPI. The section was imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm. Arrows=T-cells.

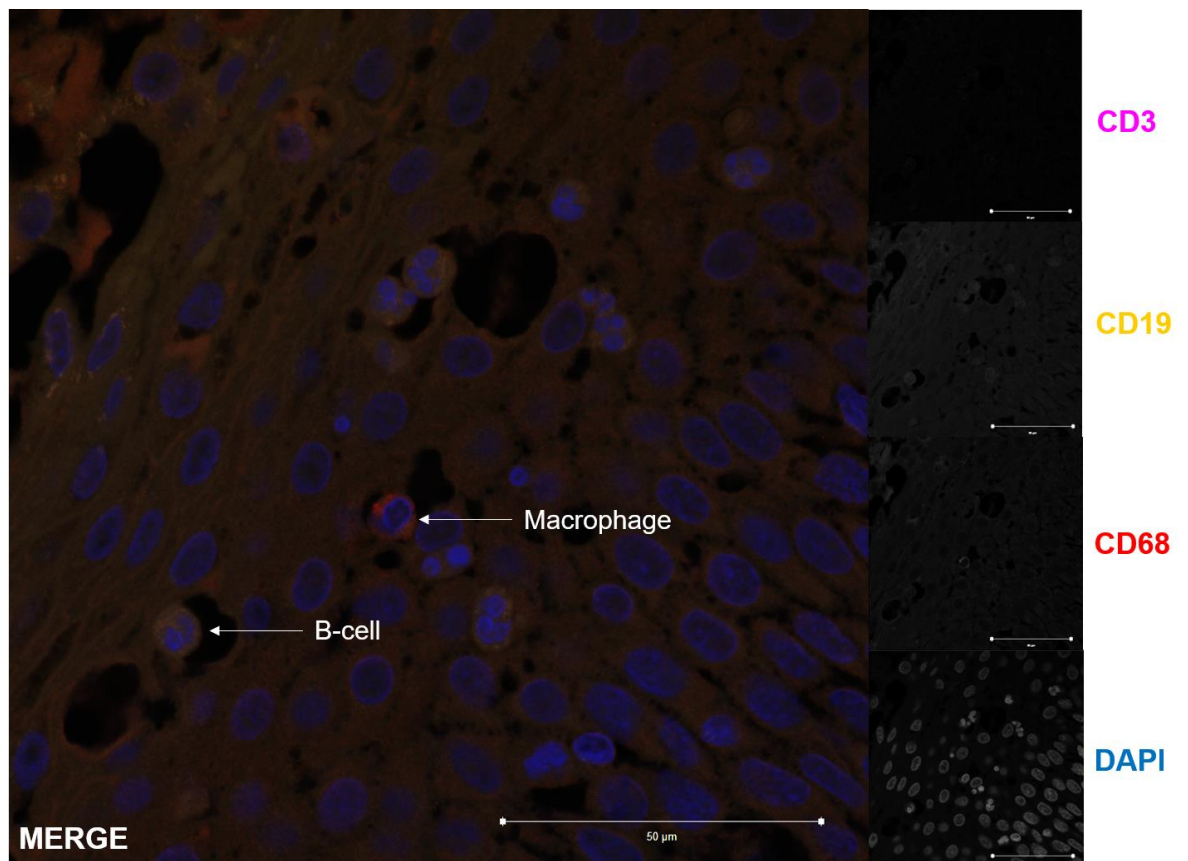


Figure 4.02. CD19 and CD68 positive control. An FFPE human tonsil section was dewaxed in xylene and antigen retrieval was carried out using five rounds of heating in citrate buffer. Section was permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. The section was stained with anti-CD3, anti-CD19, anti-CD68, RRX-conjugated secondary antibody and DAPI. The section was imaged on a Zeiss LSM 880 confocal microscope. B-cell and macrophage are indicated by arrows. Scale bar=50μm.

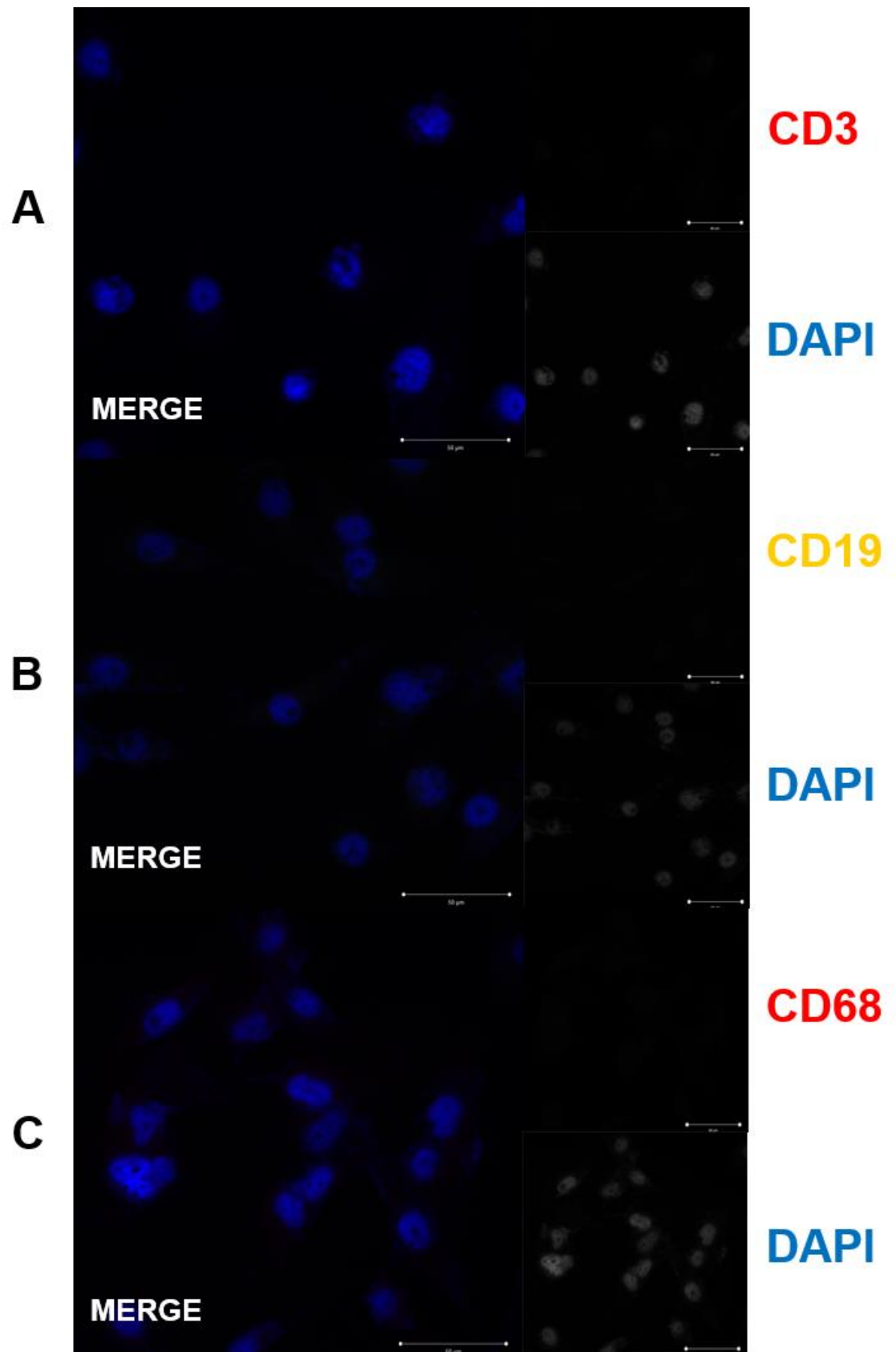


Figure 4.03. CD3, CD19 and CD68 negative controls. U251 cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-CD3 (A), anti-CD19 (B) or anti-CD68 and RRX-conjugated secondary antibody (C) and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm.

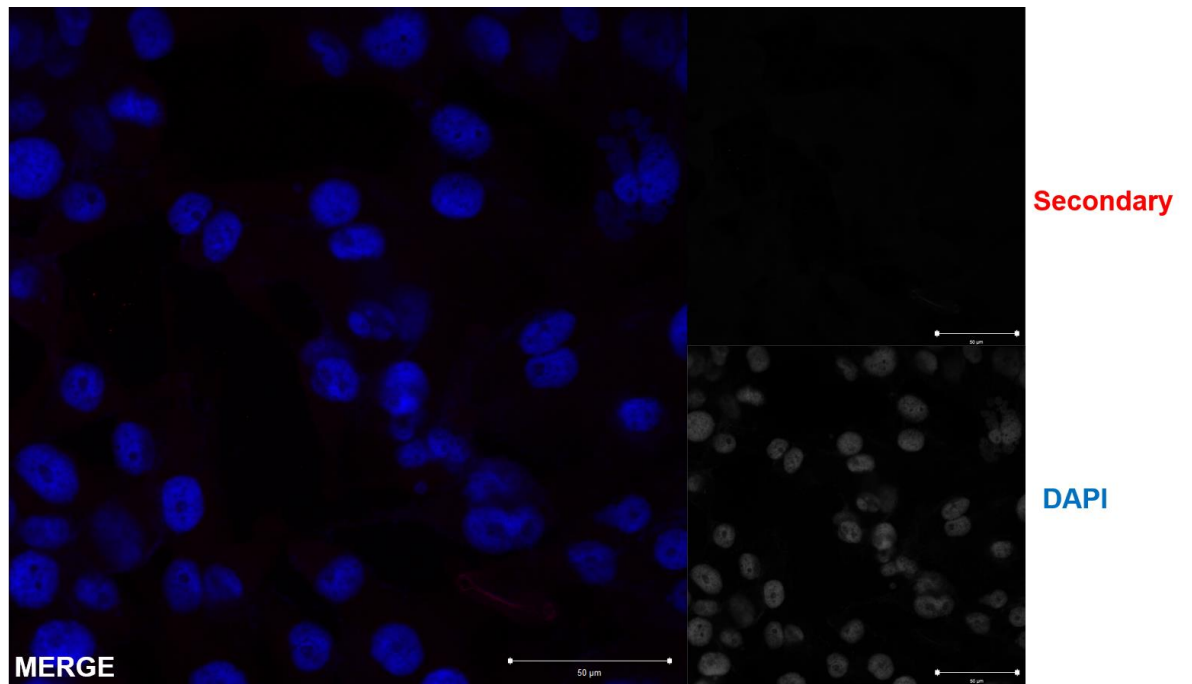


Figure 4.04. RRX-conjugated secondary antibody negative control. U251 cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Cells were stained with RRX-conjugated secondary antibody and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm.

A second panel was designed to characterise intratumoural vasculature. This panel used FITC-conjugated anti-CD34 (anti-CD34) to detect vascular endothelium, and an unconjugated rabbit antibody against LYVE1 (anti-LYVE1) to detect lymphatic endothelium. Details are given in Table 4.02.

Table 4.02. Antibody panel for characterisation of intratumoural vessels

Specificity	Cell type	Fluorophore	Host	Dilution	Clone
CD3	T-cell	AlexaFluor 647	Mouse	1:20	UCHT8
CD34	Endothelial	FITC	Mouse	1:20	WM59
LYVE1	Lymphatic vessel	N/A	Rabbit	1:150	Polyclonal
Rabbit	N/A	RRX	Donkey	1:200	Polyclonal

Table 4.02. Antibody panel for characterisation of intratumoural vessels.

LYVE1 was included in light of the finding (Aspelund et al., 2015; Louveau et al., 2015) that the central nervous system (CNS) contains lymphatic vessels (LVs). It was considered possible that the pro-angiogenic properties of GBM might promote lymph vessel formation, thus facilitating leukocyte infiltration into the tumour; to this end, anti-CD3 was included in the panel. As both anti-CD19 and anti-CD34 used the FITC fluorophore, and anti-LYVE1 and anti-CD68 were raised in rabbit, B-cell and macrophage interactions with vasculature types could not be analysed.

Positive controls for anti-CD34 and anti-LYVE1 were established using methanol-fixed human umbilical cord endothelial cells (HUVECs) and a human tonsil section; these are shown in Figures 4.05 and 4.06 respectively. Negative controls for each used methanol-fixed U251 cells, and are shown in Figure 4.07A (anti-CD34) and 4.07B (anti-LYVE1). U251 cells were negative for both markers; HUVECs expressed the endothelial cell (EC) marker CD34, and LYVE1+ vasculature was visible in the tonsil, demonstrating that these antibodies were able to detect specific vascular populations.

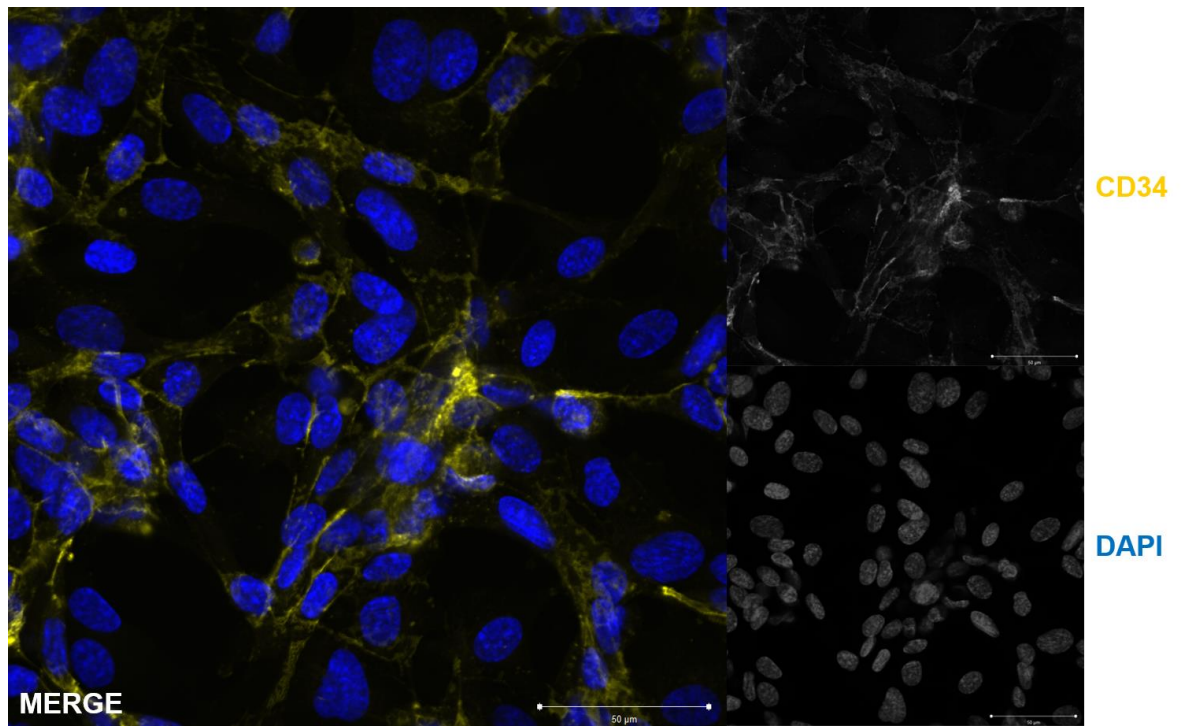


Figure 4.05. CD34 positive control. HUVEC cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were blocked with PBS 1% BSA. The section was stained with anti-CD34 and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

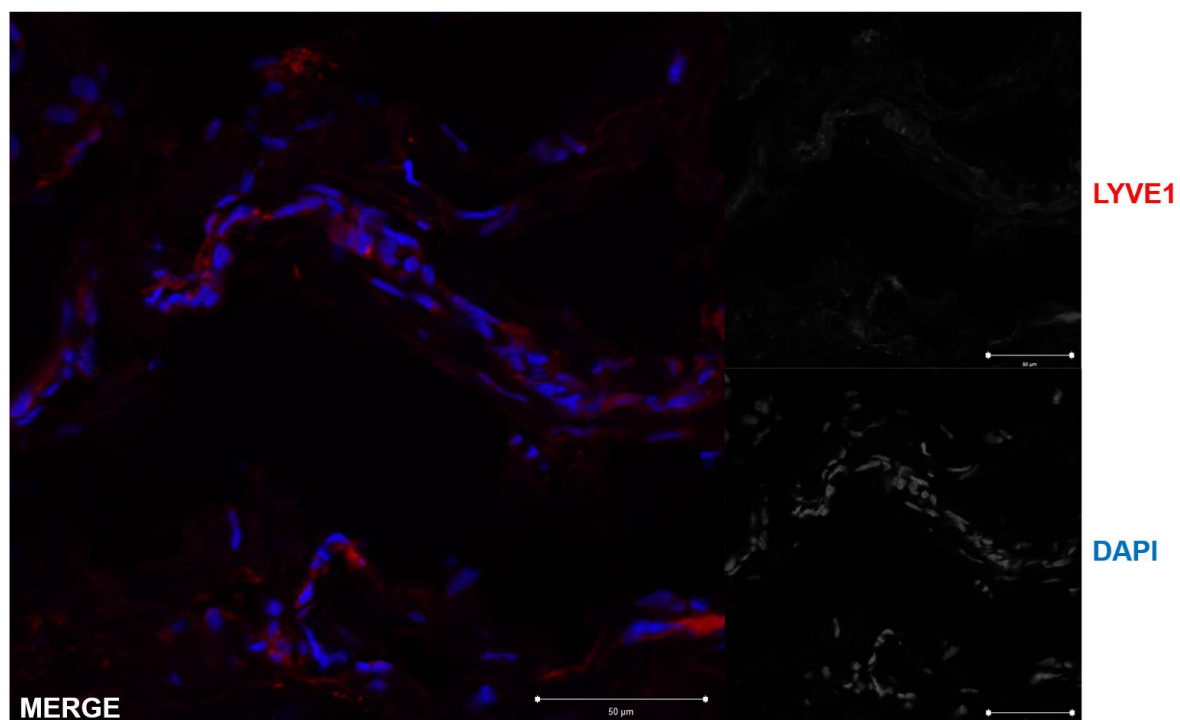


Figure 4.06. LYVE1 positive control. An FFPE human tonsil section was dewaxed in xylene and antigen retrieval was carried out using five rounds of heating in citrate buffer. Section was permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. The section was stained with anti-LYVE1, RRX-conjugated secondary antibody and DAPI. The section was imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

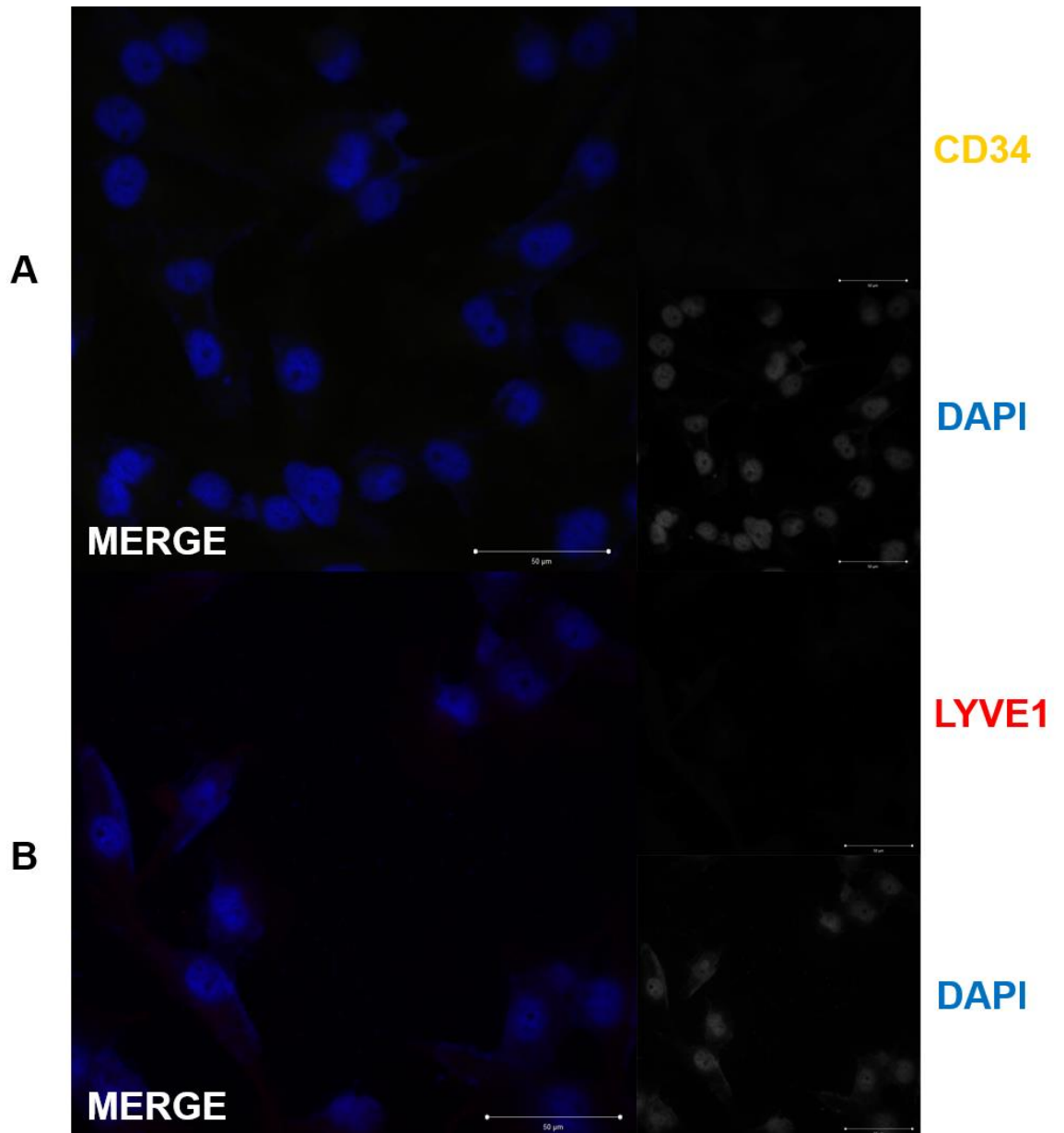


Figure 4.07. CD34 and LYVE1 negative controls. U251 cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-CD34 (A) or anti-LYVE1 and RRX-conjugated secondary antibody (B) and DAPI. Slides were imaged on a Zeiss LSM 880 confocal microscope. Scale bars=50μm.

4.2.1.2. Vascular characterisation

4.2.1.2.1. *Vessel phenotypes*

To examine the phenotype of vessels, sections from a preliminary 26 GBM were stained for CD3, CD34 and LYVE1, in an effort to determine whether the vasculature in these sections had a blood vessel CD34+LYVE- or lymphatic CD34-LYVE1+ phenotype. Additionally, this panel permitted examination of T-cells in these sections and, potentially, their co-occurrence or interactions with vasculature phenotypes. Vessel and cell type counts were not verified by another operator. The number of GBM used in this analysis was limited by their use in other panels.

All 26 GBM were determined to contain vascular structures. When stains were considered in isolation, 24 sections were CD34+ and 23 were LYVE1+. 22 contained both CD34 and LYVE1 staining, two showed only CD34 positivity, one showed only LYVE1 positivity and one was negative for both markers; this may have been an example of vascular mimicry. These data are summarised in Table 4.03. Representative images of the other three phenotypes are shown in Figure 4.08 (CD34+LYVE1+), Figure 4.09 (CD34+LYVE1-) and Figure 4.10 (CD34-LYVE1+).

Table 4.03. Vasculature phenotypic markers observed in 26 GBM

Phenotype	Number of positive GBM	% of positive GBM
CD34+	24/26	92.3
LYVE1+	23/26	88.5
CD34+LYVE1+	22/26	84.6
CD34+LYVE1-	2/26	7.7
CD34-LYVE1+	1/26	3.8
CD34-LYVE1-	1/26	3.8

Table 4.03. Vasculature phenotypic markers observed in 26 GBM. Percentages are given to one decimal place.

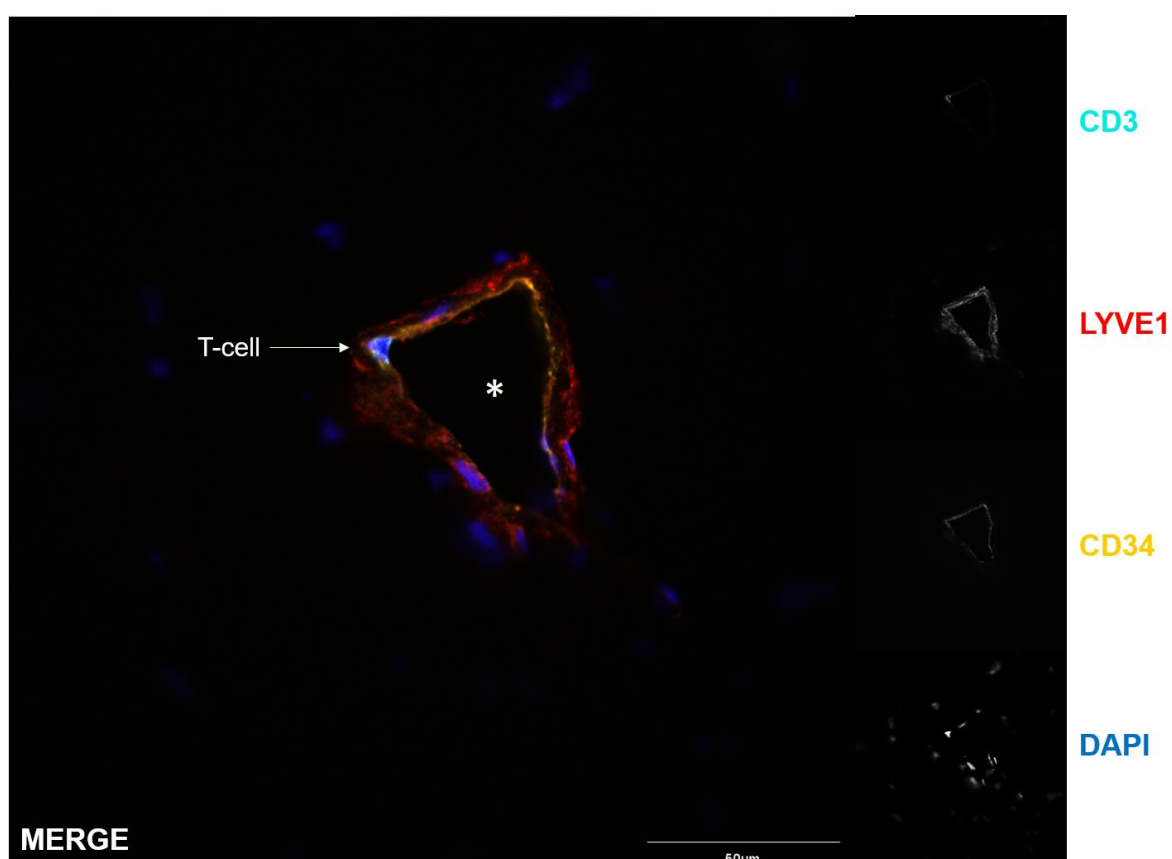


Figure 4.08. Representative image of CD34+LYVE1+ vessel with associated T-cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD34, anti-LYVE1, RRX-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm. Arrow=T-cell. Asterisk=vessel lumen.

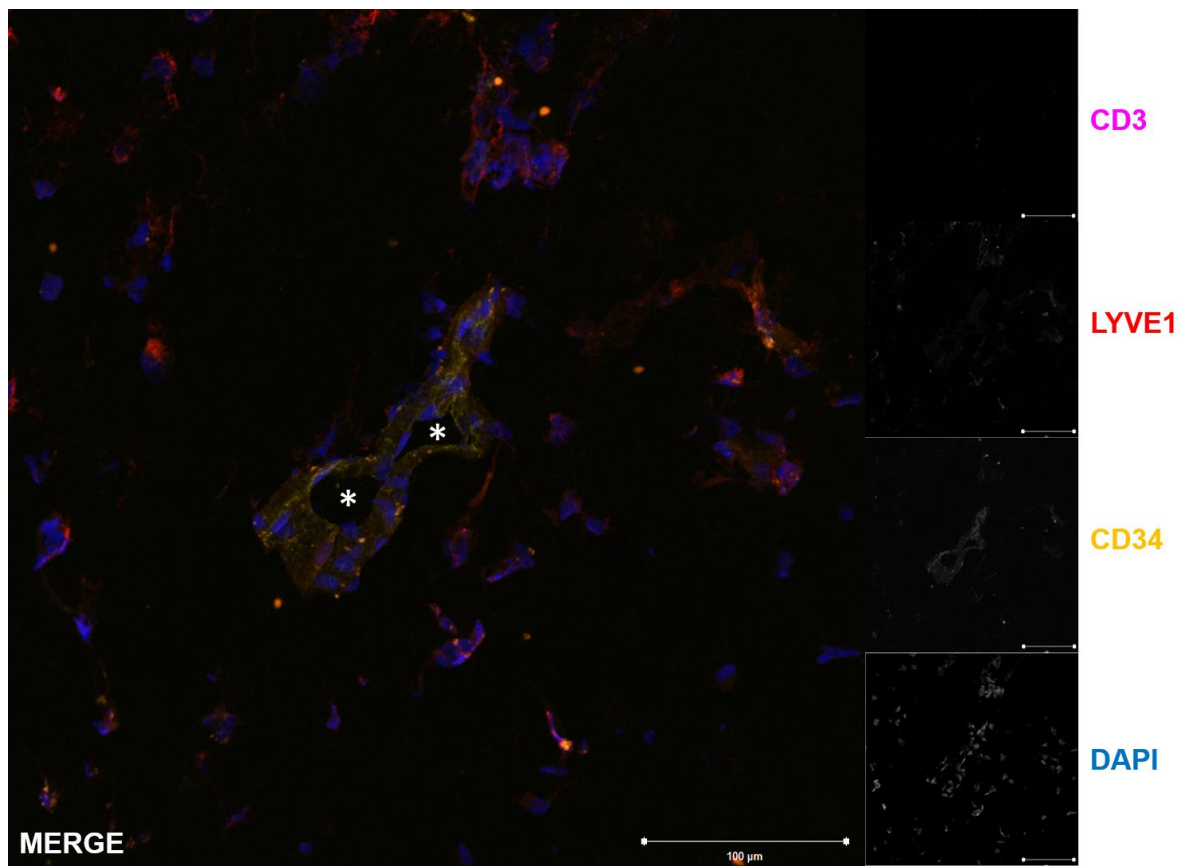


Figure 4.09. Representative image of CD34+LYVE1- vessels. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD34, anti-LYVE1, RRX-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=100μm. Asterisks=vessel lumens.

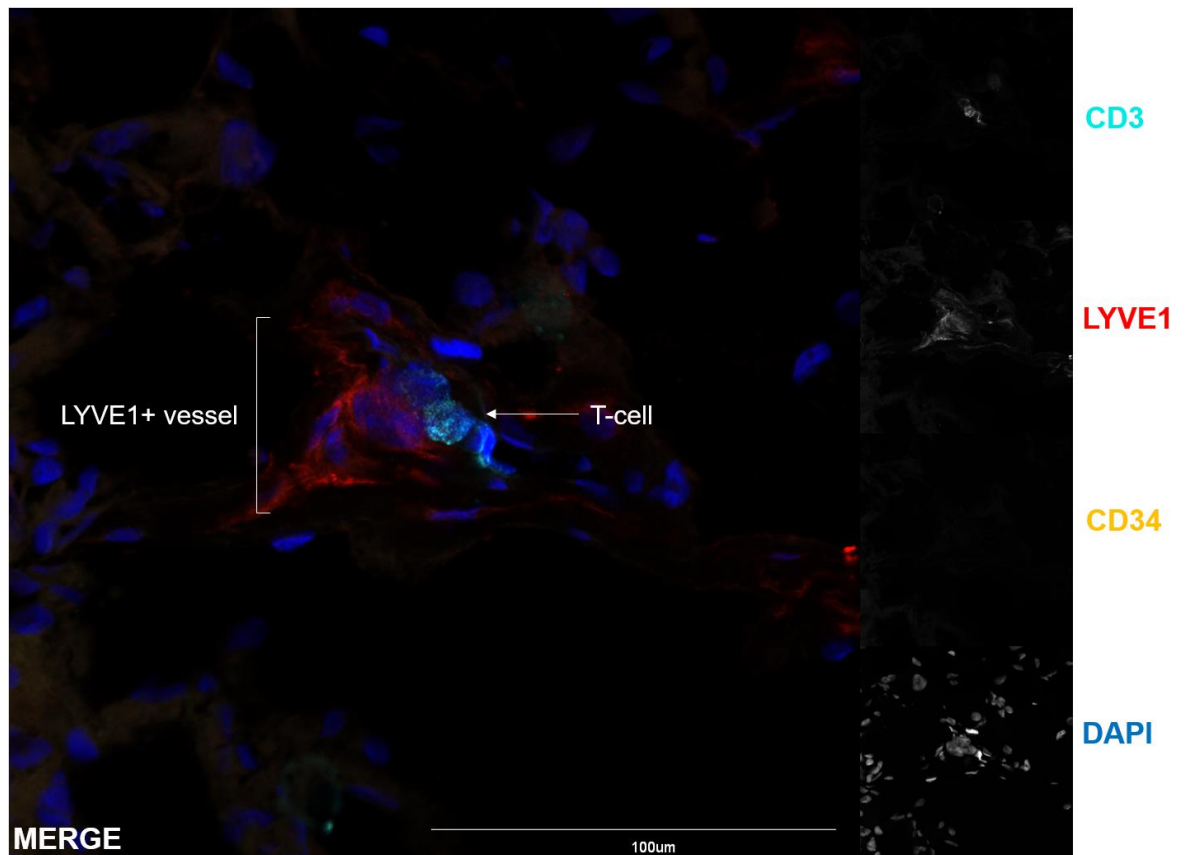


Figure 4.10. Representative image of CD34-LYVE1+ vessel with associated T-cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD34, anti-LYVE1, RRX-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=100µm. Arrow=T-cell. Square bracket=vessel cross-section.

In line with the literature, vasculature was widespread in these sections. CD34 staining, indicating EC presence, was common, which is to be expected. However, the degree of LYVE1 positivity was surprising. Thus, the phenotype of each vessel observed in the 26 sections was categorised.

102 vessels were counted across the 26 sections. A majority – 70 of 102, or 68.6% – expressed both markers, and the next most common phenotype was CD34+LYVE1-, at 21 or 20.6% of vessels. The other two phenotypes were rare, with nine, or 8.8%, of vessels CD34-LYVE1+, and two vascular structures that lacked both markers, accounting for 2.0%. These data are summarised in Table 4.04.

When averaged across all 26 GBM, fewer than three vessels of each phenotype were apparent per tumour. However, there was an order of magnitude between the most (double positive; 2.9 vessels per tumour) and least common (double negative; 0.1 vessels per tumour). When these data were analysed by one-way analysis of variance (ANOVA) with Welch's correction, a significant difference in means ($p < 0.0001$) was apparent; Dunnett's T3 multiple comparisons test identified the significantly diverging pairs of means to be double positive vessels compared to every other phenotype ($p = 0.0002$ for CD34+LYVE1- and CD34-LYVE1-; $p = 0.0003$ for CD34-LYVE1+) (Figure 4.11).

It appeared, from this analysis, that mature blood vessels were common within GBM, that a small proportion of lymphatic vessels may exist, and that most vascular structures displayed markers associated with both vessel types. Vascular mimicry was potentially present, but accounted for a clear minority of vessels in this dataset.

Table 4.04. Phenotypic characterisation of 102 vessels in 26 GBM

Phenotype	Number of vessels	% of vessels	Average vessels per tumour
CD34+LYVE1+	70/102	68.6	2.7
CD34+LYVE1-	21/102	20.6	0.8
CD34-LYVE1+	9/102	8.8	0.3
CD34-LYVE1-	2/102	2.0	0.1

Table 4.04. Phenotypic characterisation of 102 vessels in 26 GBM. Percentages and mean averages are given to one decimal place.

Phenotypic characterisation of 102 vessels in 26 GBM

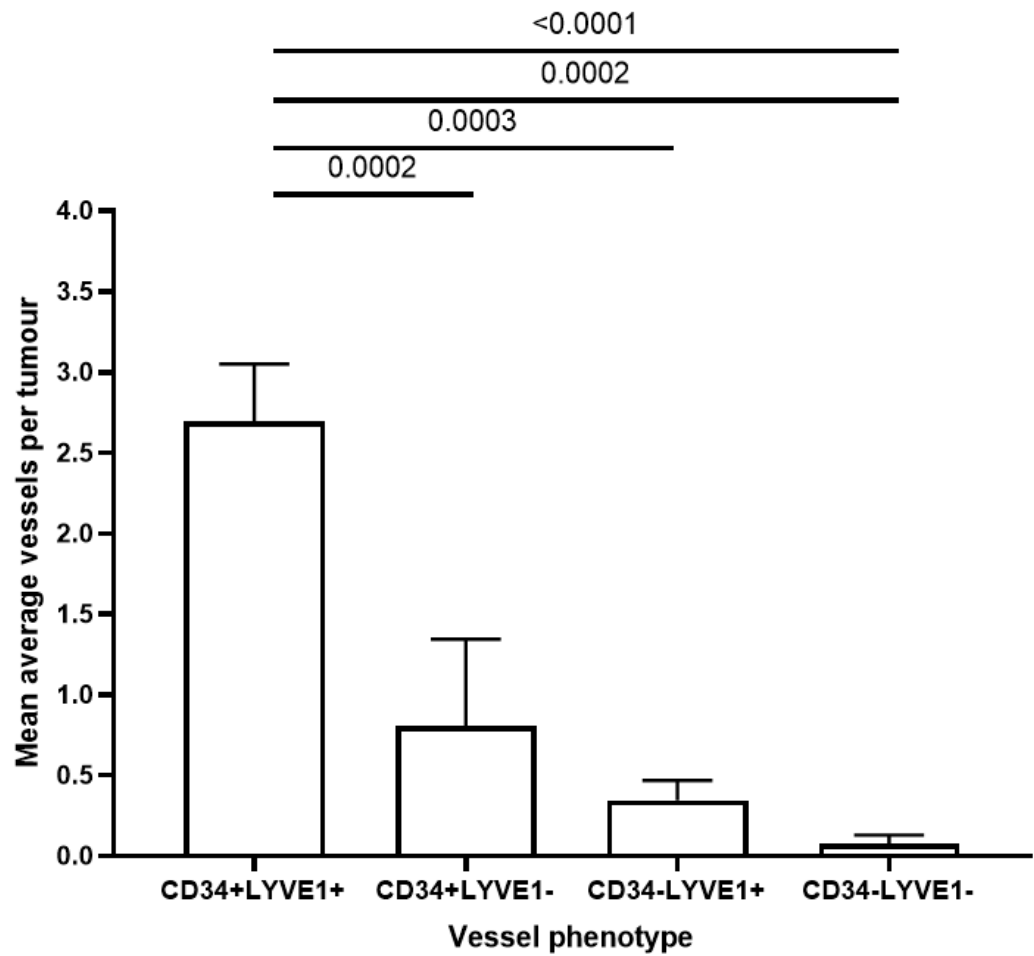


Figure 4.11. Phenotypic characterisation of 102 vessels in 26 GBM. Sections were stained for CD34 and LYVE1 and the mean average number of vessels of each phenotype was graphed. Data were analysed by one-way ANOVA with Welch's correction (upper line) and Dunnett's T3 multiple comparisons test (lower lines). Error bars=standard error of the mean (SEM).

4.2.1.2.2. *T-cell and vessel interactions*

As all 26 GBM were also stained with anti-CD3, the presence of T-cells around vessels of different phenotypes was examined next.

While all 26 GBM were T-cell+, T-cells were only observed in contact with vessels in 18 tumours, across which 33 T-cell associated vessels were apparent. 30, or 90.9%, were double-positive; one double-negative vessel (3.0%) was observed, and two, or 6.1%, were CD34-LYVE1+. Data are summarised in Table 4.05.

When these data were averaged over the 18 GBM, an order of magnitude difference between the most (double positive; 1.6 vessels per tumour) and least prevalent (double negative; 0.1 vessels per tumour) was apparent. A significant difference was found by one-way ANOVA ($p < 0.0001$). Tukey's multiple comparisons test revealed the significantly different groups to be double-positive vessels compared to every other phenotype ($p < 0.0001$ for all) (Figure 4.12). Although ANOVA does not assume equal group sizes, these results may have been affected by the low prevalence of other phenotypes, particularly CD34-LYVE1+ vessels. Only nine vessels of this phenotype were observed, of which two were T-cell-associated. Additionally, these data did not follow a normal distribution according to the Brown-Forsythe test ($p = 0.0047$) but because one column was empty, ANOVA with Welch's correction could not be performed. Tukey's multiple comparisons test assumes a normal distribution, and so these data must be regarded as preliminary and in need of verification in a larger dataset.

Table 4.05. Phenotypic characterisation of 33 T-cell-associated vessels in 18 GBM

Phenotype	Number of vessels	% of vessels	Average vessels per tumour
CD34+LYVE1+	30/33	90.9	1.7
CD34+LYVE1-	0/33	0.0	0.0
CD34-LYVE1+	2/33	6.1	0.1
CD34-LYVE1-	1/33	3.0	0.1

Table 4.05. Phenotypic characterisation of 33 T-cell-associated vessels in 18 GBM. Percentages and mean averages are given to one decimal place.

Phenotypic characterisation of 33 T-cell-associated vessels in 18 GBM

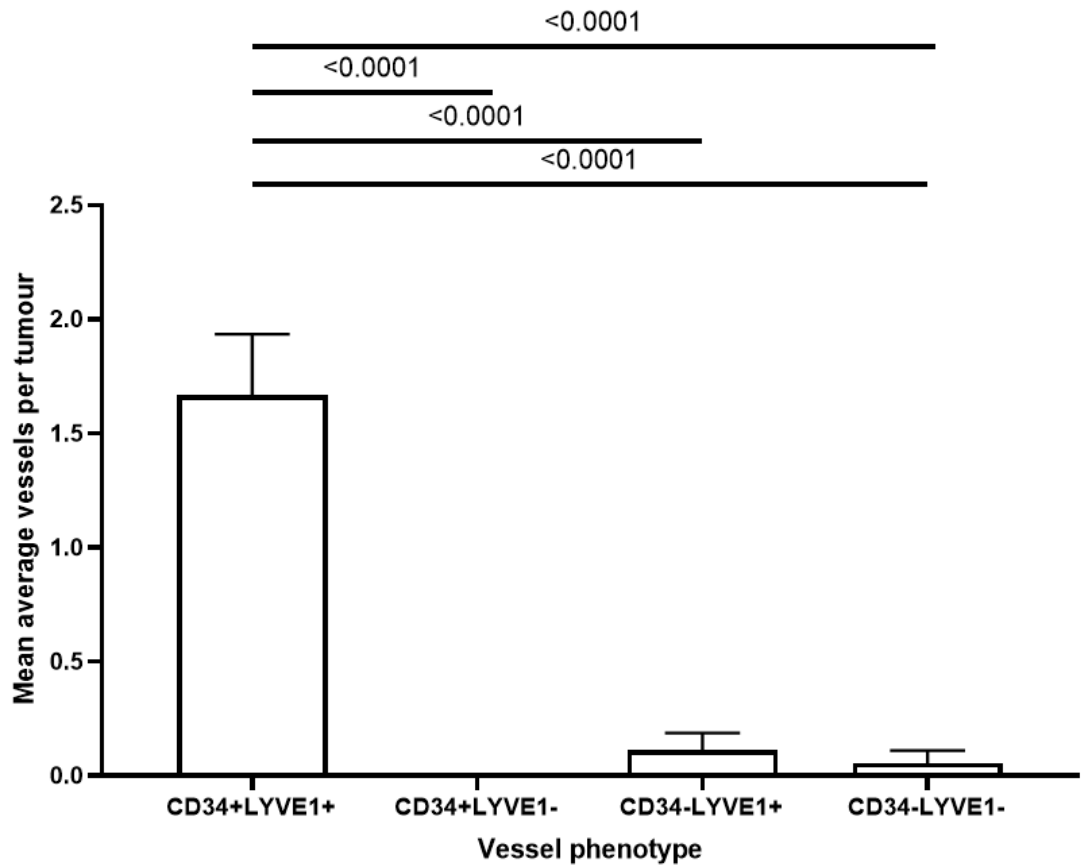


Figure 4.12. Phenotypic characterisation of 33 T-cell-associated vessels in 18 GBM. Sections were stained for CD3, CD34 and LYVE1 and the mean average number of T-cell-associated vessels of each phenotype was graphed. Data were analysed by one-way ANOVA (upper line) and Tukey's multiple comparisons test (lower lines). Error bars=SEM.

4.2.1.3. Distribution of TIL populations

4.2.1.3.1. Overall TIL presence

Following confirmation that the antibodies were suitable for detection of immune effector populations, sections were stained to examine the prevalence and interactions of macrophages, T- and B-cells. Cell type counts were not verified by another operator. Where possible, every tumour was stained for immune effector populations; the number of tumours stained with anti-CD19 was reduced due to financial constraints.

All three cell types were highly represented in the TME. The majority of tumours contained macrophages (72.0%) and T-cells (84.0%); just under half (45.8%) contained B-cells. Additionally, necrosis and vasculature, which could be observed on the basis of morphology, were almost universally present, which was in accordance with the understanding of GBM as a highly necrotic and vascularised tumour. Of particular interest was the discovery that, of the 42 T-cell+ tumours, 11, or 26.1%, contained large aggregates comprising dozens of T-cells, often in the perivascular (PV) region. These results are summarised in Table 4.06. Representative images of a PV T-cell aggregation and tumour bulk (TB) T-cells are shown in Figure 4.13A and B, respectively. PV B-cells and tumour-associated macrophages (TAMs) are shown in Figure 4.14; TB B-cells and TAMs are shown in Figure 4.15.

Table 4.06. Prevalence of macrophages, T- and B-cells, and necrosis and vasculature in 50 GBM

Population of interest	Number of tumours	% of tumours
Macrophage	36/50	72.0
T-cell	42/50	84.0
T-cell aggregation	11/50 (11/42)	22.0 (26.1)
B-cell	22/48	45.8
Necrosis	50/50	100.0
Vasculature	48/50	96.0

Table 4.06. Prevalence of macrophages, T- and B-cells, and necrosis and vasculature in 50 GBM. Tumour sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD19, anti-CD68 and an RRX-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Percentages are given to one decimal place.

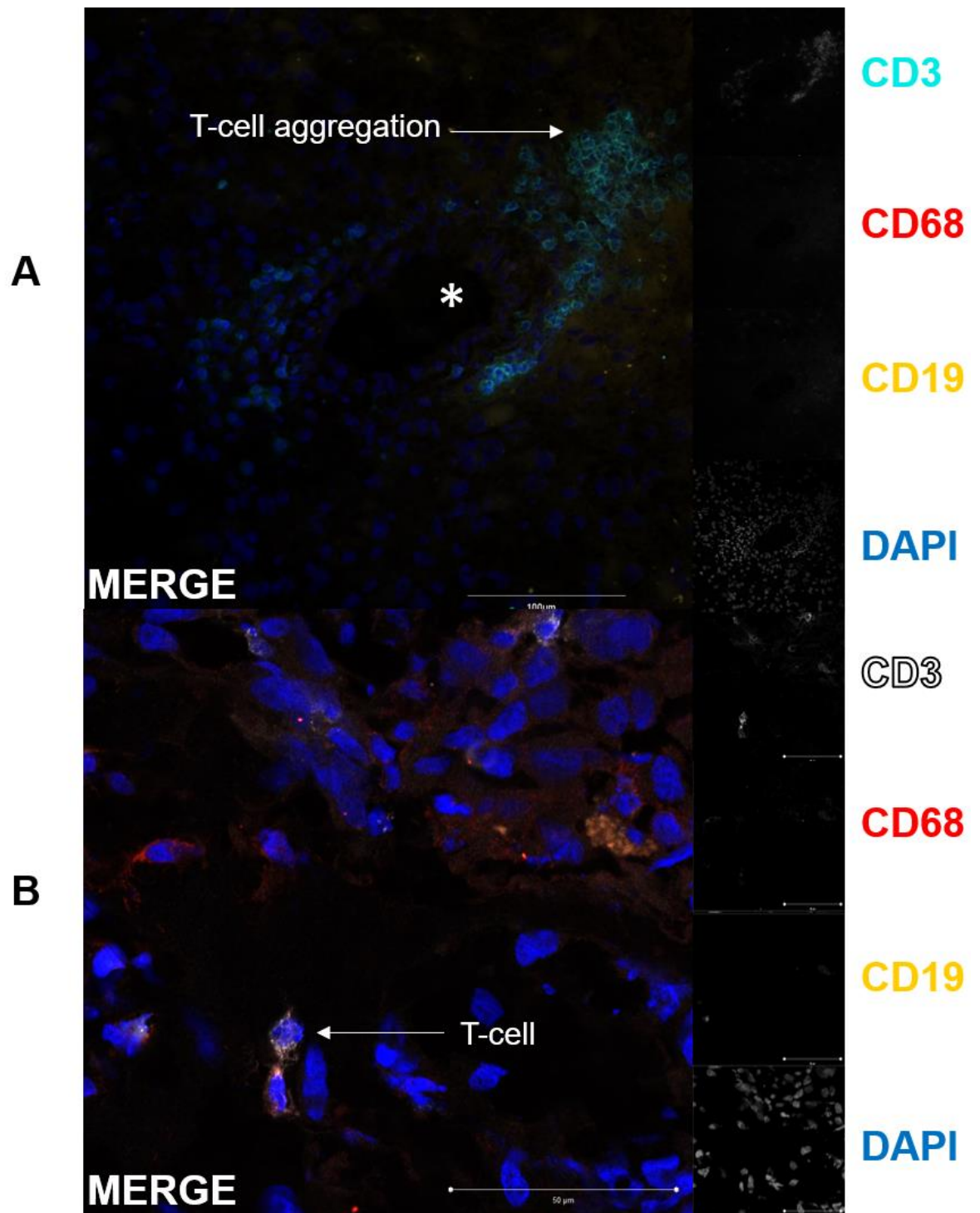


Figure 4.13. Representative images of intratumoural T-cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD19, anti-CD68, RRX-conjugated secondary antibody and DAPI. Sections imaged on a Zeiss LSM 880 confocal microscope. A: PV T-cell aggregation. Scale bar=100µm. Arrow=aggregation. Asterisk=vessel lumen. B: TB T-cells. Scale bar=50µm. Arrow=T-cell.

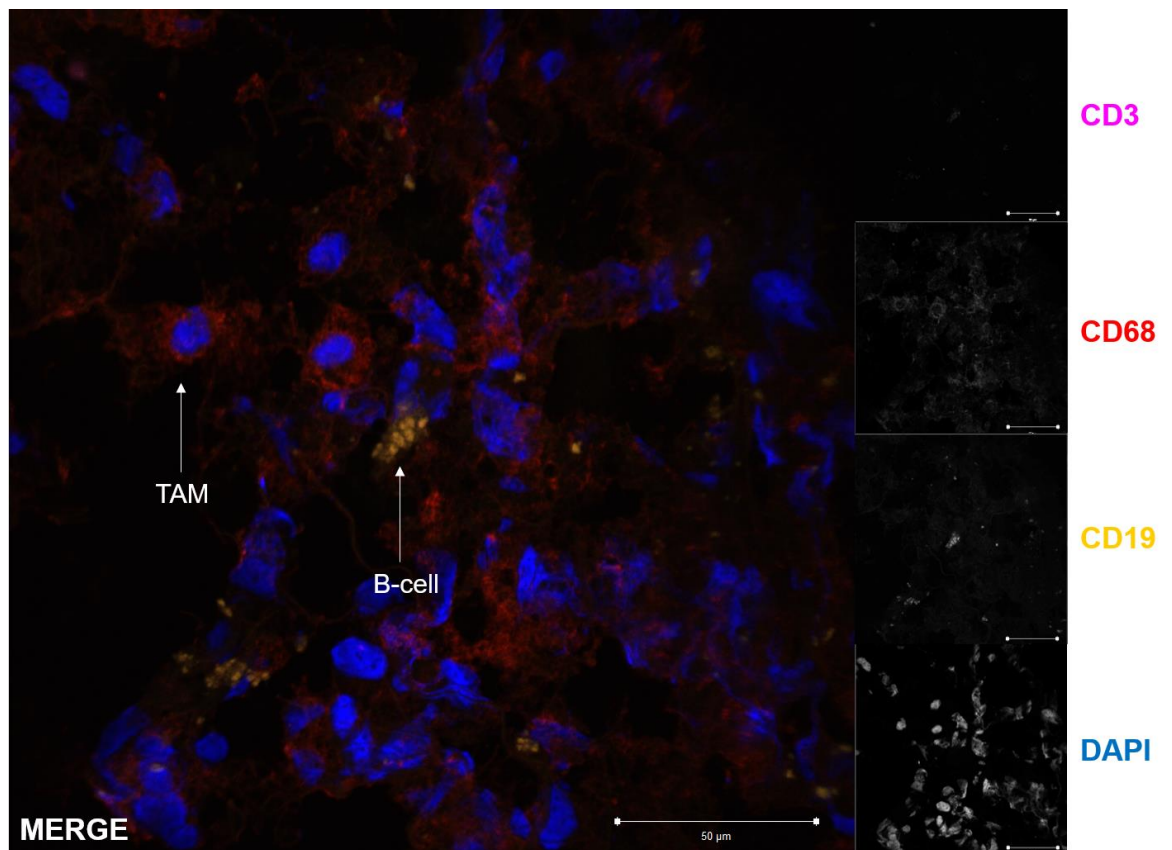


Figure 4.14. Representative image of intratumoural B-cells and TAMs in TB. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD19, anti-CD68, RRX-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrows indicate B-cell and TAM.

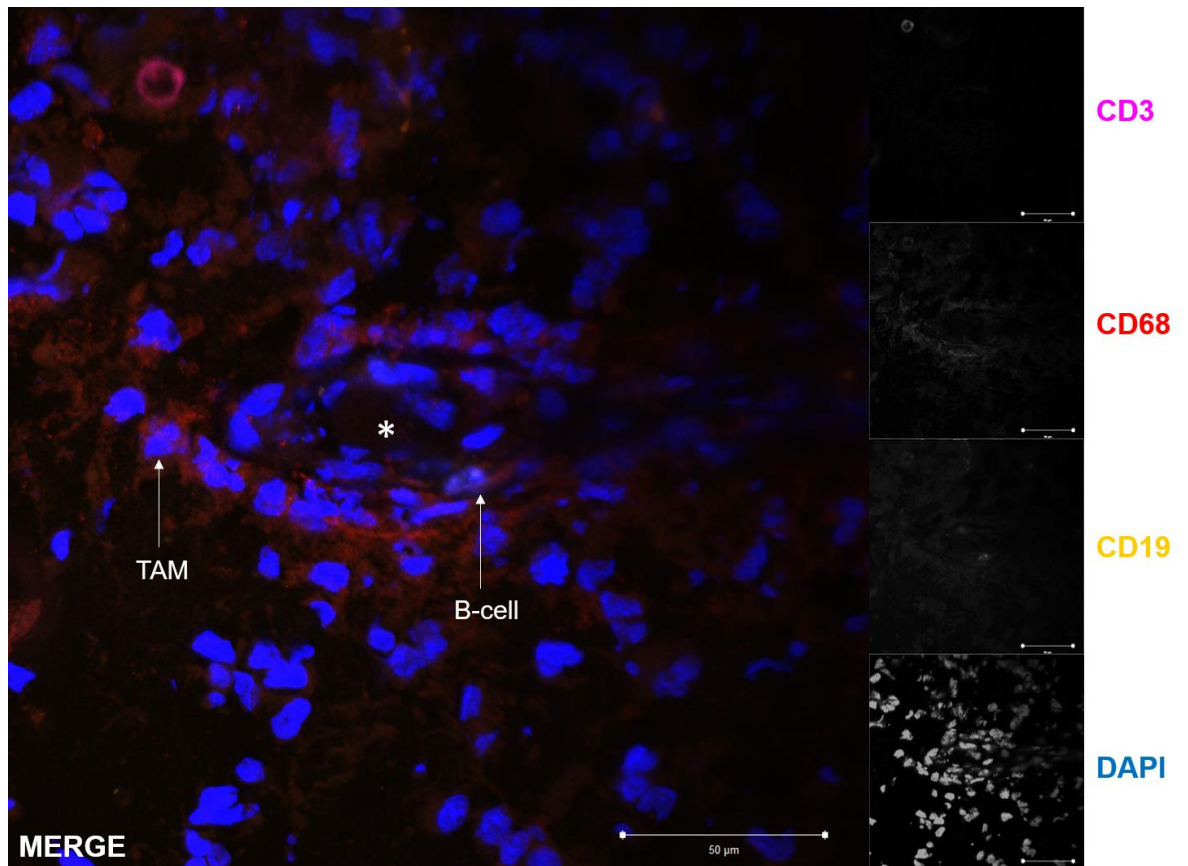


Figure 4.15. Representative image of intratumoural B-cells and TAMs in PVN. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD19, anti-CD68, RRX-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrows indicate B-cell and TAM. Asterisk=vessel lumen.

4.2.1.3.2. Distribution of T-cells

In the above section, it was shown that T-cells could be detected in frozen GBM sections. Next, the intratumoural distribution of T-cells in the 42 T-cell+ GBM was studied. Sections from 4.2.1.5., 4.2.2. and 4.2.3. were also included in this analysis. Cell counts were not verified by another operator.

T-cells were categorised by their location in either the perivascular niche (PVN) or the TB. The PVN was defined as the region within 100µm of a vessel. Images using the 40X and 63X objective lenses were used for this analysis, as images taken using the 10X or 20X objectives had lower resolution, making it difficult to distinguish adjacent T-cells. One exception was made for an image using the 20X objective, in which seven T-cells could be easily distinguished. Concurrently, the number of T-cells in physical contact in each image was recorded, to permit analysis of the size of T-cell clusters across different intratumoural niches.

2235 T-cells were found across the 42 positive GBM, giving a mean average of 53.2 T-cells per positive tumour. The majority of T-cells – 1527, or 68.3% – were located in the PVN, with the remaining 708 or 31.6% in the TB. This trend approached, but did not reach, significance ($p=0.0932$, unpaired 2-tailed t-test with Welch's correction); data are shown in Figure 4.16. Analysis of T-cell clustering found no significant difference in the sizes of T-cell clusters in the PVN versus the TB ($p=0.2237$, unpaired 2-tailed t-test). Clusters were mostly small, with a mean average of 1.8 cells per PV cluster and 1.6 cells per TB cluster. These data are shown in Figure 4.17.

The nearly-significant presence of T-cells in the PVN was of interest. T-cells typically either migrate into the tissue, or exit back into a vessel if their cognate antigen is not present. Pathway analysis results in 3.2.1.1.3. showed T-cell activation-related events such as downstream T-cell receptor (TCR) signalling; however, if T-cells are inhibited to some extent from leaving the PVN, this could reduce the efficacy of anti-tumoural activity.

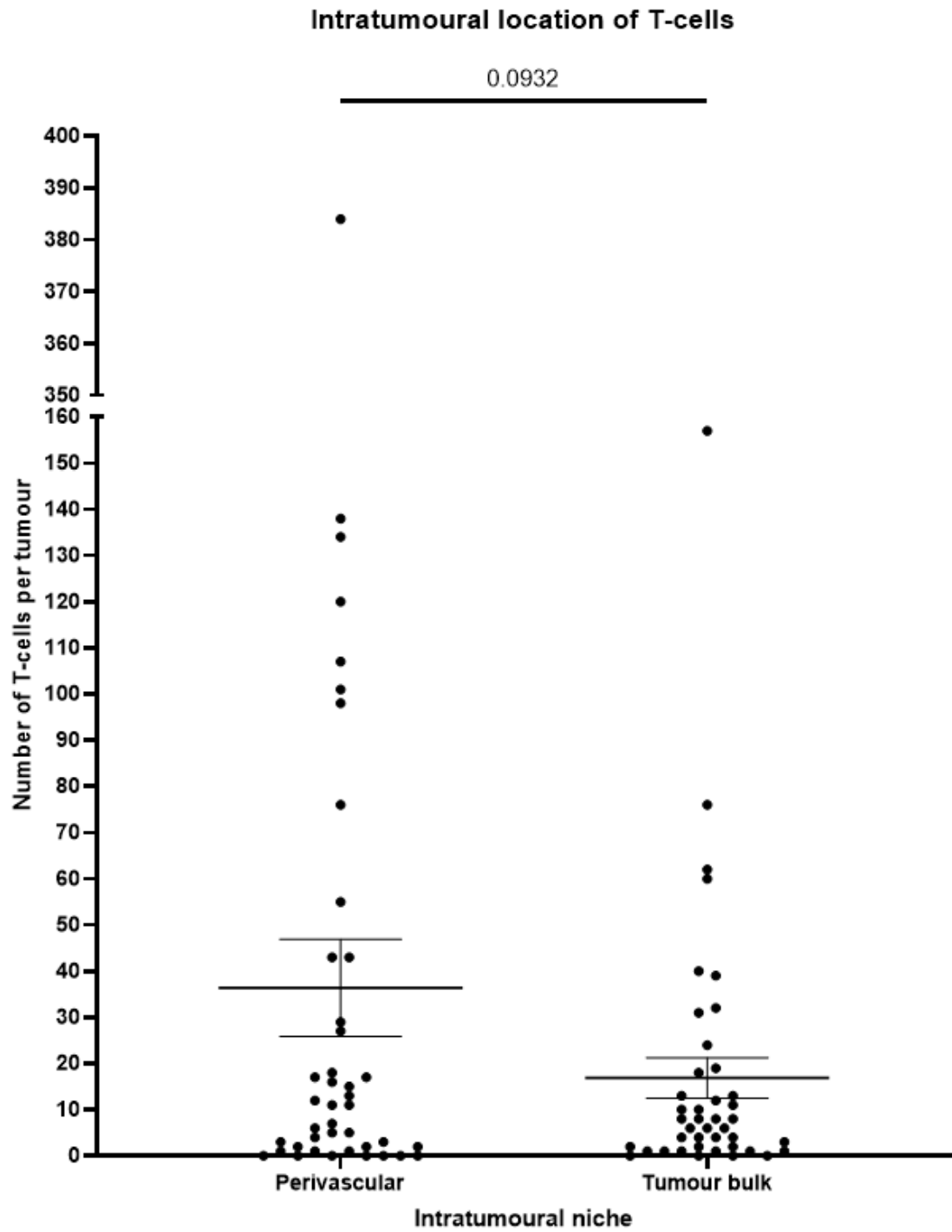


Figure 4.16. Intratumoural location of T-cells. 2235 T-cells were counted in 42 GBM stained with anti-CD3 and their location was categorised as perivascular (within 100 μ m of a vessel) or tumour bulk. Data were analysed using unpaired 2-tailed t-test with Welch's correction. Error bars=mean $\pm$ standard error of the mean (SEM).

4.2.1.3.3. Distribution of B-cells

After describing the distribution and clustering of T-cells, the same analysis was performed for B-cells. It is not implausible that many TILs would be located in the PVN shortly after extravasation, which may facilitate cell-cell interactions; conversely, if T-cells are uniquely prevalent in this region, a cell-type-specific mechanism of suppression might be indicated.

171 B-cells were counted in the 22 B-cell+ GBM, for a mean average of 7.8 B-cells per positive tumour. Cell niches were characterised as PV or TB using the same approach as for T-cells, and numbers of B-cells in physical contact with one another were noted. As with T-cells, only images taken using the 40X or 63X objective lens were used for this analysis. While T-cells were preferentially found in the PVN, B-cells were almost exclusively located in the TB. 160, or 93.6%, of B-cells were in this niche; this distribution was found to be statistically significant ($p=0.0053$) by unpaired 2-tailed t-test. These data are shown in Figure 4.18. B-cell clusters in the TB were also found to be significantly ($p=0.0295$, unpaired 2-tailed t-test) larger than PV clusters, at a mean average of 1.4 versus 1.1 cells respectively (Figure 4.19).

While the much smaller sample size of B-cells versus T-cells may have affected these results, it appeared likely that B-cells may not be subject to the same inhibited migration that may exist for T-cells. Instead, the significant presence of B-cells in the TB suggested that B-cell suppression may be mediated through separate mechanisms.

4.2.1.3.4. Distribution of TAMs

The initial analysis of immune effector distribution and clustering was concluded by examination of TAMs. The above sections found that T-cells were preferentially located in the PVN, whereas B-cells were significantly more likely to be found in the TB. Using images taken with the 40X or 63X objective lens, 1318 macrophages were counted in the 36 positive GBM, giving a mean average of 36.6 cells per positive tumour. Sections from 4.2.2. were also included in this analysis.

As with B-cells, the majority of TAMs (977, or 74.1%) were located in the TB. This was found to be significant ($p=0.0087$) by unpaired 2-tailed t-test, as shown in Figure 4.20. Unlike B-cells, TAMs did not show a significant difference in cluster sizes between the PVN and TB, at 2.0 and 1.8 cells respectively ($p=0.6031$), which data are shown in Figure 4.21.

To conclude, TILs were found throughout GBM tumours. However, TAMs and B-cells were largely absent from the PVN, where T-cells are most present. These data suggested that T-cells specifically may be prevented from migrating into the tumour. Furthermore, it implied that interactions between T-cells and either of these antigen-presenting populations were rare. However, given the increased numbers of TAMs and slightly reduced prevalence of PV B-cells compared to TAMs (6.4% compared to 25.9% of cells), interactions between T-cells and macrophages were potentially more likely to occur than T-cell and B-cell interactions. The antigen-presenting capacity of TAMs was suggested both by pathway analysis (3.2.1.1.3.) and the retention of T-cells within GBM tissue. However, this activation did not appear sufficient to allow T-cell migration into the tumour. Overall, B-cells and TAMs may

have been at least partially exempted from a migration-inhibitory mechanism in the PVN.

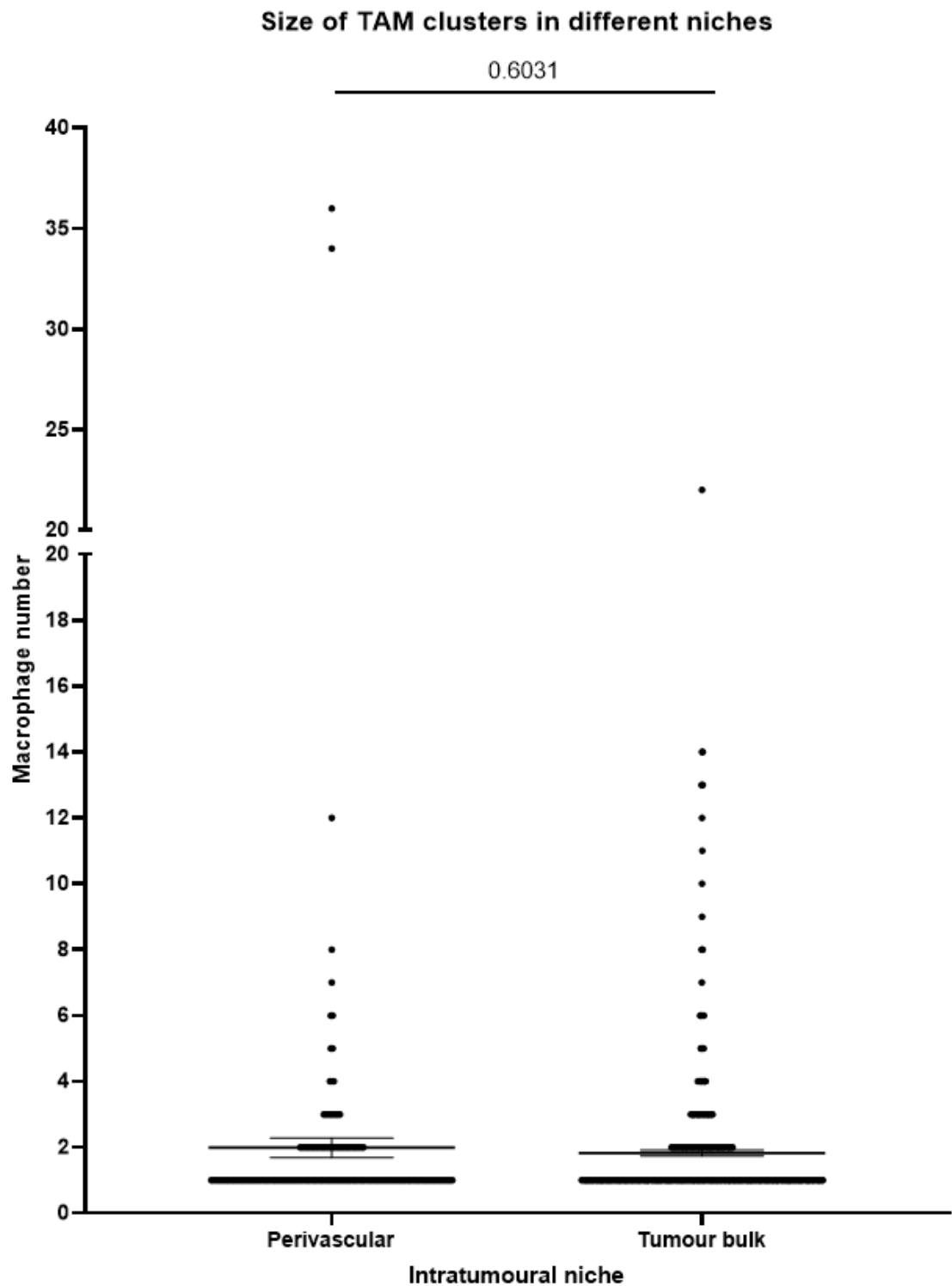


Figure 4.21. Size of TAM clusters in different niches. 1318 TAMs were counted in 36 GBM stained with anti-CD68 and the number of macrophages in physical contact with each other in each image was recorded. Their location was categorised as perivascular (within 100µm of a vessel) or tumour bulk. Data were analysed using unpaired 2-tailed t-test with Welch's correction. Error bars=mean ± SEM.

4.2.1.3.5. Relative prevalence of TILs

The above section described the intratumoural locations of T-cells, B-cells and macrophages. Next, slides that had been stained for all three markers were examined to assess the relative prevalence of TIL phenotypes while avoiding over- or under-representation.

Across 36 slides stained for all three markers, there were 412 T-cells, 160 B-cells and 562 TAMs, for mean averages of 11.4, 4.4 and 15.6 cells per tumour respectively. The data are summarised in Table 4.07.

Kruskal-Wallis test identified a significant difference in means ($p=0.0093$). Dunn's multiple comparisons test revealed that B-cells were significantly under-represented compared to TAMs ($p=0.0114$), and the difference between T-cells and TAMs approached significance ($p=0.0646$), though T- and B-cells were not significantly different ($p=>0.9999$). Thus, TAMs were over-represented compared to both other phenotypes. While both TAMs and B-cells were preferentially located in TB (4.2.1.3.3. and 4.2.1.3.4.), B-cell numbers were significantly lesser than TAMs. These data suggested that a B-cell-specific mechanism of suppression may exist, either by limiting B-cell lifespan or function within the TB or preventing B-cells from extravasating in the first place. It appeared likely that T-cells, by contrast, are suppressed in the PVN.

Table 4.07. TIL numbers observed in 36 GBM

Population	Total cells	Total tumours	Mean average cells per positive tumour
T-cell	412	36	11.4
B-cell	160	36	4.4
Macrophage	562	36	15.6

Table 4.07. TIL numbers observed in positive GBM. Mean averages are given to one decimal place.

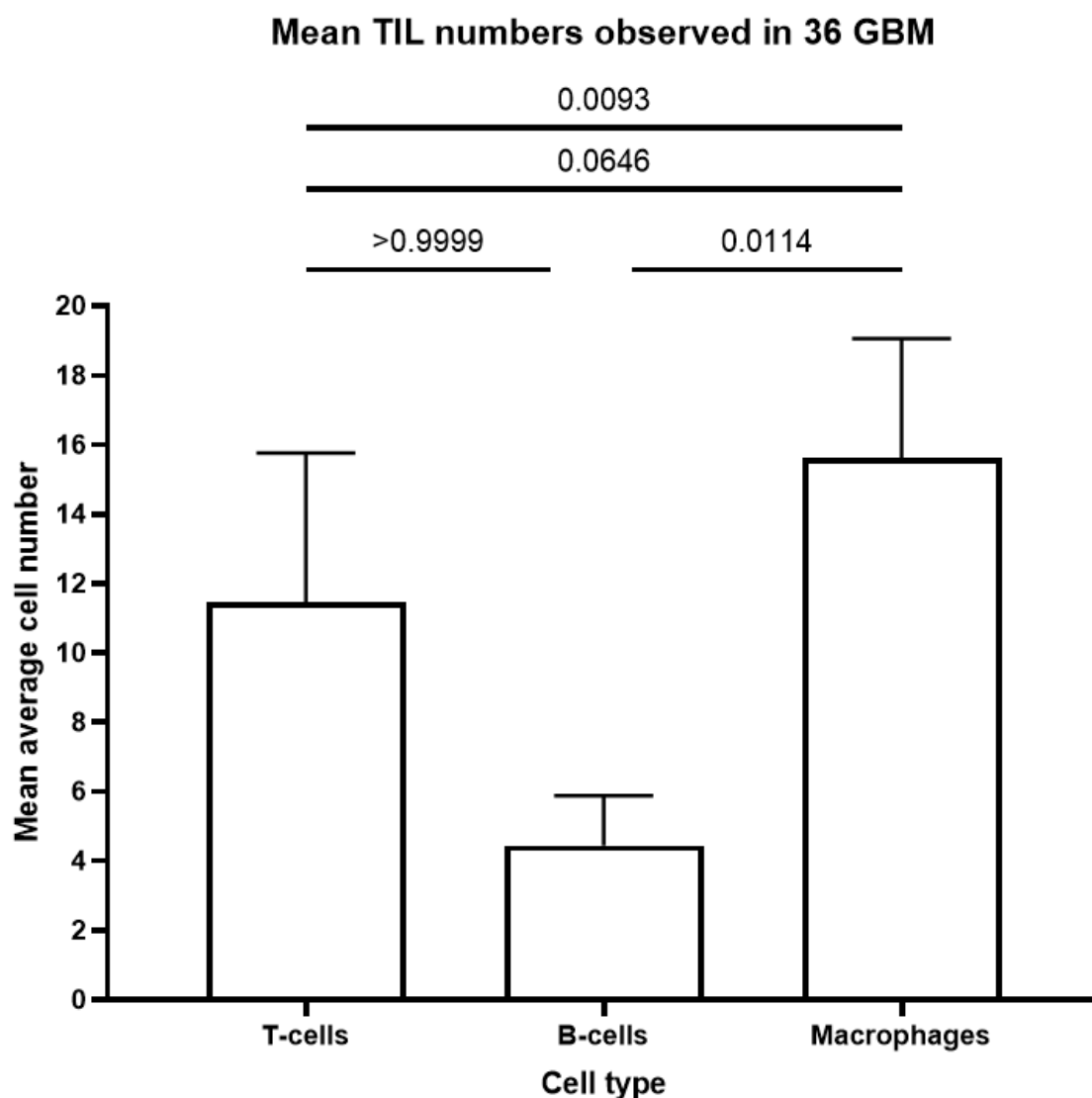


Figure 4.22. TIL numbers observed in positive GBM. The total number of T-cells, B-cells and macrophages was counted in sections stained simultaneously for all three populations and divided by the number of tumours. Data were analysed by Kruskal-Wallis test (upper line) and Dunn's multiple comparisons test (lower lines). Error bars=SEM.

Fisher's exact test was then used to examine whether presence of one TIL population correlated with the presence of either of the others. No significant correlations between the presence of different TILs were noted. Additionally, within the T-cell+ subset of tumours, there was no significant correlation between T-cell aggregations and the presence of either B-cells or macrophages. No corrections for multiple comparisons were performed because no statistically significant results were observed. These data are summarised in Table 4.08.

Table 4.08. Correlations between TILs

Comparison	N	P-value (Fisher's exact test)
T-cell and B-cell	48	0.4290
T-cell and macrophage	50	0.3835
B-cell and macrophage	48	0.7456
T-cell aggregation and B-cell	41	0.3058
T-cell aggregation and macrophage	43	0.1136

Table 4.08. Correlations between TILs. GBM were scored for the presence of different TIL populations and correlations were examined using Fisher's exact test. For T-cell aggregation correlations, only T-cell+ tumours were considered in the analysis.

4.2.1.4. TIL interactions

Next, cell-cell interactions were studied. These could provide further information about adaptive immune cell function beyond the location and relative prevalence data described above. In particular, both TAMs and B-cells can act as antigen-presenting cells (APCs), and thus the presentation of antigen to T-cells could be inferred; and TAMs may also engage in phagocytic behaviour, which could be a method of immune evasion if directed towards other TILs or a mechanism of anti-tumoural immunity if directed towards GBM cells. Cell interaction numbers were not verified by another operator.

It is worth noting that this analysis was limited to physical cell-cell interactions and did not provide any detail of paracrine signalling. The criteria for defining a cell-cell interaction were: both TIL markers must be present; both cells must have a nucleus; and there must be clear contact between the cells, with no intervening other cell bodies, nuclei or extracellular space. This analysis was also limited to a two-dimensional plane, whereas cell-cell interactions occur in three dimensions, and so this reduction in detail may have rendered certain interactions ambiguous.

4.2.1.4.1. T-cell and B-cell interactions

Interactions between T- and B-cells were of interest because B-cells have some APC capacity; thus, documenting interactions between these two cell types would shed light on intratumoural antigen presentation.

To examine T-cell and B-cell interactions, double-positive tumours were selected from the 50 GBM dataset. Of the 22 tumours stained for CD19, 19 also contained CD3 staining in at least one section. 12 sections from these tumours contained both CD3⁺ and CD19⁺ cells. However, T- and B-cells rarely appeared in the same images and no definitive interactions between the two cell types were observed. Given that B-cells were preferentially found in the TB ($p=0.0053$) and T-cells in the PVN ($p=0.0932$) as described in 4.2.1.3., it is understandable that interactions between these TILs were rare. It appeared unlikely that B-cells played a major role in presenting antigen to T-cells from these data.

4.2.1.4.2. T-cell and macrophage interactions

Next, interactions between T-cells and macrophages were examined. Although T-cell priming usually occurs via interactions with dendritic cells (DCs) in lymph nodes (LNs), it can also occur *in situ*; furthermore, recognition of the cognate antigen is required for T-cell retention in tissue following extravasation, and for these reasons, documentation of T-cell and macrophage interactions was important.

31 tumours contained both cell types; from these tumours, 33 sections were confirmed double-positive. Across all the images from these sections, 36 interactions between T-cells and macrophages were counted. In two interactions, there was a large area of cell-cell contact with CD3 clustering, which may have been formation of an immunological synapse (IS); an example is shown in Figure 4.23. Another interaction appeared potentially phagocytic in nature (Figure 4.24). The other 33 interactions could not be easily categorised.

In conclusion, clear instances of either antigen presentation or phagocytosis directed toward T-cells were rare; however, cell-cell contacts between macrophages and T-cells were not uncommon, averaging 1.1 occurrences per double-positive tumour. It is entirely possible that many of these contacts were simply a case of two cells moving past one another, with no long-term interaction occurring.

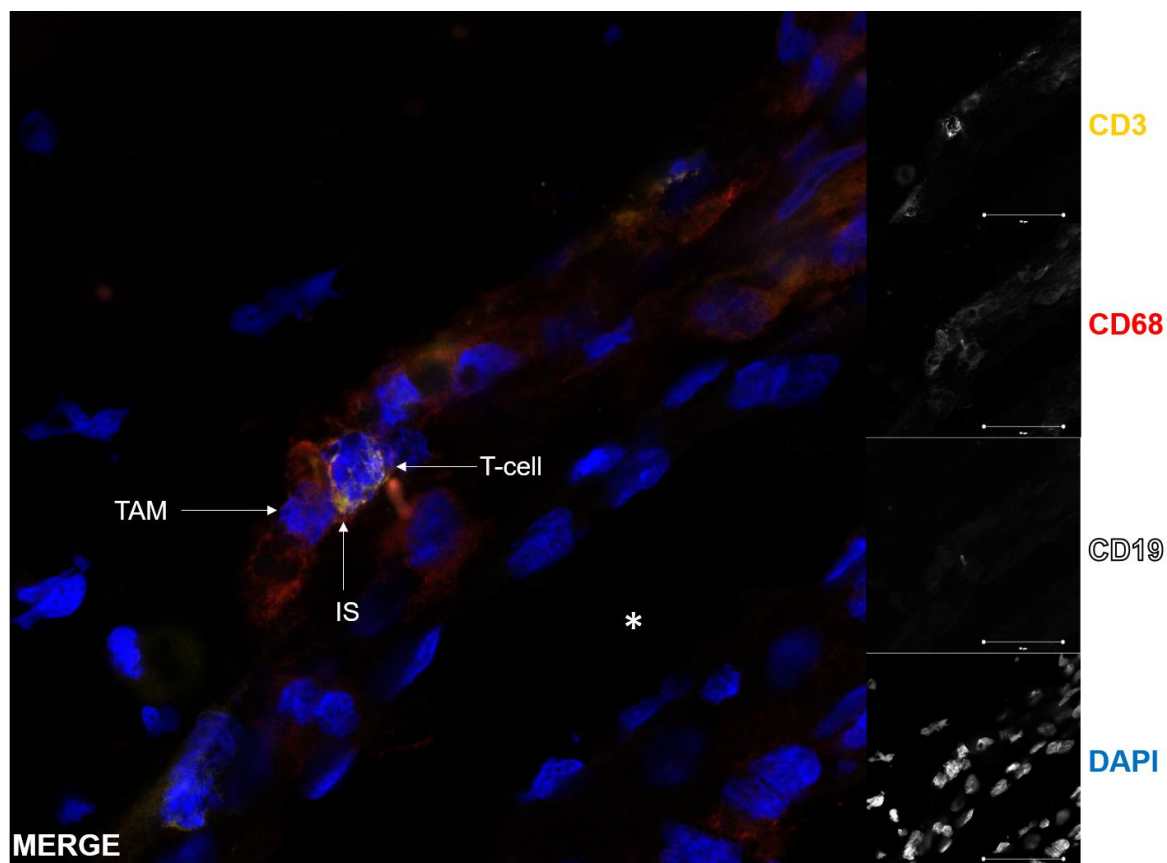


Figure 4.23. Potential IS formed between TAM and T-cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD19, anti-CD68, RRX-conjugated secondary antibody and DAPI. Sections were stained imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50 μ m. Arrows indicate T-cell, TAM and potential IS. Asterisk=vessel lumen.

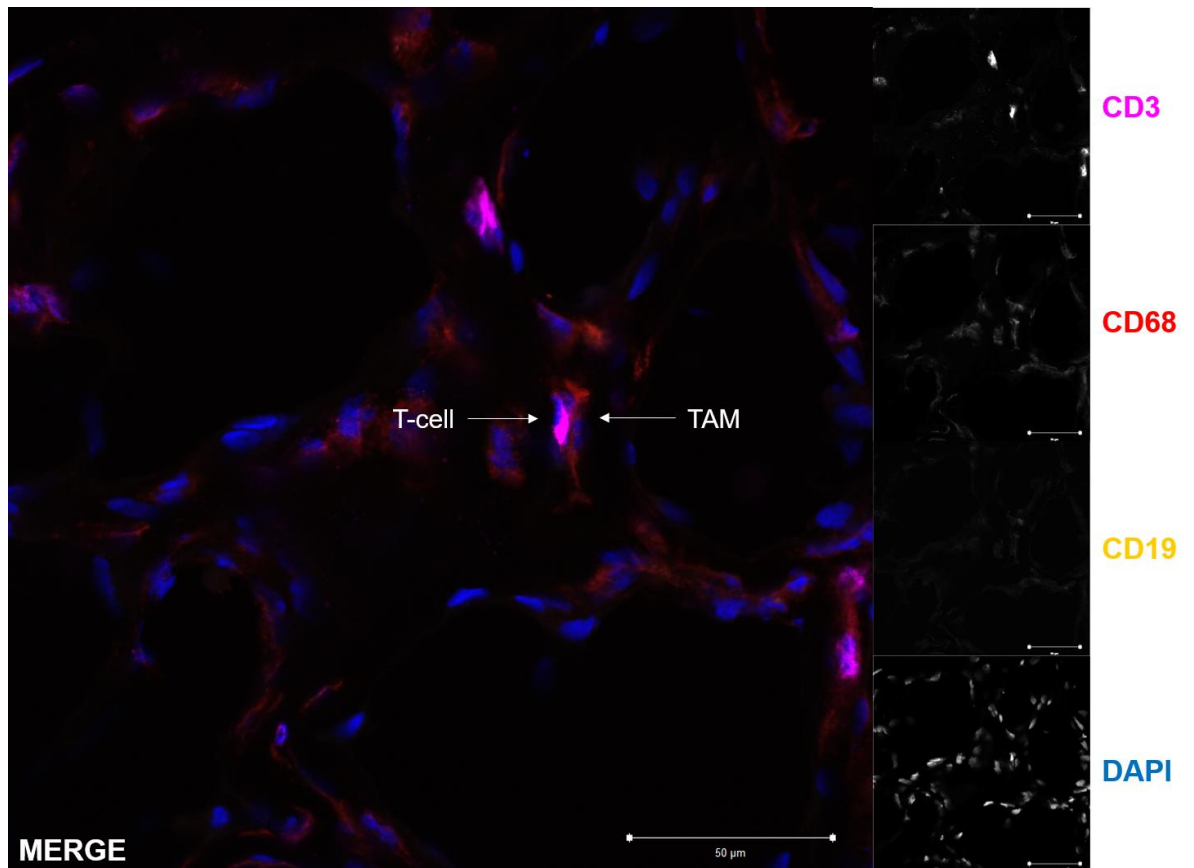


Figure 4.24. Potential phagocytic interaction between TAM and T-cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD19, anti-CD68, RRX-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrows indicate T-cell and TAM.

4.2.1.4.3. B-cell and macrophage interactions

Finally, the relationship between B-cells and macrophages was considered. Of the 48 tumours stained for CD19, 17 contained both CD19 and CD68 positivity; both markers were present in 15 sections. Contact between B-cells and macrophages was observed in eight cases, in five of which the B-cell appeared unhealthy or dying; 10 cases were observed where CD19 staining appeared within macrophages (Figure 4.25).

Based on these data, it appeared likely that TAMs adversely affect B-cell function. No clear incidences of phagocytosis directed towards B-cells were observed, although due to the limited numbers of B-cells, these may be rare. It is also possible that B-cells were less resistant than TAMs to factors such as hypoxia, nutrient depletion, cytotoxic secreted proteins or others, resulting in B-cell death, followed by scavenging by nearby TAMs. Either theory would explain the occurrences of CD19 positivity within macrophages. The images of clearly unhealthy B-cells not in contact with TAMs lent weight to the latter, although the dataset was not sufficient to draw any firm conclusion. Rather, it may be an avenue for future research. In addition, death and scavenging by TAMs could be the B-cell specific suppressive mechanism that occurs away from the PVN.

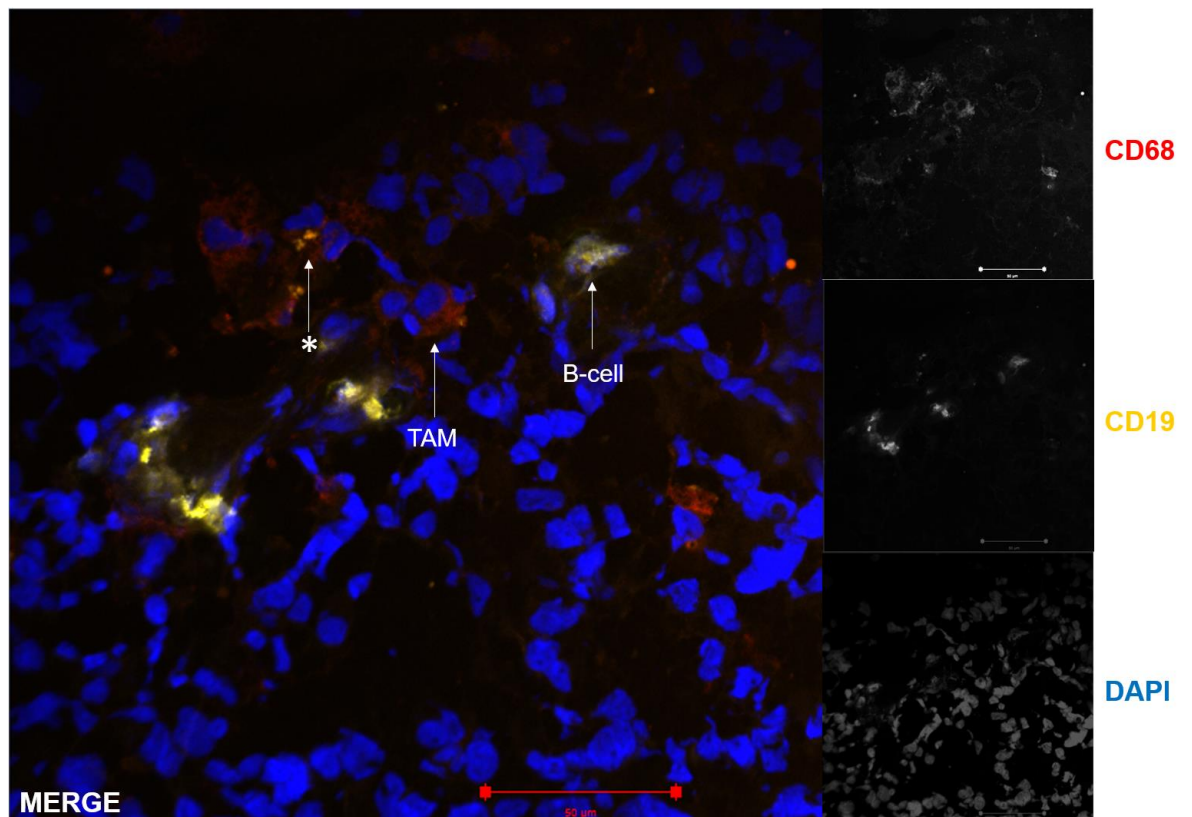


Figure 4.25. Dying B-cells in proximity to TAMs and TAMs with CD19 positivity. Frozen human GBM Sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD19, anti-CD68, RRX-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrows indicate TAMs and B-cells. Arrow with asterisk indicates CD68 and CD19 co-localisation.

4.2.1.5. T-cell and CNS cell interactions

4.2.1.5.1. Preliminary work

To conclude the analysis of cell-cell interactions, the interactions of T-cells with tumour cells and astrocytes were examined. Human astrocytes may have some APC capability (reviewed in Sutter and Crocker, 2022) and tumour cells may be able to directly suppress TIL function through expression of cell surface markers or paracrine signalling through secreted proteins; thus, documenting these populations was of interest. Additionally, formation of an IS between CD8⁺ T-cells and their target cells is necessary for cytolytic activity, and so documenting these cell populations and any interactions was also of interest.

To this end, sections were stained with unconjugated antibodies against CD56 (anti-CD56) – a tumour cell marker – and the astrocyte marker glial fibrillary astrocyte protein (GFAP) (anti-GFAP). A FITC-conjugated anti-goat antibody was obtained for use with anti-GFAP, in addition to the previously-described anti-rabbit secondary. Details are given in Table 4.09. CD56 is also expressed on natural killer (NK) cells and neural tissue. CD56⁺ cells in these sections were observed to be rounded in shape, with no projections resembling axons and neurites, and so were unlikely to be neural. While NK cells could theoretically account for large areas of CD56⁺ tissue, these cells are often rare in GBM, and are decreased in the peripheral compartment following treatment (Fadul et al., 2011); however, the possibility that some CD56⁺ cells were NK cells could not be ruled out.

Positive controls were established using methanol-fixed U251 cells for anti-CD56 (Figure 4.26A) and methanol-fixed normal human astrocytes (NHA) for anti-GFAP

(Figure 4.26B). HUVECs were used as an anti-CD56 negative control (Figure 4.27A) U251 was used as the negative control for anti-GFAP (Figure 4.27B). As these results showed, marker expression was restricted to cells of the expected lineage; thus, these stains were considered appropriate for use in GBM sections. A secondary-only control for the RRX-conjugated secondary antibody is shown in Figure 4.4; a secondary-only control for the FITC-conjugated antibody is shown in Figure 4.28.

Table 4.09. Antibody panel for identification of T-cells, tumour cells and astrocytes

Specificity	Cell type	Fluorophore	Host	Dilution	Clone
CD3	T-cell	AlexaFluor 647	Mouse	1:20	UCHT8
CD56	Tumour cells	N/A	Rabbit	1:100	JF1021
GFAP	Astrocyte	N/A	Goat	1:30,000	Polyclonal
Goat	N/A	FITC	Donkey	1:100	Polyclonal
Rabbit	N/A	RRX	Donkey	1:200	Polyclonal

Table 4.09. Antibody panel for identification of T-cells, tumour cells and astrocytes.

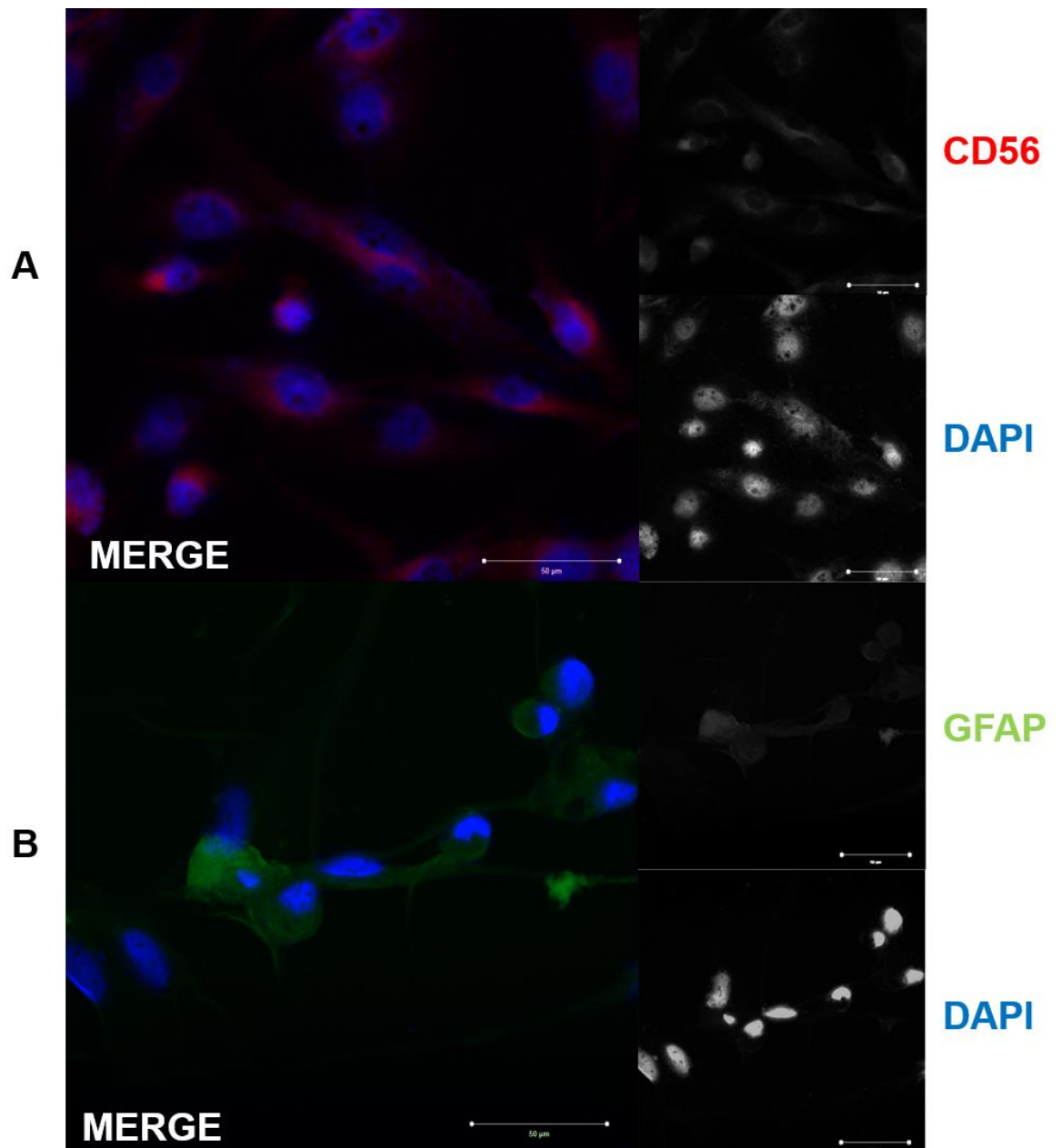


Figure 4.26. CD56 and GFAP positive controls. U251 and NHA cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. U251 cells were stained with anti-CD56 and RRX-conjugated secondary antibody (A) and NHA cells were stained with anti-GFAP and FITC-conjugated secondary antibody (B) and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bars=50μm.

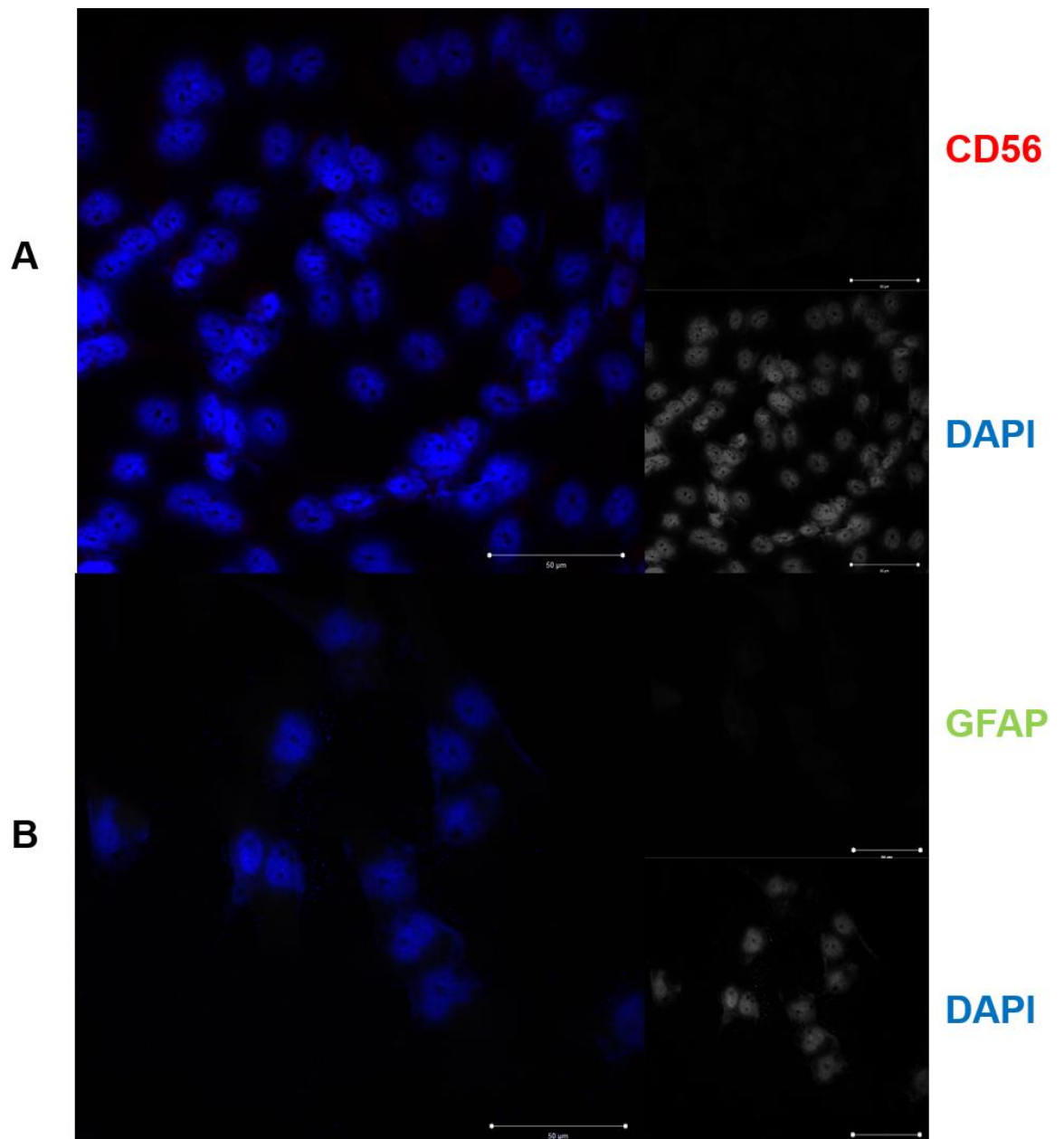


Figure 4.27. CD56 and GFAP negative controls. U251 and HUVEC cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. HUVEC cells were stained with anti-CD56 and RRX-conjugated secondary antibody (A) and U251 cells were stained anti-GFAP and FITC-conjugated secondary antibody (B) and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bars=50μm.

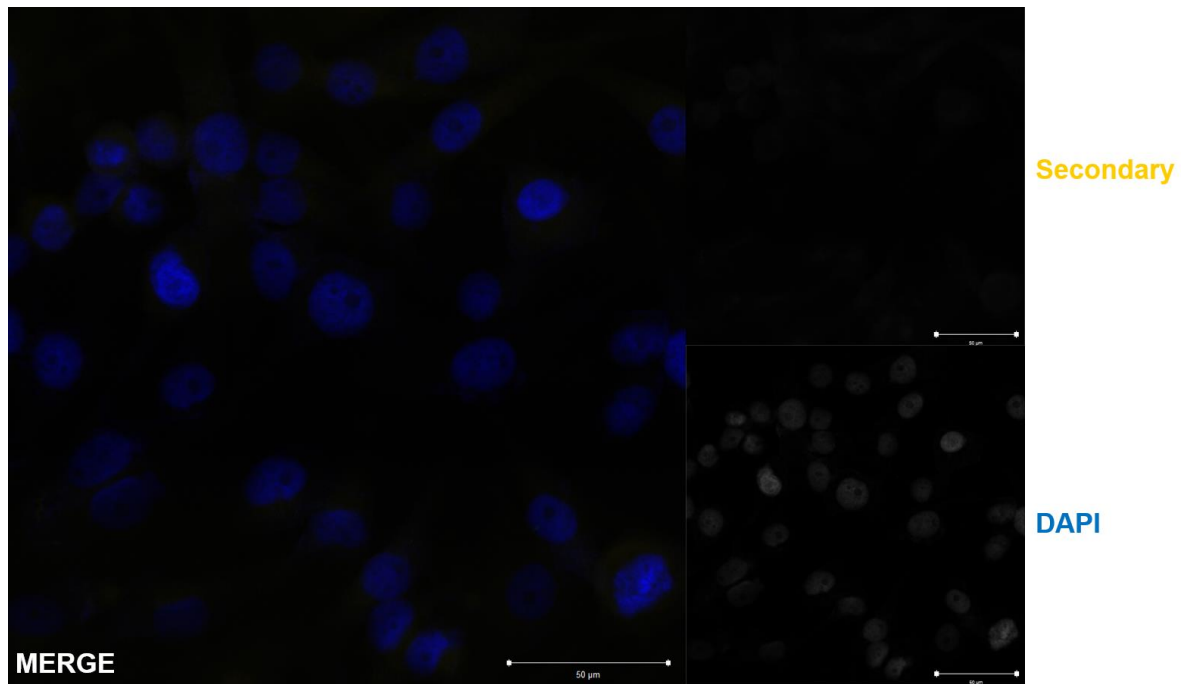


Figure 4.28. FITC-conjugated secondary antibody negative control. U251 cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Cells were stained with FITC-conjugated secondary antibody and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

4.2.1.5.2. T-cell and tumour cell or astrocyte interactions

In order to examine the interactions of T-cells with astrocytes and tumour cells, 35 sections from separate GBM were stained with the CD3-CD56-GFAP panel. Cell counts were not verified by another operator. The number of GBM used was limited by their use in other panels.

According to either these or previous sections from the same tumours stained with anti-CD3, all 35 tumours were T-cell+. GFAP was present in 22, or 62.9% of tumours examined; CD56 was observed in 34, or 97.1 percent. The majority of tumours were triple-positive (60.0%); 13, or 37.1%, were CD3+CD56+ but lacked GFAP, and one (2.9%) was GFAP+CD56-. These data are summarised in Table 4.10.

Table 4.10. Prevalence and co-occurrence of T-cells, astrocytes and tumour cells in 35 GBM

Phenotype	Number of tumours	% of tumours
CD3+	35/35	100.0
CD56+	34/35	97.1
GFAP+	22/35	62.9
CD3+CD56+GFAP+	21/35	60.0
CD3+CD56-GFAP+	1/35	2.9
CD3+CD56+GFAP-	13/35	37.1

Table 4.10. Prevalence and co-occurrence of T-cells, astrocytes and tumour cells in 35 GBM. Tumour sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-GFAP, RRX- and FITC-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Percentages are given to one decimal place.

Of the 35 tumours stained with this panel, T-cell positivity was directly observed in 12; confirmation that the other tumours were T-cell positive came from previous slides also stained with CD3. For three of the 12, the only images of T-cells were taken using the 20X objective lens, making analysis of their interactions difficult due to poor resolution. Thus, for analysis of T-cell and astrocyte or tumour cell interactions, the dataset was limited to images from nine GBM.

Images from these nine GBM contained a total of 148 T-cells. No observations with astrocytes were observed. 47 cells, or 31.8%, were observed interacting with CD56+ cells or tissue; an example of T-cells not interacting with CD56+ cells is shown in Figure 4.29. An average of 5.2 CD3+/CD56+ interactions were observed per double-positive tumour; while this is lower than the average 11.2 T-cells that were not interacting with CD56+ cells or tissue per tumour, the difference did not reach significance (Figure 4.30).

In conclusion, T-cell and astrocyte interactions in this dataset were rare in nature; in light of the lack of T-cell/B-cell interactions and rarity of potential IS formation between T-cells and TAMs documented in 4.2.1.4., that antigen presentation in GBM may be rare, or may occur largely through a population not examined here such as ECs. Additionally, interactions between T-cells and CD56+ cells were rare. Overall, it appeared that T-cells that successfully infiltrate the tumour may rarely form contacts with tumour cells, with resulting poor levels of anti-tumoural immunity.

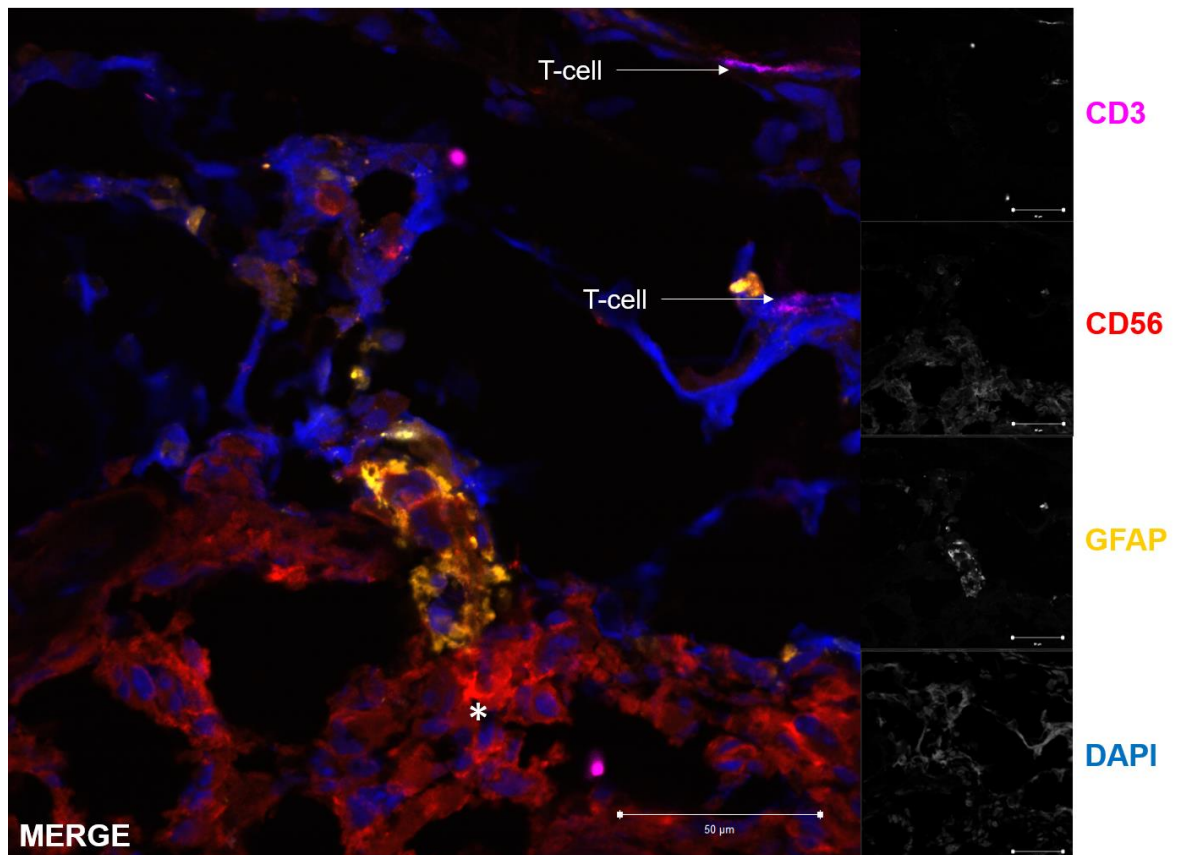


Figure 4.29. Representative image of T-cells not interacting with CD56+ cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-GFAP, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=T-cell. Asterisk=CD56+ tissue.

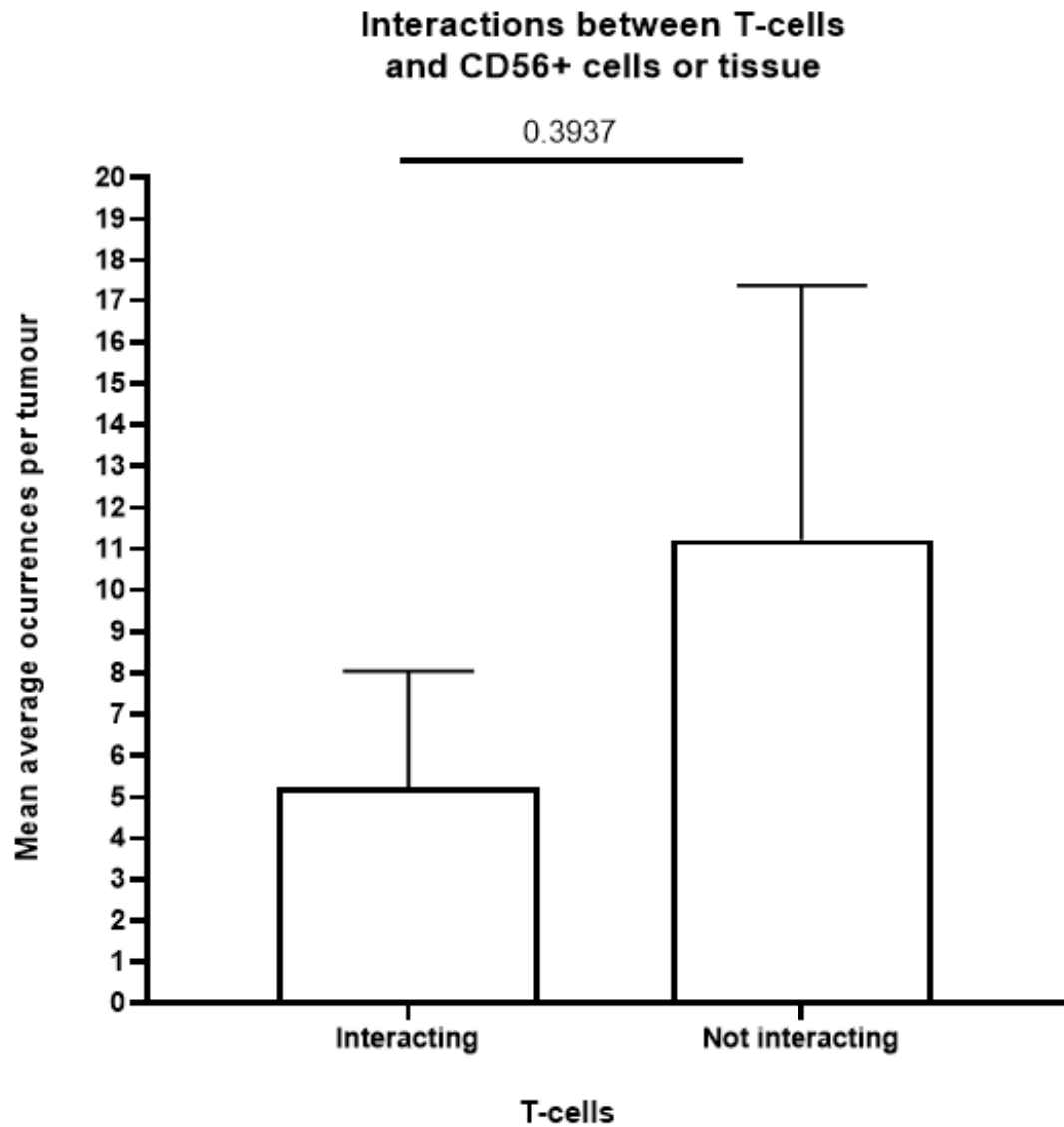


Figure 4.30. Interactions between T-cells and CD56+ cells or tissue. 148 T-cells were counted in nine GBM stained with anti-CD3 and anti-CD56. The number of T-cells in physical contact with CD56+ cells or tissue in each image was recorded. Data were analysed using unpaired 2-tailed t-test with Welch's correction. Error bars=SEM.

4.2.2. Characterisation of target proteins

In the above section, the distribution and interactions of three TIL populations were described. T-cells were primarily found in the PVN and may be inhibited from migrating into the tumour; conversely, both B-cells and TAMs were significantly more present in the TB, although both populations were rarer than T-cells. Additionally, T-cell aggregations were found in a large minority of GBM. Furthermore, T- and B-cell interactions were rare. T-cell and TAM interactions were more common, but could not be broadly categorised as either phagocytic or stimulatory in nature. Additionally, T-cell interactions with tumour cells were rare.

The next series of experiments focused on the soluble proteins of interest described in 3.2.1.2.1. IF would not only allow characterisation of their distribution and cellular sources, but would also show any cell-protein interactions that may explain some of the TIL behaviours outlined above. The galectins were prioritised for these experiments, due to their documented immunosuppressive and synergistic effects; CTGF, another protein with immunosuppressive effects, was also included.

4.2.2.1. Preliminary work

Unconjugated antibodies against galectins 1, 3, 4 and 7 and CTGF were obtained. Anti-CD56 and anti-CD68 were used to ascertain whether target proteins were directly secreted by tumour cells and TAMs. Sections were not co-stained for CD19, as all target proteins used an anti-goat FITC-conjugated secondary. Details are given in Table 4.11.

Table 4.11. Antibodies for identification of target proteins and potential cellular sources

Specificity	Cell type	Fluorophore	Host	Dilution or concentration ($\mu\text{g/mL}$)	Clone
CD3	T-cell	AlexaFluor 647	Mouse	1:20	UCHT8
CD56	Tumour cells	N/A	Rabbit	1:100	JF1021
CD68	Macrophage	N/A	Rabbit	1:1000	Polyclonal
CTGF	N/A	N/A	Goat	10	Polyclonal
Galectin-1	N/A	N/A	Goat	10	Polyclonal
Galectin-3	N/A	N/A	Goat	10	Polyclonal
Galectin-4	N/A	N/A	Goat	10	Polyclonal
Galectin-7	N/A	N/A	Goat	10	Polyclonal
Goat	N/A	FITC	Donkey	1:100	Polyclonal
Rabbit	N/A	RRX	Donkey	1:200	Polyclonal

Table 4.11. Antibodies used for identification of target proteins and potential cellular sources.

Differentiated macrophages were identified as suitable negative controls for galectin-3 (gal-3), galectin-4 (gal-4), galectin-7 (gal-7) and CTGF, which required development of a suitable protocol for differentiating monocyte cell lines to macrophages. Available protocols for macrophage differentiation using phorbol 12-myristate-13-acetate (PMA) were varied: concentrations of PMA ranged from under 10 up to 100ng/mL for varying lengths of time. As high concentrations of PMA are cytotoxic and no more than 24 hours' differentiation was required, cells from the THP1 and Monomac-6 lines were initially treated with 20ng/mL PMA for 24 hours, then imaged using an Evos FL Auto 2 microscope. This protocol has previously been found sufficient to induce THP1 differentiation (Valle and Guiney, 2005).

Images showed that this protocol was sufficient to induce macrophage differentiation without obvious cytotoxicity. Representative images are shown in Figure 4.31A (THP1) and B (Monomac-6). Compared to untreated controls in panels C (THP1) and D (Monomac-6), cells were visibly more granular, and could be seen clustering and adhering to the plate. As PMA was supplied diluted in dimethyl sulfoxide (DMSO), an identical experiment was performed using equivalent volumes of DMSO to confirm PMA alone was responsible for differentiation. Representative images are shown in Figure 4.31E (THP1) and F (Monomac-6), showing that DMSO at these levels promoted neither differentiation nor cell death. This was further supported by the cells' strong CD68 expression, as noted in 4.2.2.2., following treatment with PMA. Henceforth, monocytes were treated with 20ng/mL PMA for 24 hours for differentiation to macrophages.

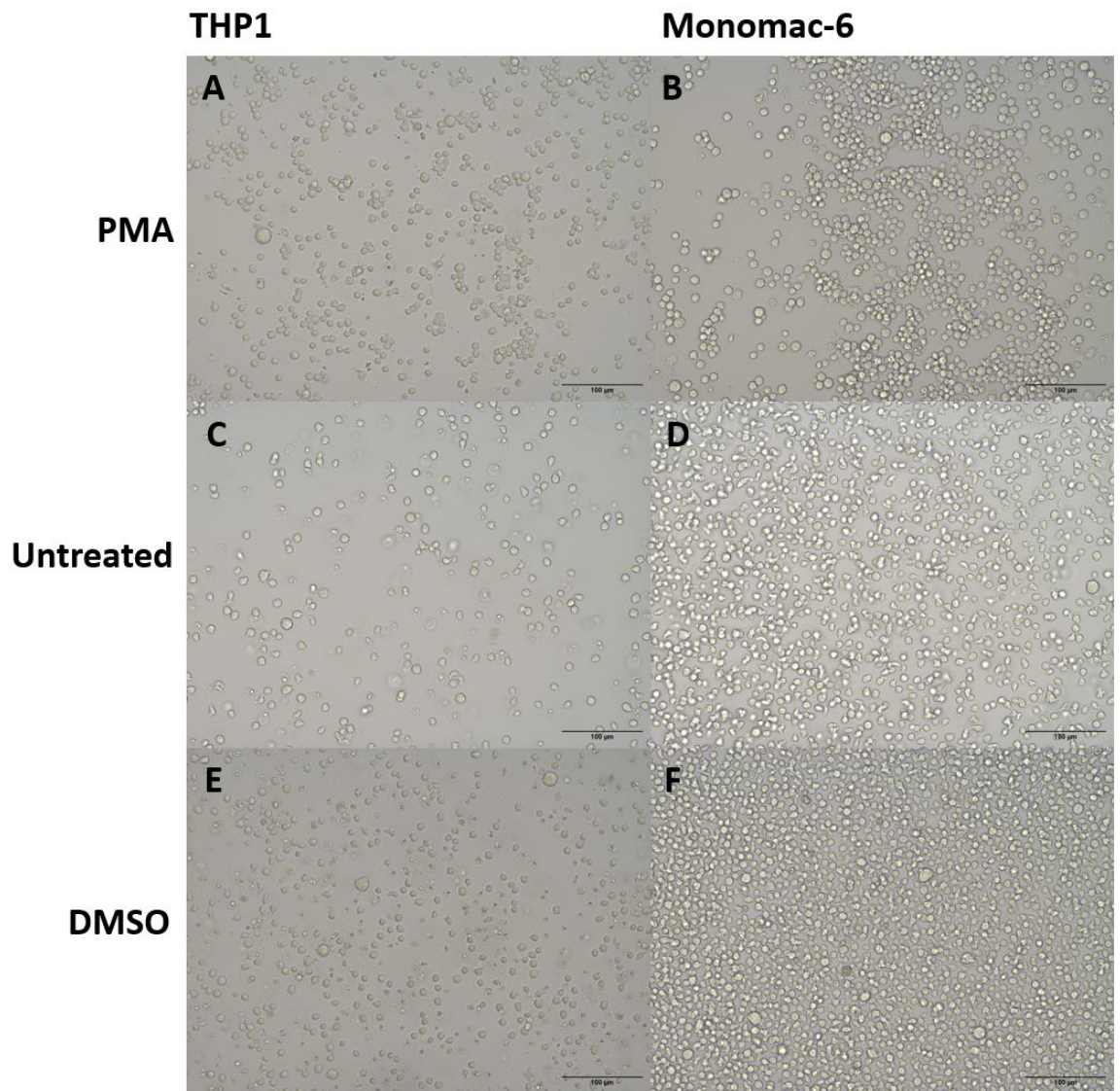


Figure 4.31. Differentiation of monocyte cell lines using 20ng/mL PMA for 24 hours. Cells were seeded at a minimum density of 500,000/mL. A: THP1 cells treated with 20ng/mL PMA. B: Monomac-6 cells treated with 20ng/mL PMA. C: Untreated THP1 cells. D: Untreated Monomac-6 cells. E: THP1 cells treated with DMSO. F: Monomac-6 cells treated with DMSO. Cells were imaged using an Evos FL Auto 2. Scale bar=100µm.

Monocytes were differentiated directly on glass slides and used as negative controls for galectins 3, 4 and 7 and CTGF. These stains are shown in Figures 4.32, 4.33, 4.34 and 4.35 respectively. No positivity was observed in any of the cells; thus the antibodies were considered acceptable for use in GBM sections. As other human tissues were not available, appropriate positive controls for these proteins could not be performed; regardless, a negative secondary-only control for the FITC-conjugated anti-goat did not reveal any positive staining (Figure 4.28), suggesting that the stains observed in GBM sections were genuine.

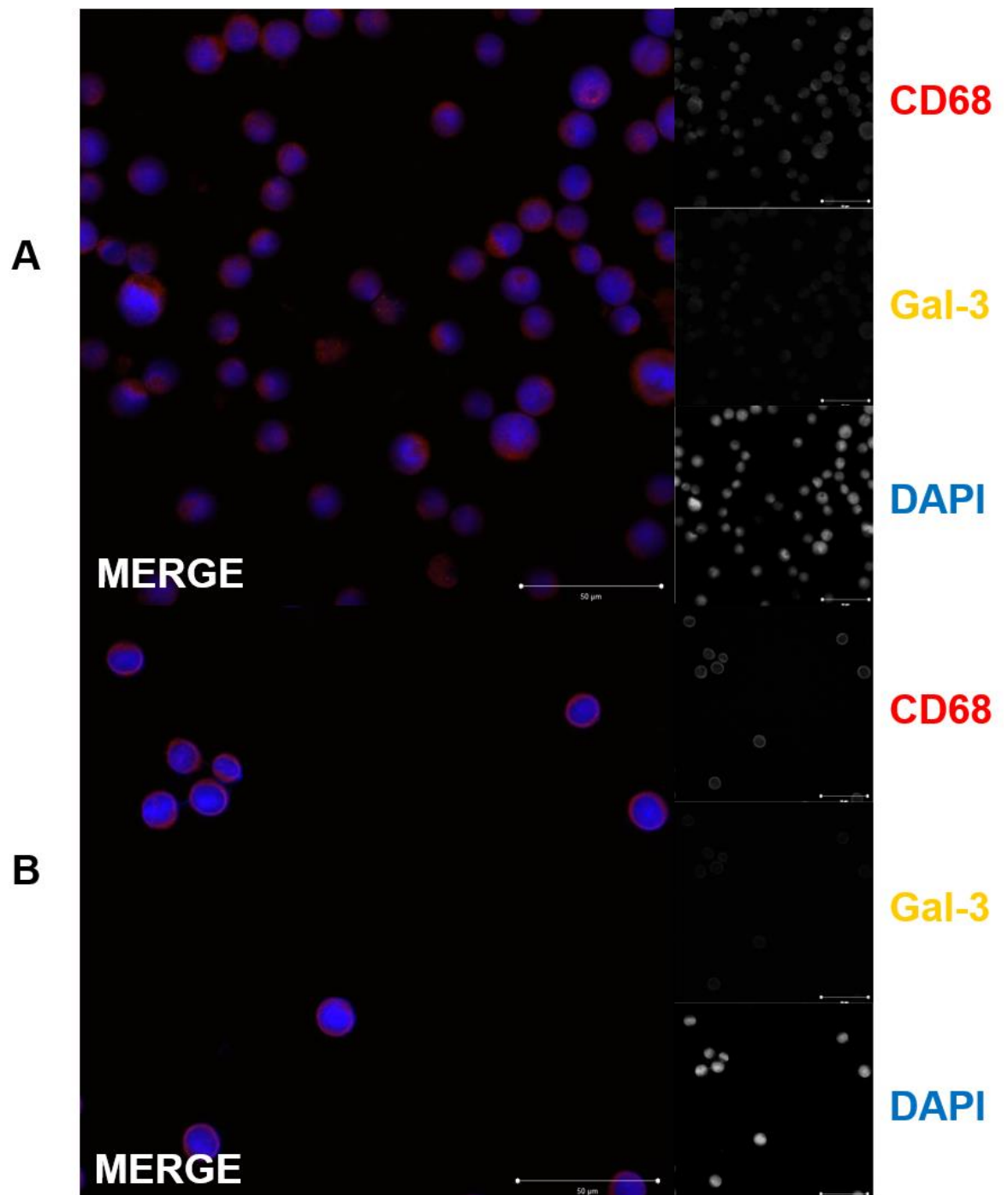


Figure 4.32. Galectin-3 negative control. THP1 (A) and Monomac-6 (B) cells were grown on glass slides with 20ng/mL PMA and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-CD68, anti-galectin-3, FITC and RRX-conjugated secondary antibodies and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm.

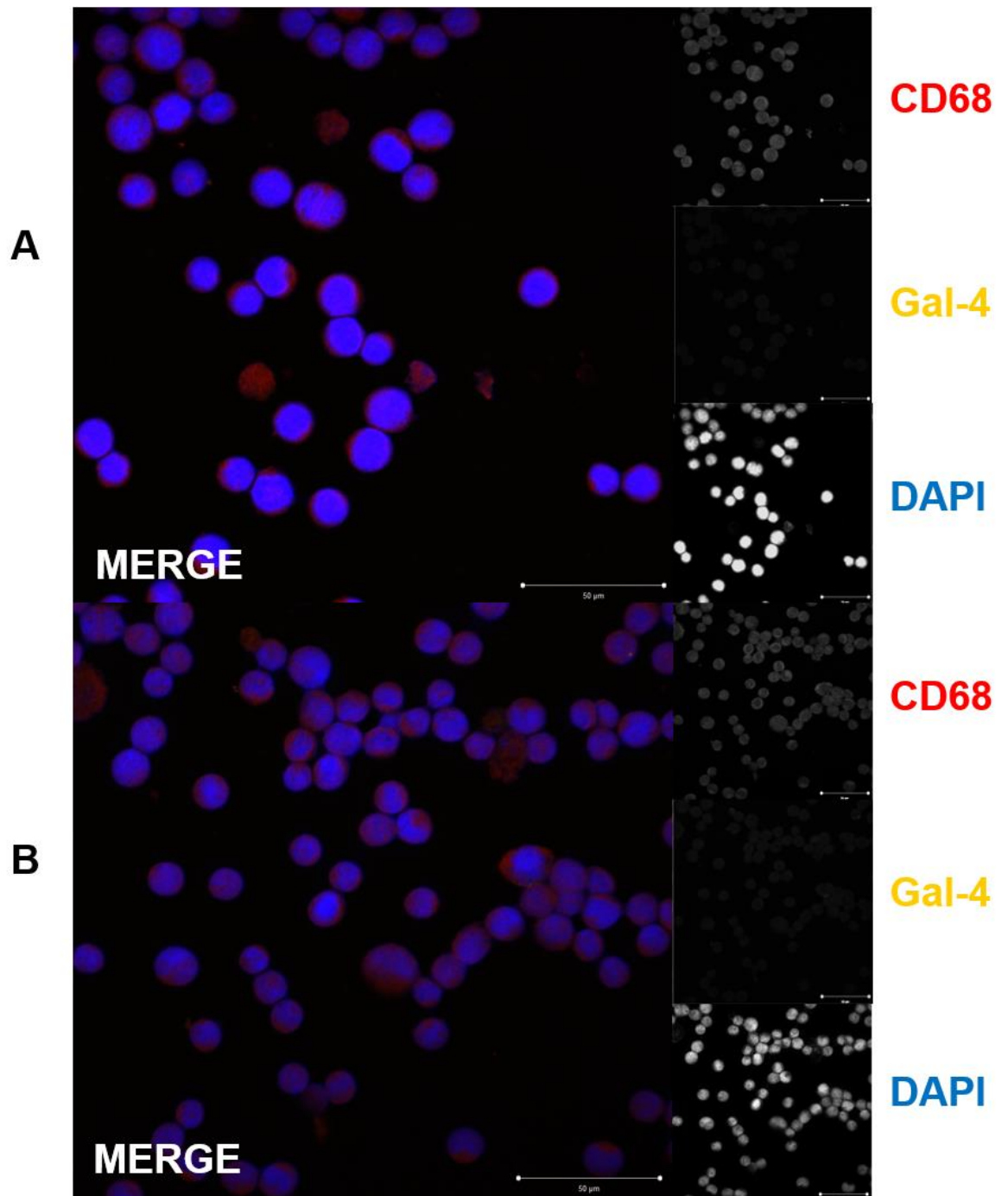


Figure 4.33. Galectin-4 negative control. THP1 (A) and Monomac-6 (B) cells were grown on glass slides with 20ng/mL PMA and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-CD68, anti-galectin-4, FITC and RRX-conjugated secondary antibodies and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm.

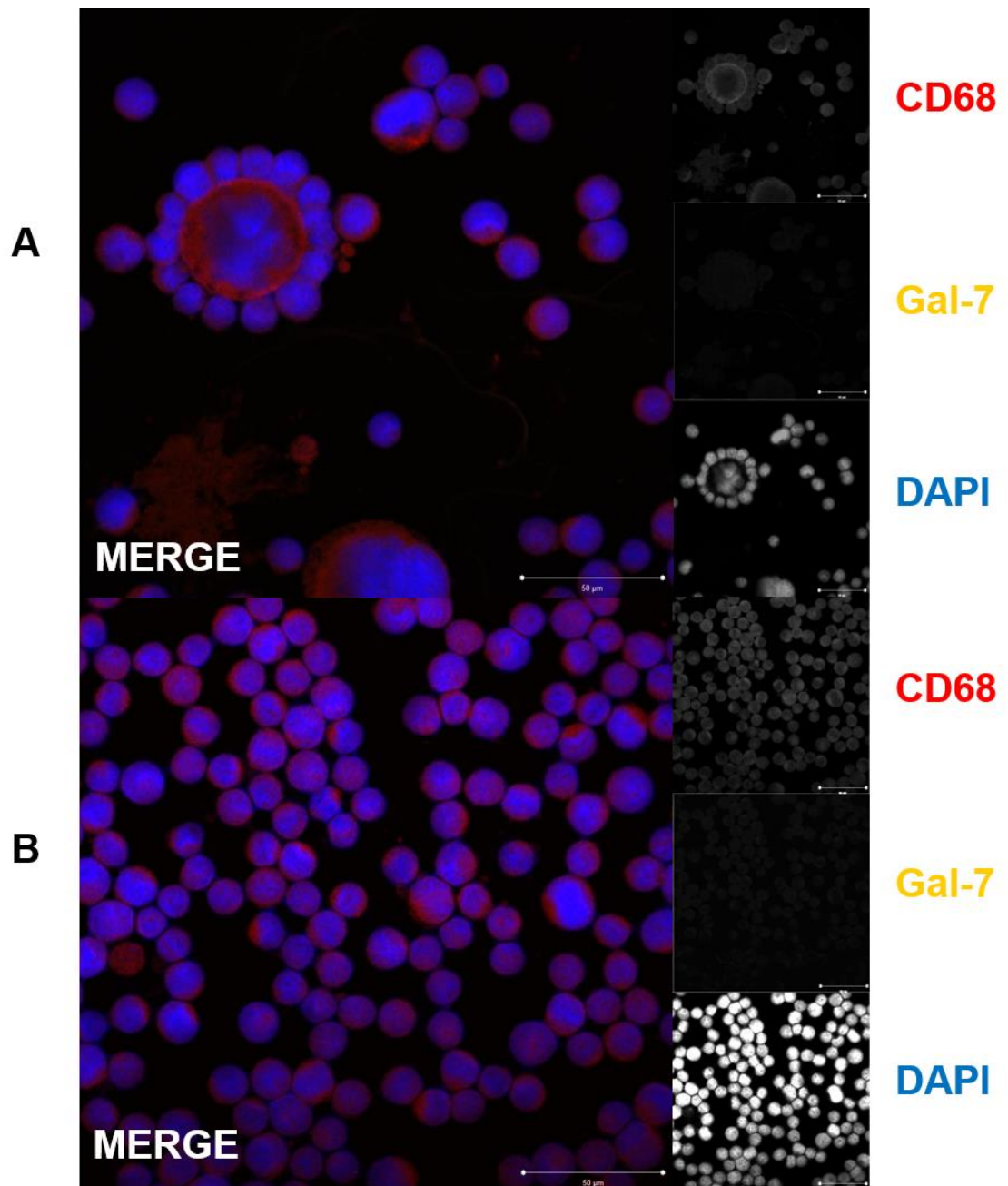


Figure 4.34. Galectin-7 negative control. THP1 (A) and Monomac-6 (B) cells were grown on glass slides with 20ng/mL PMA and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-CD68, anti-galectin-7, FITC and RRX-conjugated secondary antibodies and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

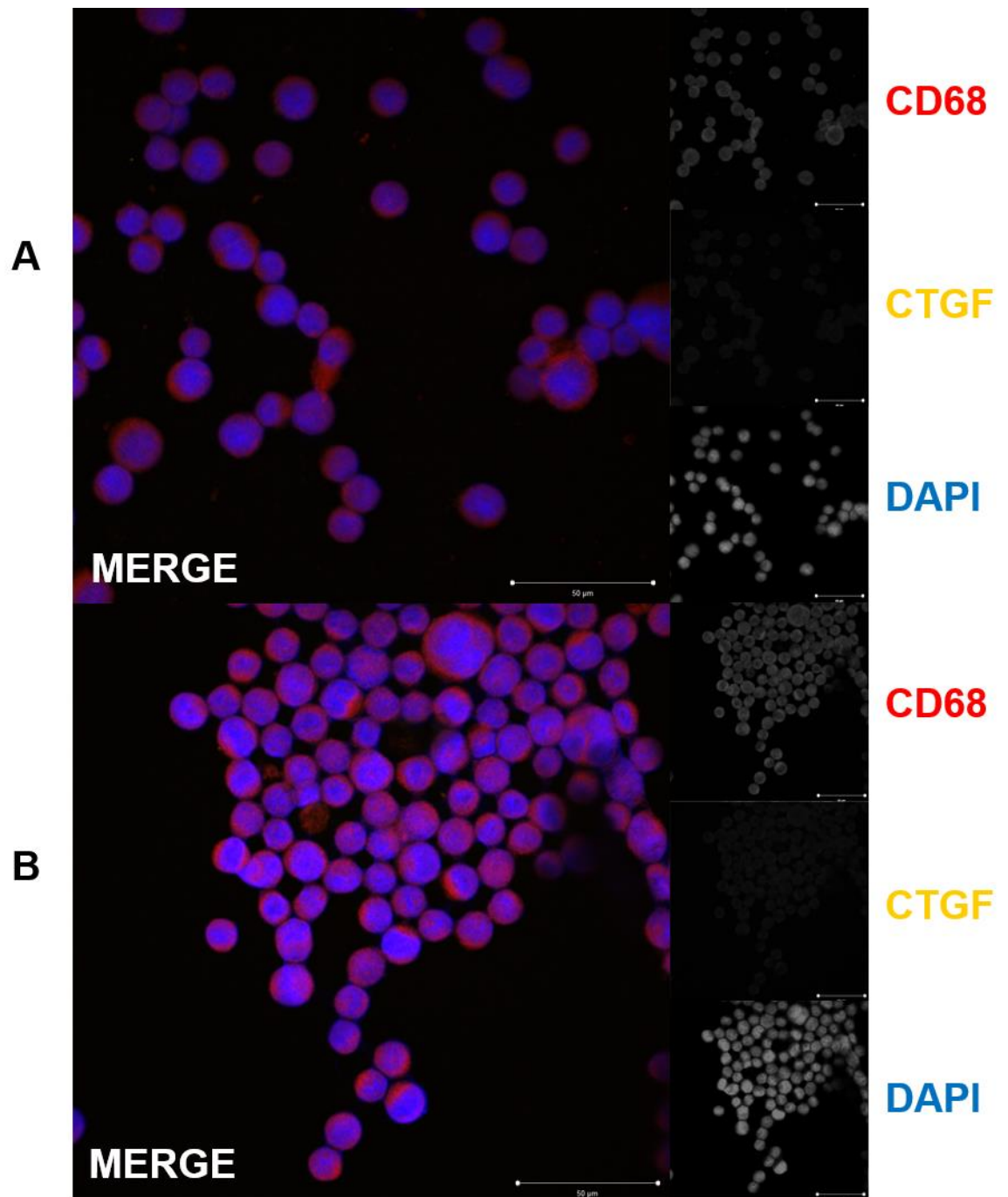


Figure 4.35. CTGF negative control. THP1 (A) and Monomac-6 (B) cells were grown on glass slides with 20ng/mL PMA and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-CD68, anti-CTGF, FITC and RRX-conjugated secondary antibodies and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm.

Human tonsil was used as a negative control for anti-galectin-1 (Figure 4.36A); regional galectin-1 (gal-1) positivity was observed but staining was absent in most cells. While not ideal, when combined with the lack of staining observed with the secondary antibody when the primary was omitted (Figure 4.28), and the lack of staining observed with an isotype-matched control antibody (Figure 4.36B), the specificity of this antibody appeared acceptable.

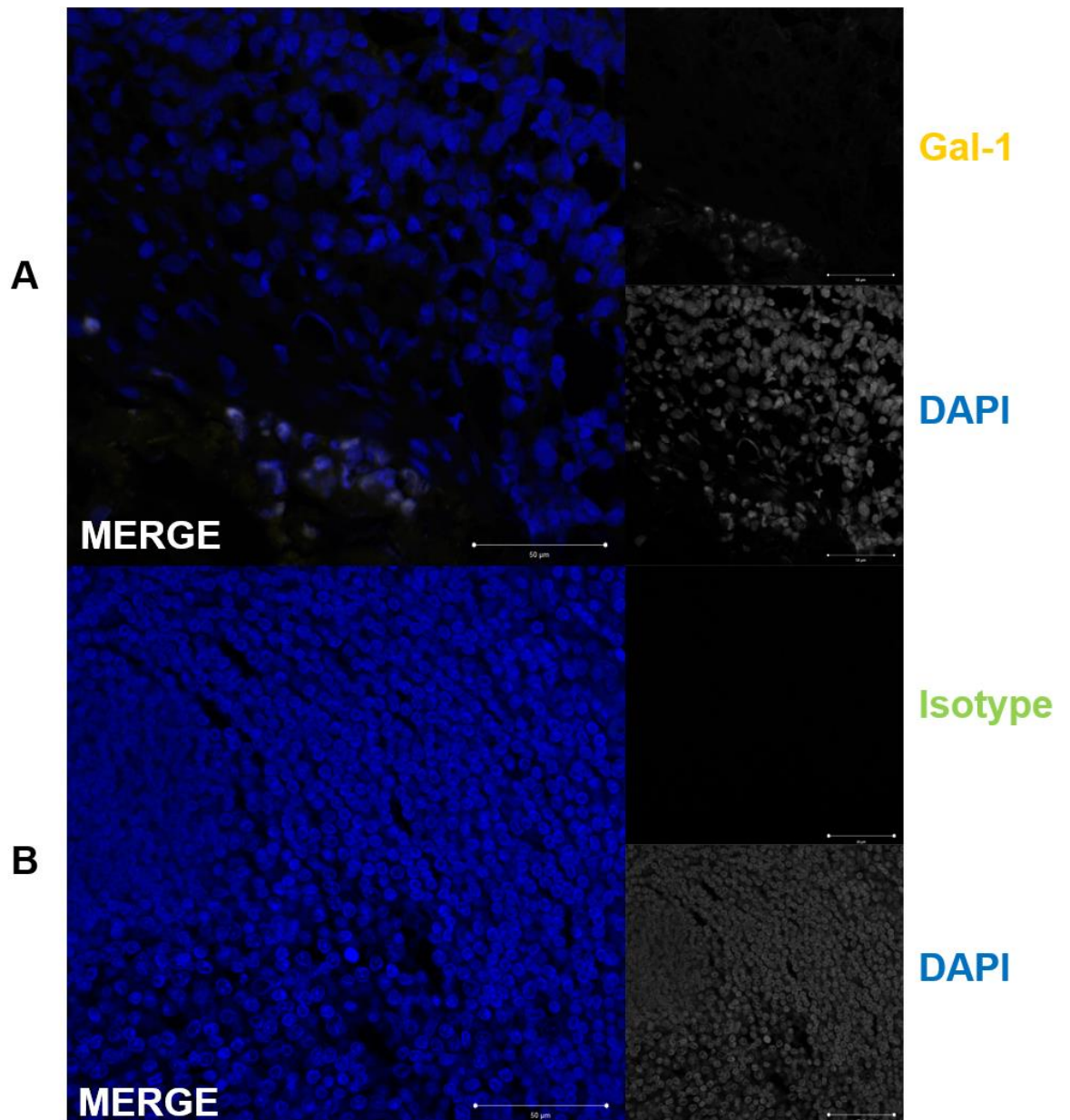


Figure 4.36. Galectin-1 negative control. FFPE human tonsil sections were dewaxed in xylene and antigen retrieval was carried out using five rounds of heating in citrate buffer. Sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-galectin-1 (A) or isotype control (B), FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

4.2.2.2. Expression of target proteins in cell lines

Given that all five proteins were detectable to some extent in GBM TM samples, and some in U251 and U373 cell line supernatants, U251 and U373 cell cultures were stained to examine the presence of the five proteins. Not only would this provide insight into whether the proteins were present at levels detectable both by ELISA and IF, but it would reveal if target proteins undetectable in cell line supernatant were synthesised but not secreted. Additionally, as differentiated macrophages were not used as a negative control for gal-1, these cells were examined for the presence of this protein.

4.2.2.2.1. *Galectin-1*

Initially, differentiated macrophages were examined for the presence of galectin-1 (gal-1). Both THP1-derived macrophages (Figure 4.37A) and Monomac-6-derived macrophages (Figure 4.37B) were gal-1+. Additionally, both cells appeared to deposit extracellular gal-1 onto the slides. It appeared that TAMs may be a source of intratumoural gal-1, although TAMs likely behaved differently from cell line-derived macrophages.

Next, glioma cell lines were stained for gal-1. Both U251 (Figure 4.38A) and U373 (Figure 4.38B) cells were also gal-1+. This was in line with the results seen in 3.2.2.2.2., where both cell lines were found to secrete gal-1 in the nanogram range. However, not all cells were clearly gal-1+, indicating heterogeneity within the cell lines and suggesting that the high levels of secreted gal-1 in culture supernatant samples were produced by a minority population.

As both glioma and macrophage cell lines expressed galectin-1, this protein was likely to have multiple intratumoural sources. This was in line with the literature, where galectin-1 has been documented to be produced by a wide variety of cell types and tissues.

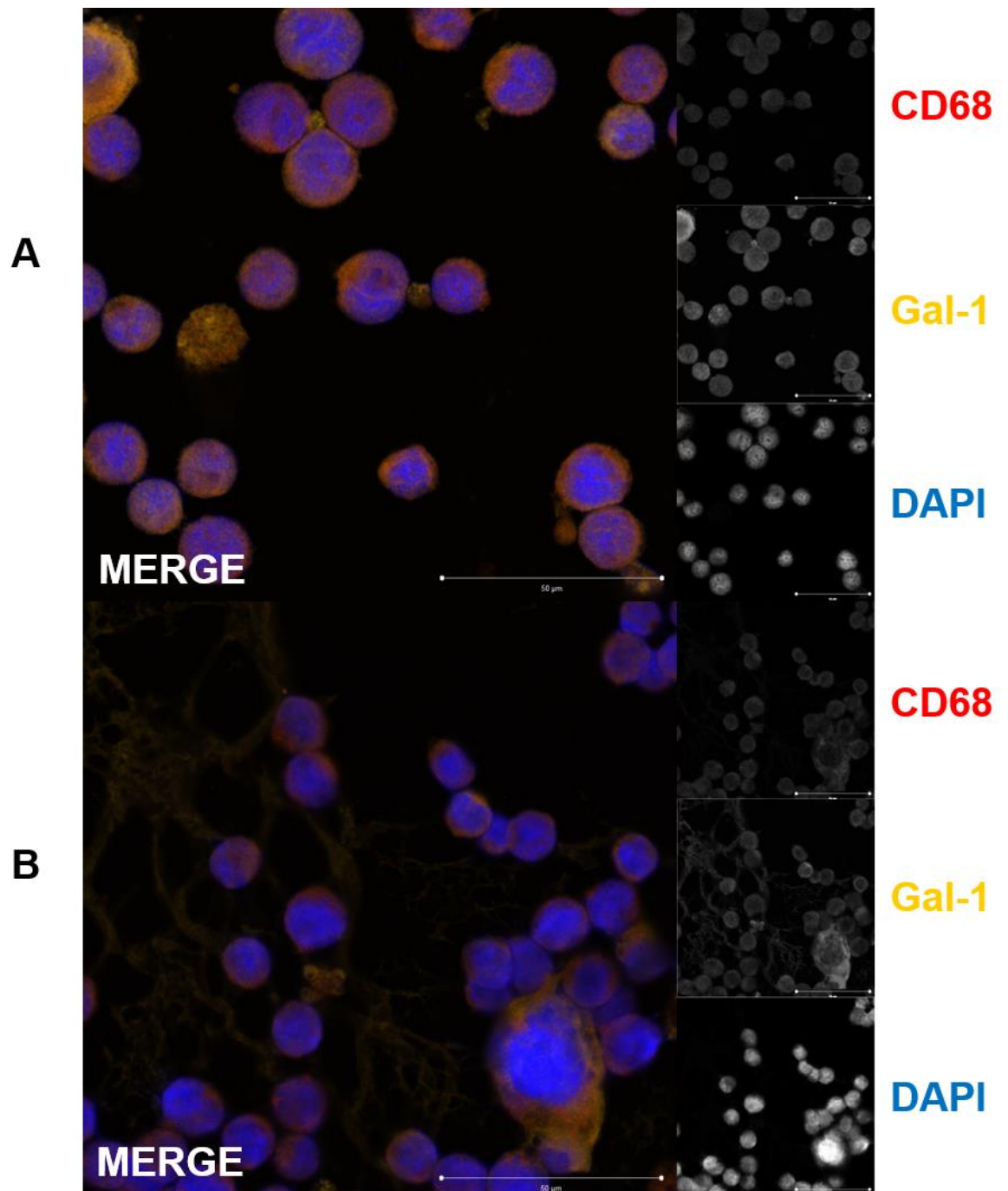


Figure 4.37. Galectin-1 in differentiated macrophages. THP1 (A) and Monomac-6 (B) cells were grown on glass slides with 20ng/mL PMA and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-CD68, anti-galectin-1, FITC- and RRX-conjugated secondary antibodies and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm.

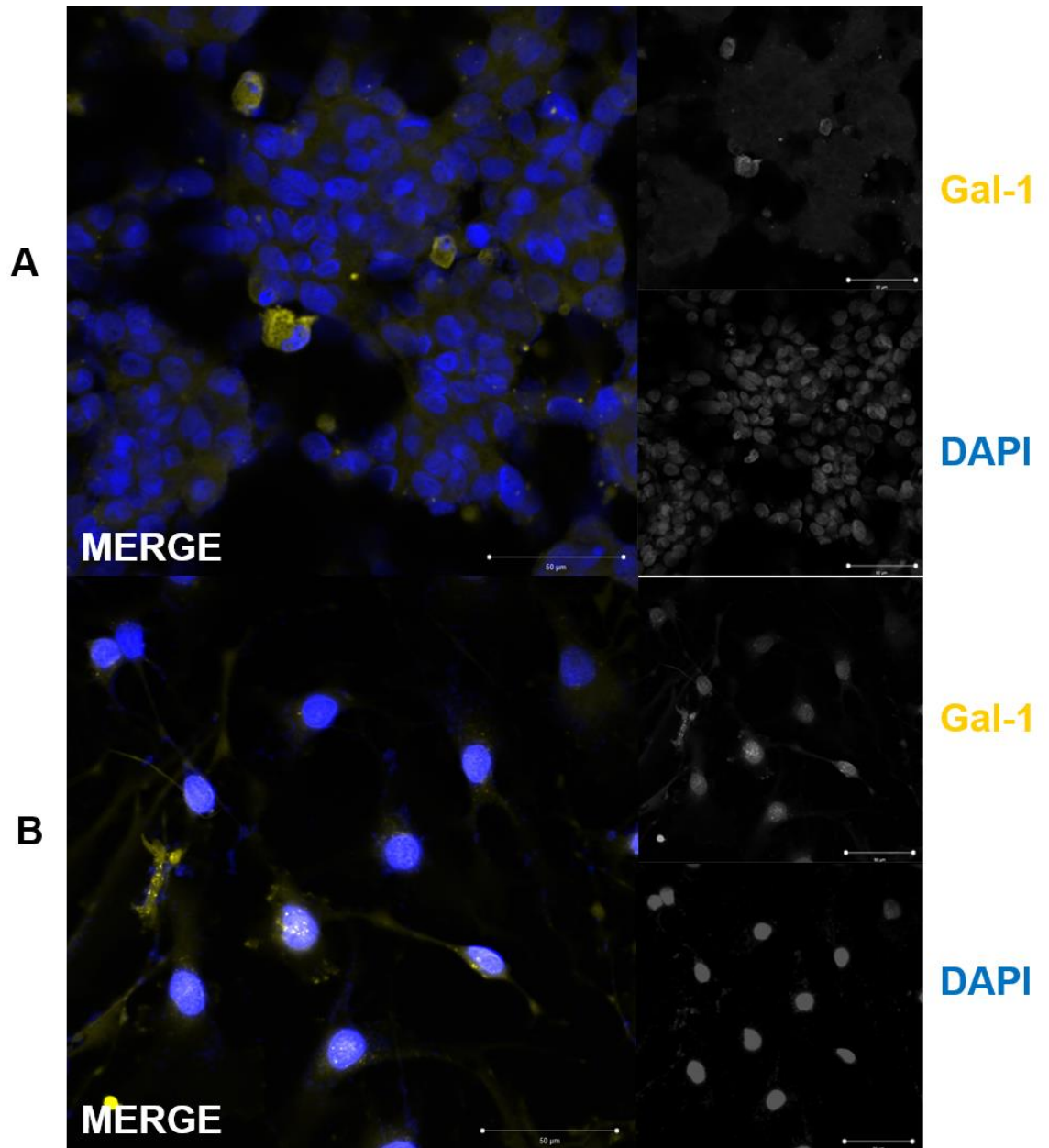


Figure 4.38. Galectin-1 in glioma cell lines. U251 (A) and U373 (B) cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-galectin-1, FITC-conjugated secondary antibody and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50 μ m.

4.2.2.2.2. *Galectin-3*

Glioma cell lines were then stained for the presence of galectin-3 (gal-3). In contrast to the results seen in 3.2.2.2.3., where only U373 supernatant contained detectable gal-3, cells from both U251 (Figure 4.39A) and U373 (Figure 4.39B) lines contained gal-3. Notably, the majority of U251 cells were clearly gal-3+. These results indicated that gal-3 is likely produced by glioma cells, and furthermore, that it is produced but not secreted in the U251 cell line.

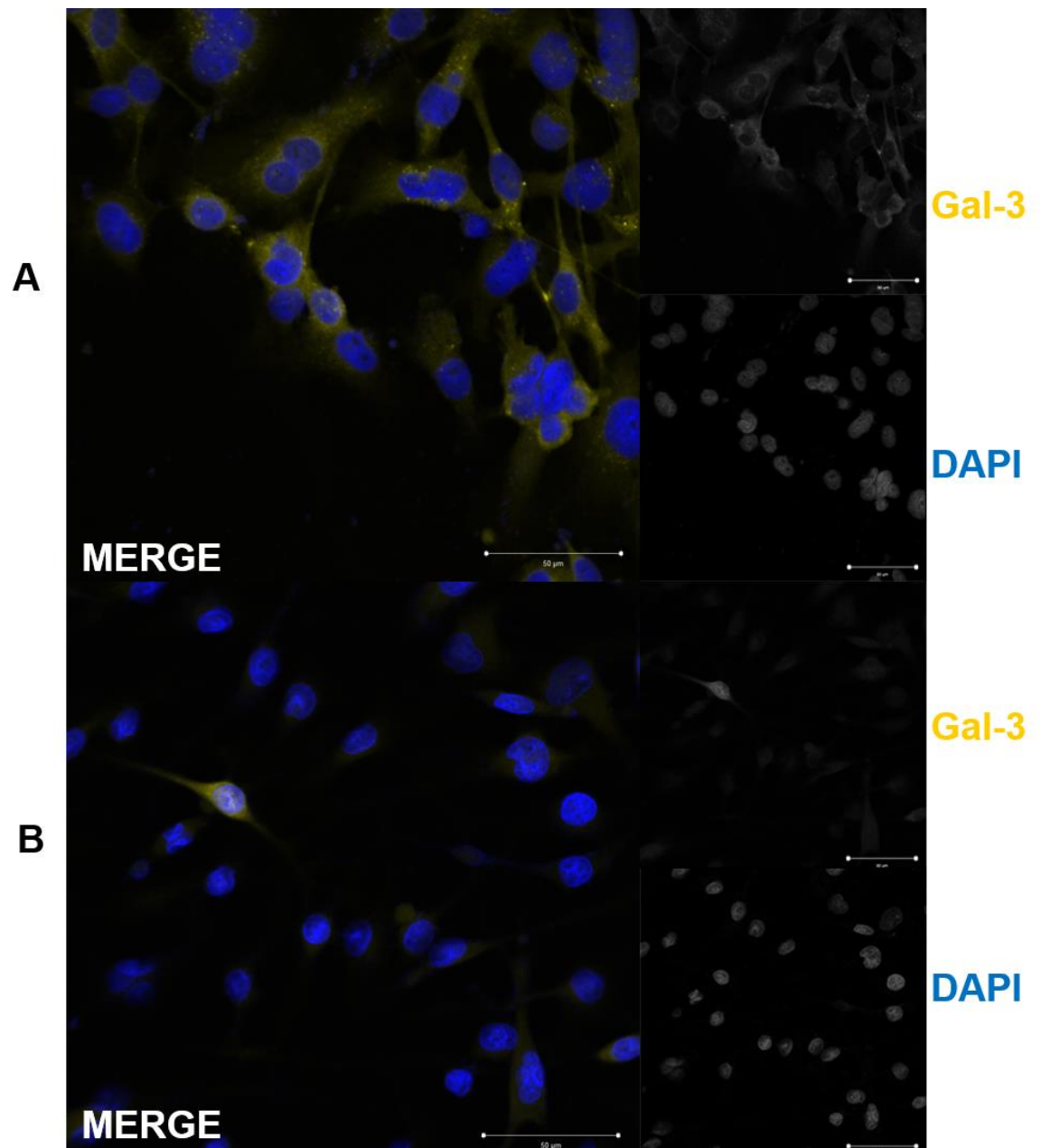


Figure 4.39. Galectin-3 in glioma cell lines. U251 (A) and U373 (B) cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-galectin-3, FITC-conjugated secondary antibody and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

4.2.2.2.3. *Galectin-4*

Glioma cell lines were then examined for the presence of galectin-4 (gal-4), which was not found to be secreted by either cell line in 3.2.2.2.4. The results for U251 are shown in Figure 4.40A and for U373 in Figure 4.40B; both cell lines contained no detectable gal-4. This was in line with the results from ELISA experiments, and thus it can be concluded that gal-4 was unlikely to be produced by glioma cell lines. Instead, it was considered likely that intratumoural gal-4 was produced by a population other than tumour cells.

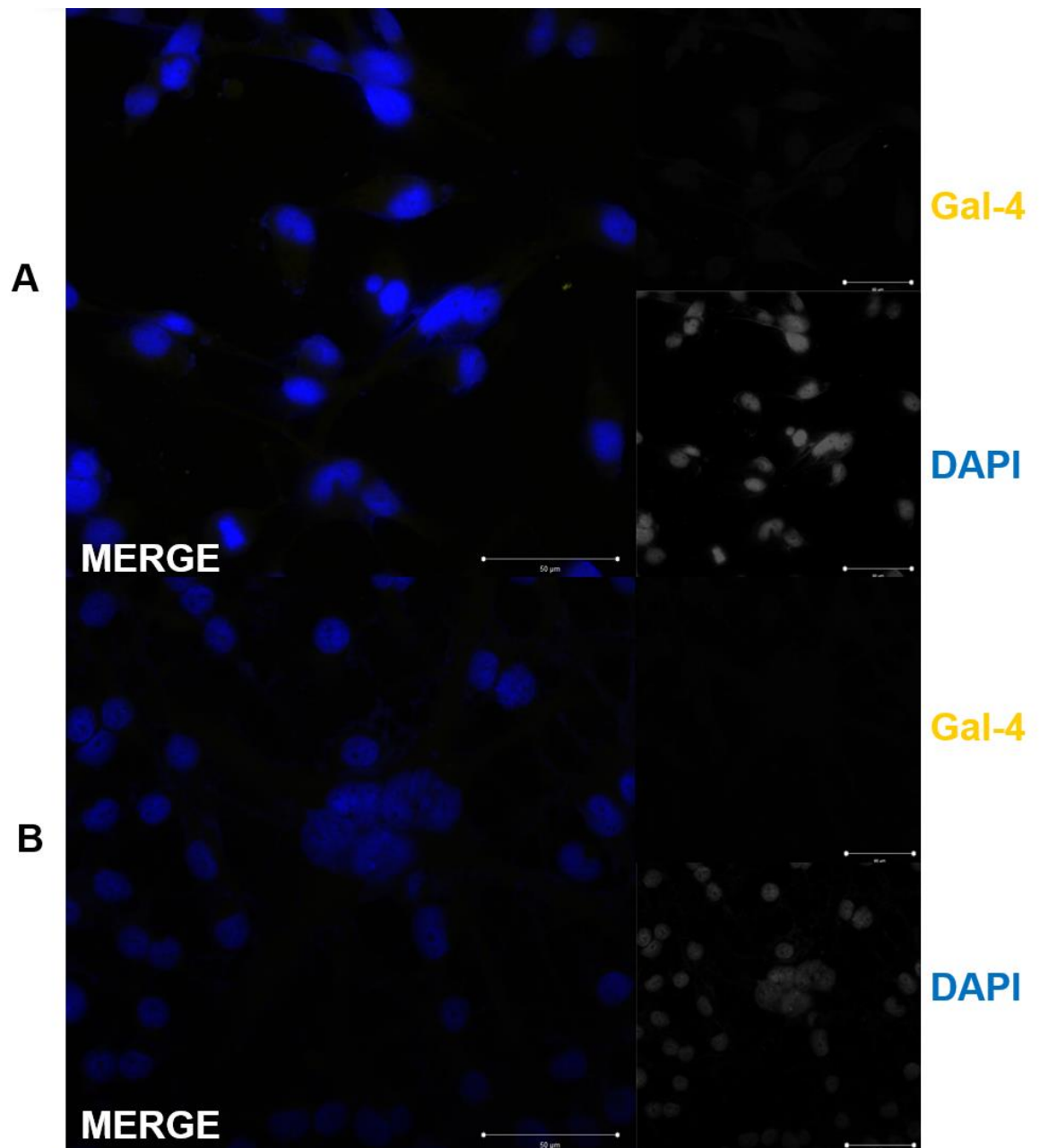


Figure 4.40. Galectin-4 in glioma cell lines. U251 (A) and U373 (B) cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-galectin-4, FITC-conjugated secondary antibody and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50 μ m.

4.2.2.2.4. *Galectin-7*

As with gal-4, galectin-7 (gal-7) was undetectable in U251 and U373 supernatants by ELISA (see 3.2.2.2.5.). To ascertain whether this protein was likely produced by glioma cell lines at all, cultures from both cell lines were stained with anti-galectin-7. No positivity was detected in U251 cells (Figure 4.41A), but a minority of U373 cells appeared to contain gal-7 (Figure 4.41B). This suggested that, as with gal-3, the protein may be produced in some cells but not secreted.

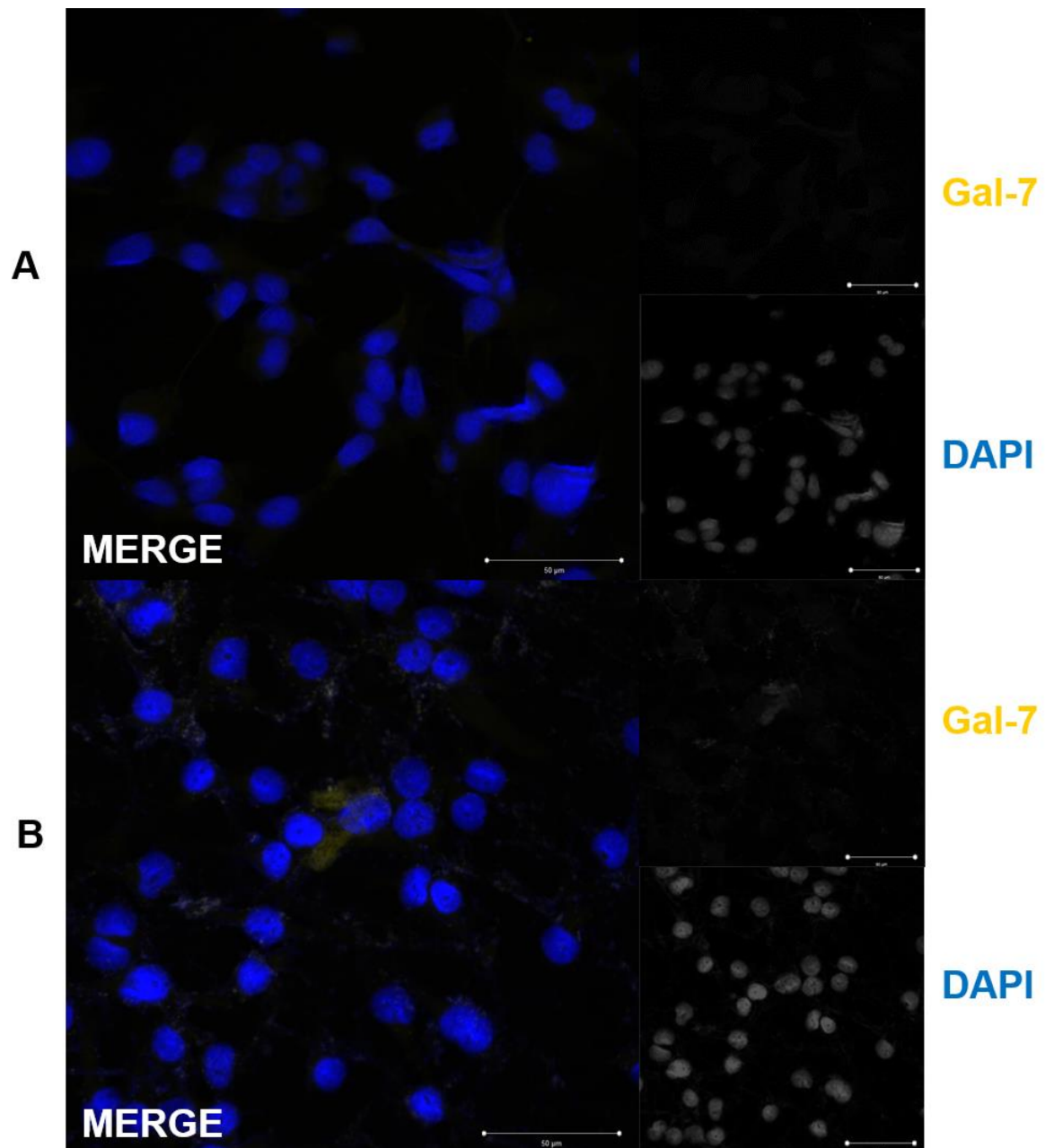


Figure 4.41. Galectin-7 in glioma cell lines. U251 (A) and U373 (B) cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-galectin-7, FITC-conjugated secondary antibody and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

4.2.2.2.5. CTGF

The experiments to detect target protein expression in cell lines were concluded by staining U251 and U373 cells for CTGF (Figure 4.42A and B respectively). In 3.2.2.2.6., CTGF was found to be present in the 1-3ng range in supernatants from both cell lines; in IF experiments, CTGF was detectable in some cells from both lines, clearly indicating synthesis and secretion. As with gal-1, most cells were CTGF-, indicating a highly secretory subset of cells are present.

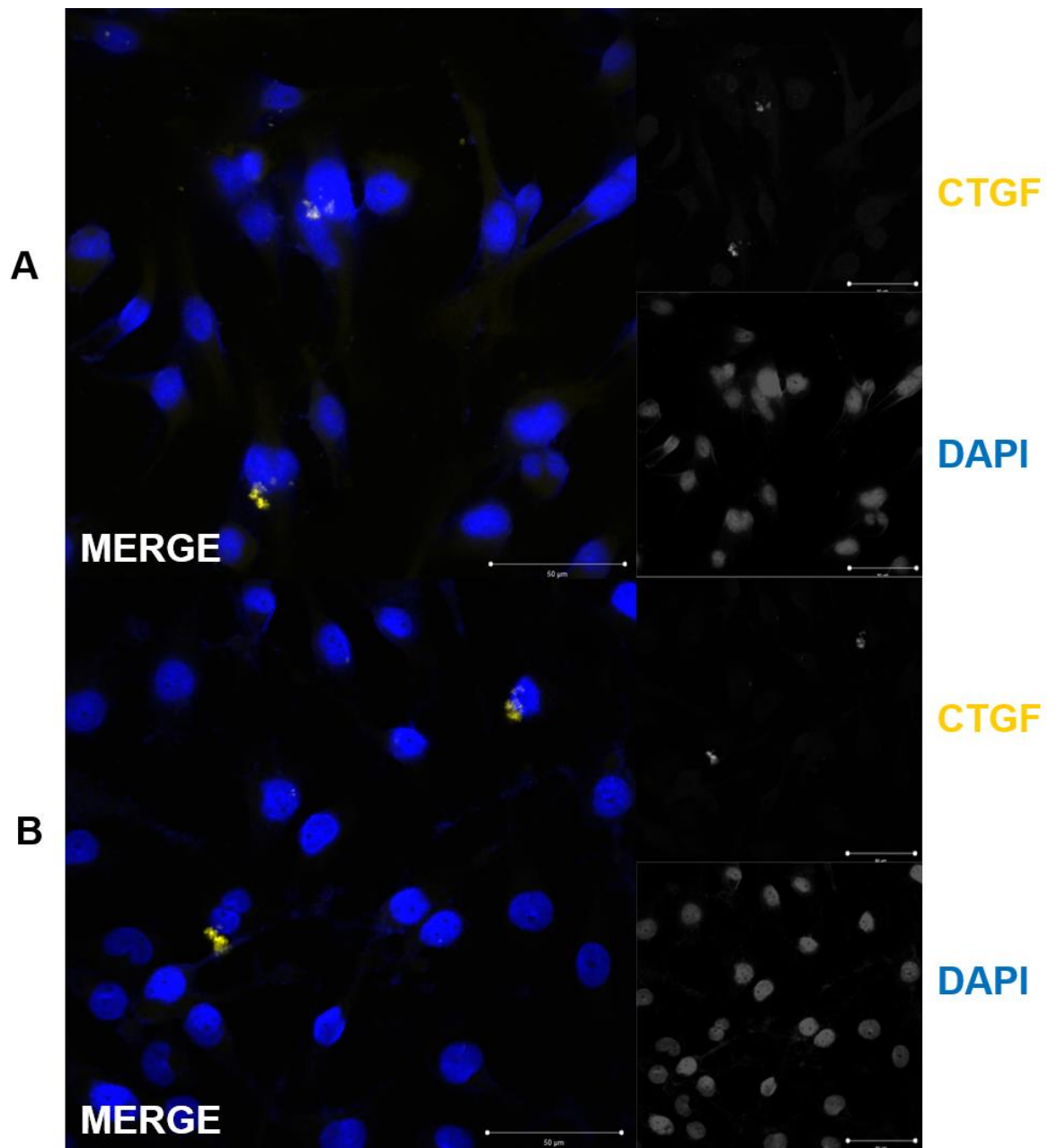


Figure 4.42. CTGF in glioma cell lines. U251 (A) and U373 (B) cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-CTGF, FITC-conjugated secondary antibody and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

4.2.2.3. Distribution of target proteins

In the above section, it was discovered that galectin-1 and -3 and CTGF were produced by U251 and U373 cells, although the results from ELISA experiments in section 3.2.2.2. and subsections therein suggested that detectable gal-3 is not secreted by U251 cells. Galectin-4 was neither produced nor secreted at detectable levels by either cell line; gal-7, though seemingly produced by some U373 cells, was not secreted. Additionally, it was discovered that differentiated THP1- and Monomac-6-derived macrophages were capable of depositing extracellular gal-1.

These results may provide some insight into potential cellular sources of target proteins. However, this was dependent on extrapolating the behaviour of *in vitro* immortalised cells to *in vivo* tumours, which comparison is not rigorous. To address the questions of distribution and cellular sources more specifically, GBM sections were stained with the same antibodies. Cell counts in these experiments were not verified by a second operator; the number of GBM used for each stain was limited by their use in other panels.

4.2.2.3.1. *Galectin-1*

Initially, the intratumoural location of galectin-1 was examined. If the protein was primarily found in the PVN, it may be more likely to interact with T-cells; conversely, location in the TB would imply greater interaction with TAMs and B-cells. These data could shed light on both potential cellular sources and likely cell interactions with this immunosuppressive protein.

To ascertain the intratumoural location of galectin-1+ cells, slides from 19 tumours were stained with anti-galectin-1. 18, or 94.7%, of these tumours contained gal-1. Additionally, 15 of the 18 contained extracellular galectin-1. These data are summarised in Table 4.12.

Gal-1 was often observed in the PVN; this is illustrated in Figure 4.43. Additionally, extracellular gal-1 was seen to form sheet-like deposits, which is shown in Figure 4.44. In both cases, gal-1 was observed in proximity to T-cells. In Figure 4.43, T-cells exiting the vessel would likely come into contact with gal-1+ cells in the PVN; in Figure 4.44, extracellular galectin-1 appears to have surrounded a T-cell aggregation and formed a barrier that the cells cannot enter or cross, thus lending further weight to the hypothesis that gal-1 inhibits T-cell motility.

Table 4.12. Summary of galectin-1 positivity in 19 GBM

Location of galectin-1	Number of positive tumours	% of positive tumours
Cellular	18/19	94.7
Extracellular	15/19	78.9

Table 4.12. Summary of galectin-1 positivity in 19 GBM. Tumour sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-galectin-1, FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Percentages are given to one decimal place.

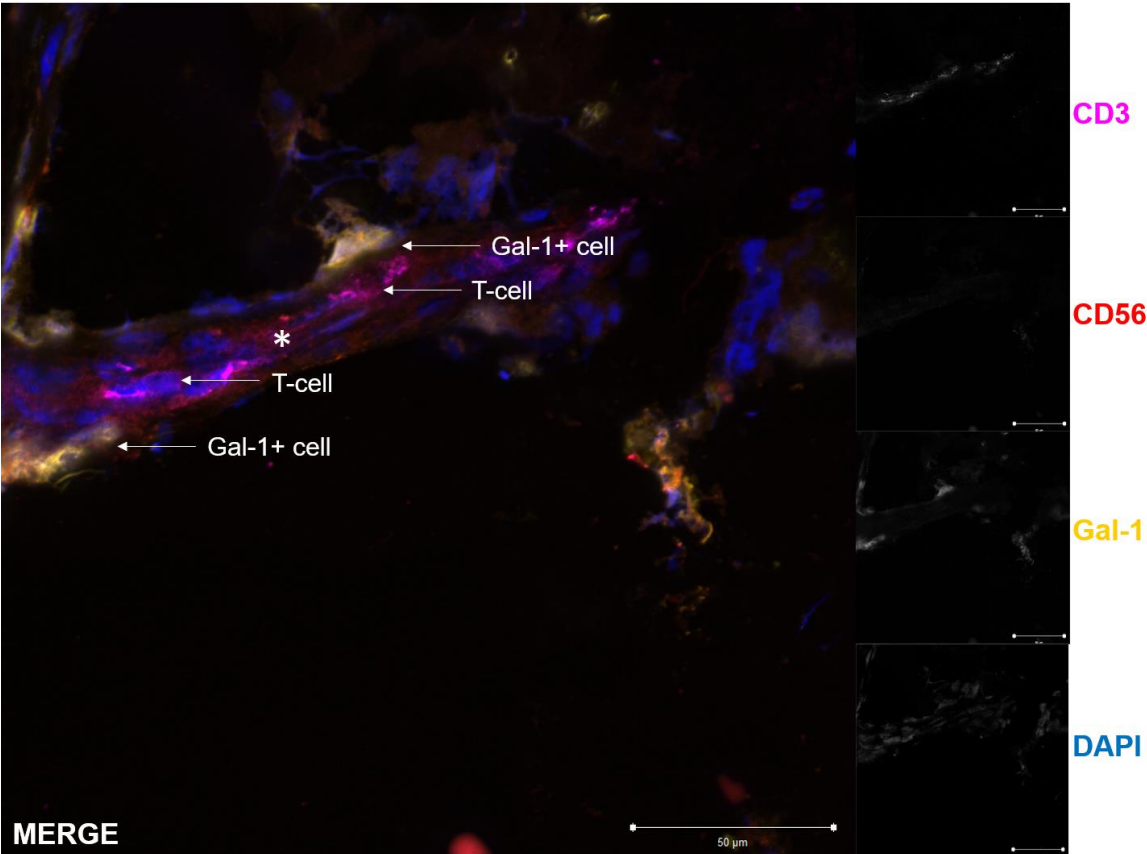


Figure 4.43. Perivascular galectin-1+ cells in proximity to intravascular T-cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-1, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrows indicate T-cells and galectin-1+ cells. Asterisk=vessel lumen.

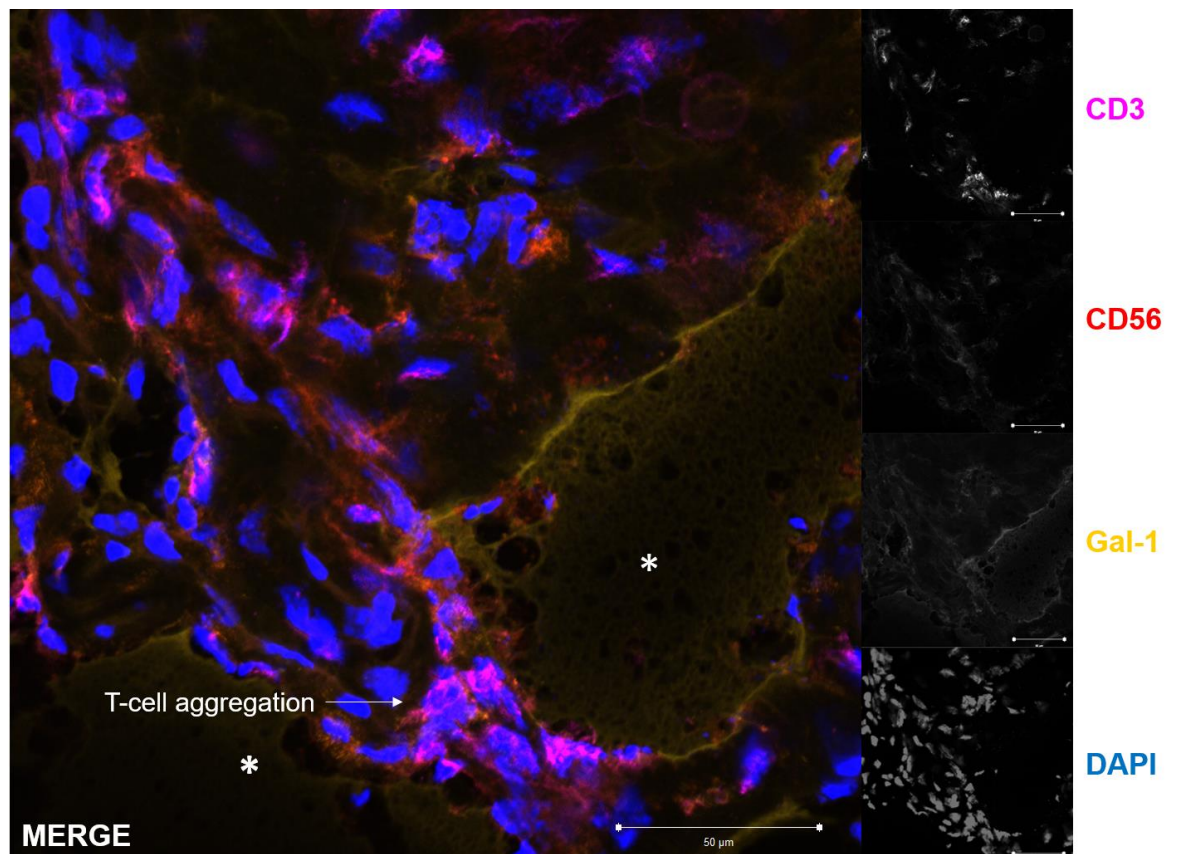


Figure 4.44. Sheetlike galectin-1 deposition surrounding aggregated T-cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-1, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=T-cell aggregation. Asterisks=galectin-1 deposits.

Next, the intratumoural location of galectin-1+ cells was considered. Cells from the 18 positive tumours were counted and categorised as either PV or TB, depending on whether they were within 100µm of the nearest vessel.

2956 galectin-1+ cells were observed. 2383, or 80.6%, were in the PVN, whereas 573, or 19.4%, were found in the TB. This distribution was found to approach, but not reach, significance ($p=0.0844$) by unpaired 2-tailed t-test with Welch's correction (Figure 4.45). The distribution was not entirely surprising, as endothelial cells are one of many populations known to express galectin-1 (Thijssen, Hulsmans and Griffioen, 2008). Clusters of gal-1+ cells in the PVN averaged 4.9 cells, whereas in the TB, mean size was 3.5 cells per cluster; this difference was found to be significant ($p=0.0171$) by unpaired 2-tailed t-test with Welch's correction, as shown in Figure 4.46.

Overall, it appeared that galectin-1-expressing cells were abundant in, and significantly more likely to aggregate with, other gal-1+ cells in the PVN as compared to the TB. These data suggested the possibility of a PV galectin-1-mediated immunosuppressive environment. In light of this protein's known immunosuppressive function and the nearly significant PV presence of T-cells (4.2.1.3.2.), this finding raised the question of whether PV galectin-1 was sufficient to inhibit T-cell motility. The increased proportion of gal-1+ cells in the PVN approached but did not reach significance; however, these data were drawn from a relatively small sample of 18 GBM. In combination with the nearly-significant presence of T-cells in the same niche, it is certainly possible that T-cells experience prolonged contact with gal-1 following and potentially during extravasation.

Intratumoural location of galectin-1+ cells

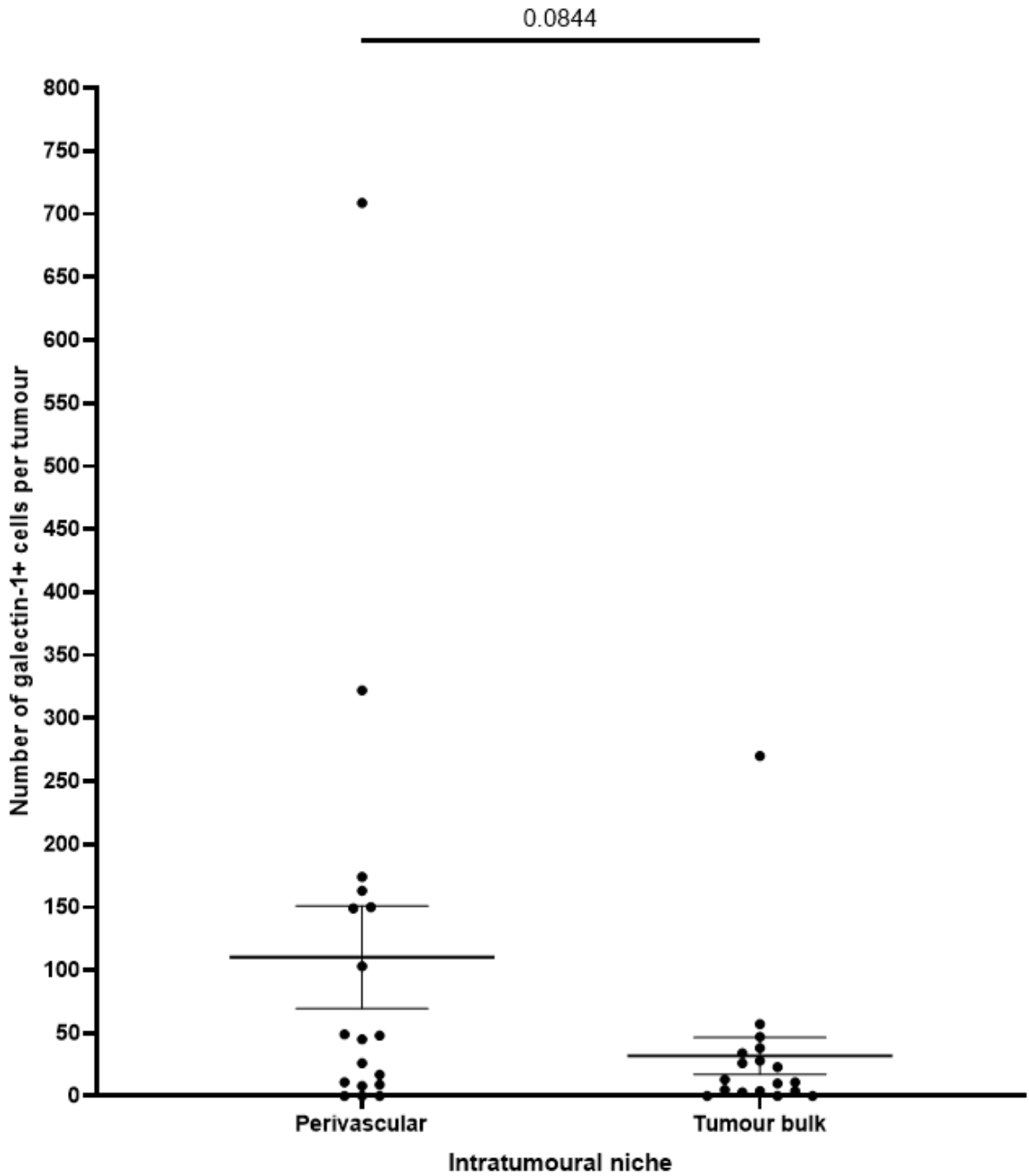


Figure 4.45. Intratumoural location of galectin-1-expressing cells. 2956 cells were counted in 18 GBM stained with anti-galectin-1 and their location was categorised as perivascular (within 100µm of a vessel) or tumour bulk. Data were analysed using unpaired 2-tailed t-test with Welch's correction. Error bars=mean ± SEM.

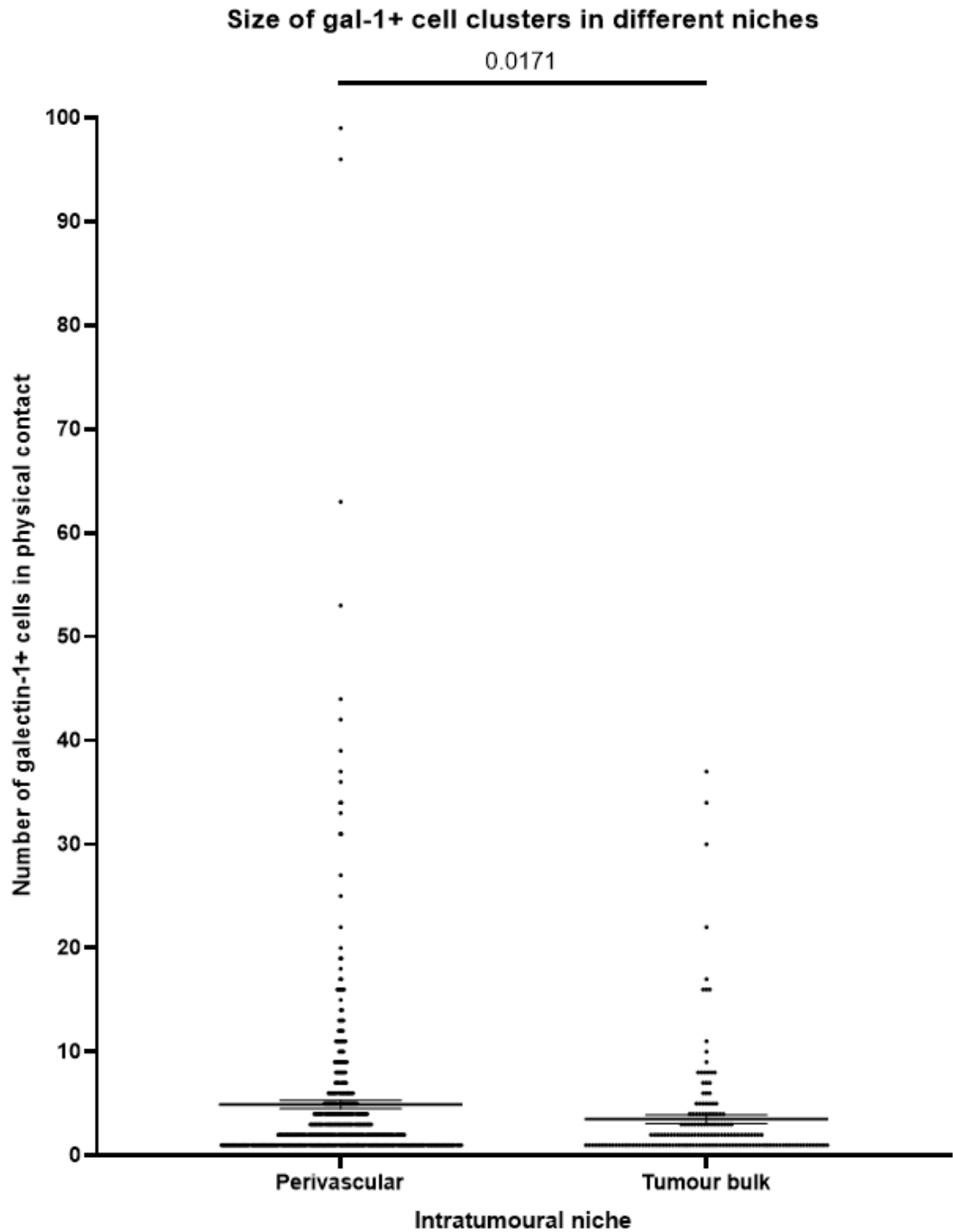


Figure 4.46. Size of gal-1+ cell clusters in different niches. 2956 cells were counted in 18 GBM stained with anti-galectin-1 and the number of gal-1+ cells in physical contact with each other in each image was recorded. Their location was categorised as perivascular (within 100µm of a vessel) or tumour bulk. Data were analysed using unpaired 2-tailed t-test with Welch's correction. Error bars=mean ± SEM.

4.2.2.3.2. *Galectin-3*

Next, the intratumoural location of galectin-3 was examined. Like galectin-1, this protein is known to be immunosuppressive, and thus categorising its location was of interest in determining its potential sources and TIL interactions with this protein.

Sections from 18 tumours were stained with anti-galectin-3. 15 of 18 tumours contained detectable gal-3; furthermore, 10 of those 15 contained extracellular gal-3. These data are summarised in Table 4.13, and a representative example of clustered gal-3+ cells is shown in Figure 4.47.

Table 4.13. Summary of galectin-3 positivity in 18 GBM

Location of galectin-3	Number of positive tumours	% of positive tumours
Cellular	15/18	83.3
Extracellular	10/18	55.5

Table 4.13. Summary of galectin-3 positivity in 18 GBM. Tumour sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-galectin-3, FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Percentages are given to one decimal place.

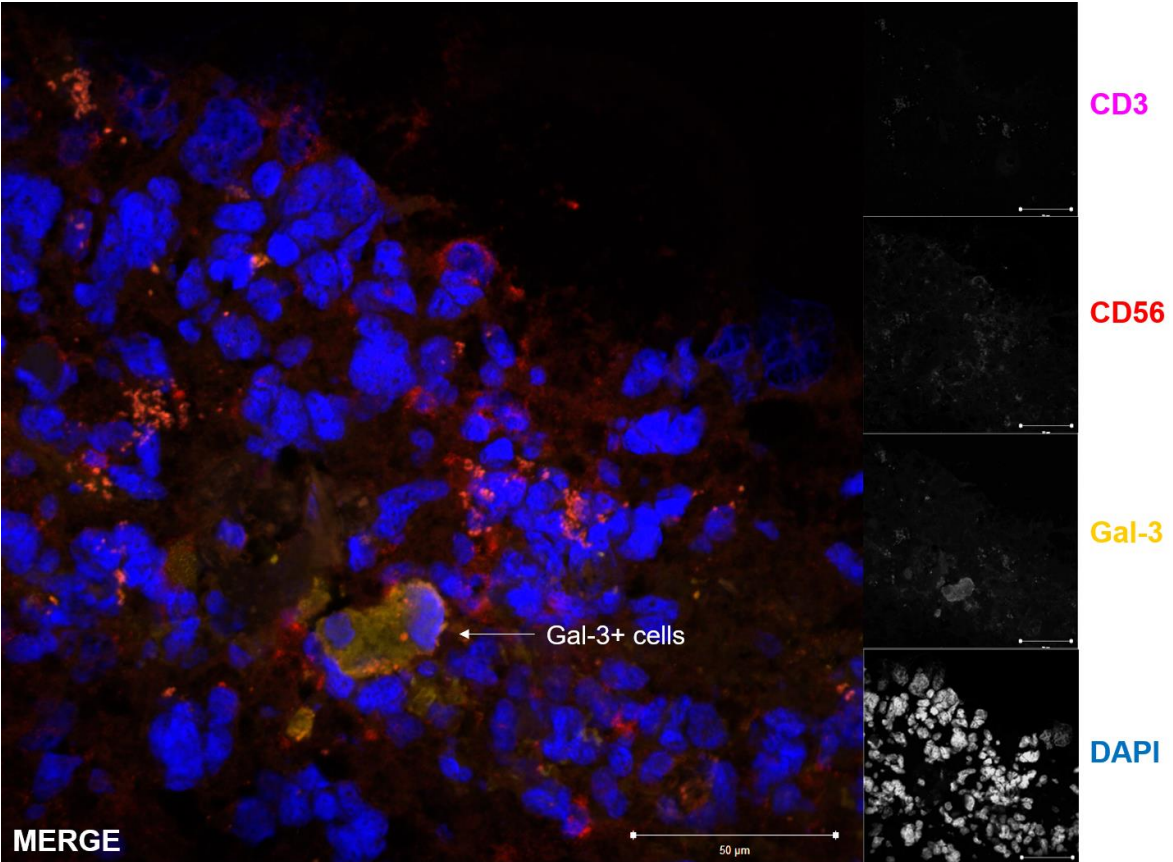


Figure 4.47. Clustered galectin-3 cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD68, anti-galectin-3, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm. Arrow=clustered gal-3+ cells.

Having confirmed the presence of galectin-3 in the majority of GBM, gal-3+ cells were counted and their intratumoural location determined.

Across the 15 positive tumours, 1204 positive cells were observed. The majority – 1137, or 94.4% – were located in the TB, whereas PV gal-3+ cells were rare. However, this did not approach significance ($p=0.1069$ by unpaired 2-tailed t-test with Welch's correction); this was likely due to the presence of four GBM with very high numbers of gal-3+ cells in the TB, leading to a wide spread of these data as seen in Figure 4.48. An example of one of these tumours is shown in Figure 4.47.

When gal-3+ cell clustering was studied, PV gal-3+ cells were found to be almost exclusively present alone or in pairs, giving a mean average cluster size of 1.7 cells; clusters in the TB were larger, at an average of 3.3 cells. This was found to be significant ($p=0.0034$) by 2-tailed t-test with Welch's correction (Figure 4.49).

Like gal-1, gal-3 was a common protein in the GBM TME, but unlike gal-1, did not show a clear tendency towards either niche. Additionally, gal-3+ cells in the TB formed larger clusters than those in the PVN; the reasons for this were unclear, and could not be addressed in this series of experiments.

Intratumoural location of galectin-3+ cells

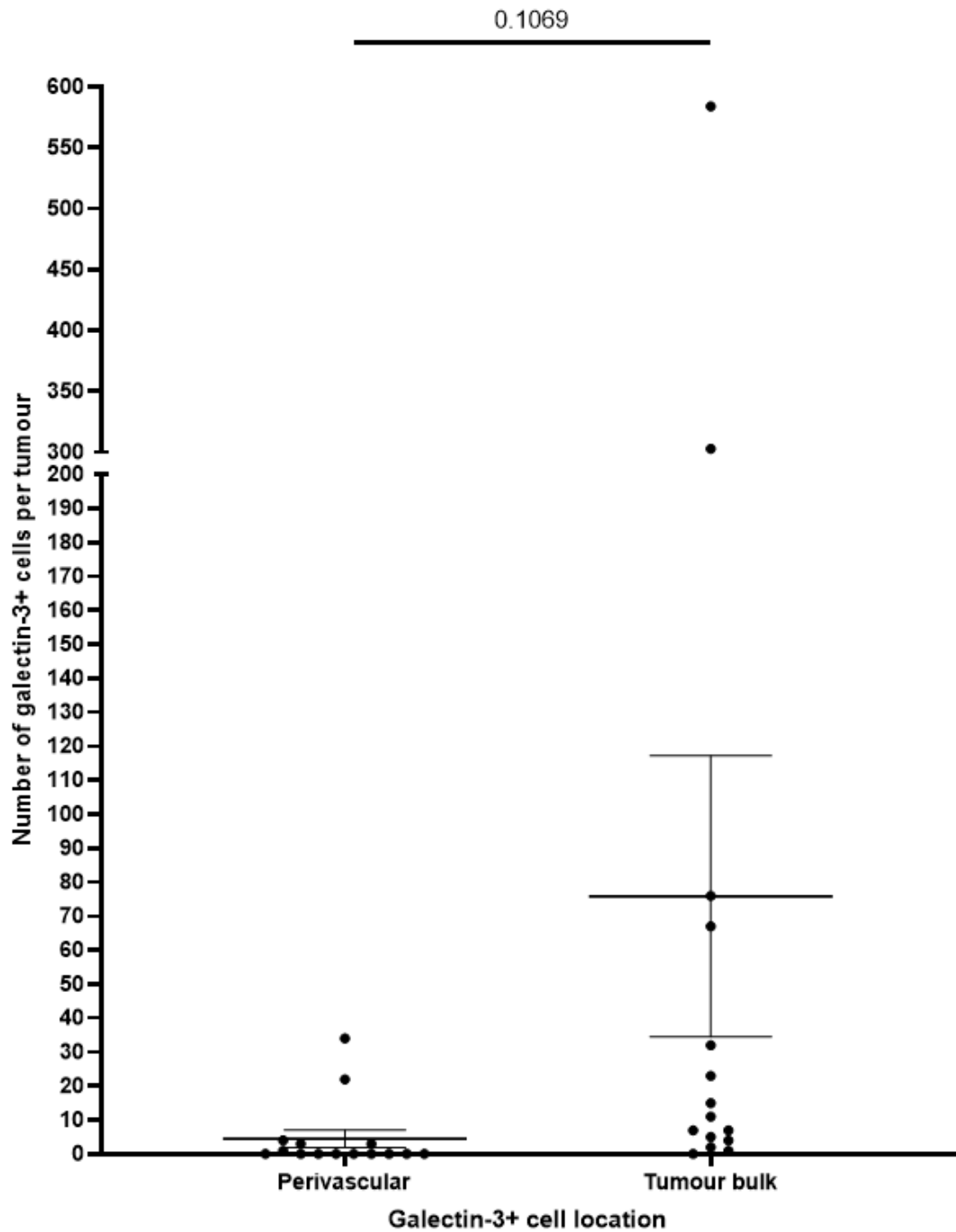


Figure 4.48. Intratumoural location of galectin-3-expressing cells. 1204 cells were counted in 15 GBM stained with anti-galectin-3 and their location was categorised as perivascular (within 100µm of a vessel) or tumour bulk. Data were analysed using unpaired 2-tailed t-test with Welch's correction. Error bars=mean ± SEM.

4.2.2.3.3. *Galectin-4*

The same analysis of intratumoural location and cell clustering was performed for galectin-4. Unlike gal-1 and gal-3, this protein has not been widely studied in GBM; data on its distribution and immunosuppressive effects in this context was extremely limited, rendering this analysis a valuable contribution to the field.

Across 13 gal-4+ GBM, 11 were found to be positive, containing a total of 336 gal-4+ cells. Five of these tumours also contained extracellular gal-4; these data are summarised in Table 4.14. A representative image of gal-4+ cells is shown in Figure 4.50.

Table 4.14. Summary of galectin-4 positivity in 13 GBM

Location of galectin-4	Number of positive tumours	% of positive tumours
Cellular	11/13	84.6
Extracellular	5/13	38.4

Table 4.14. Summary of galectin-4 positivity in 13 GBM. Tumour sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-galectin-4, FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Percentages are given to one decimal place.

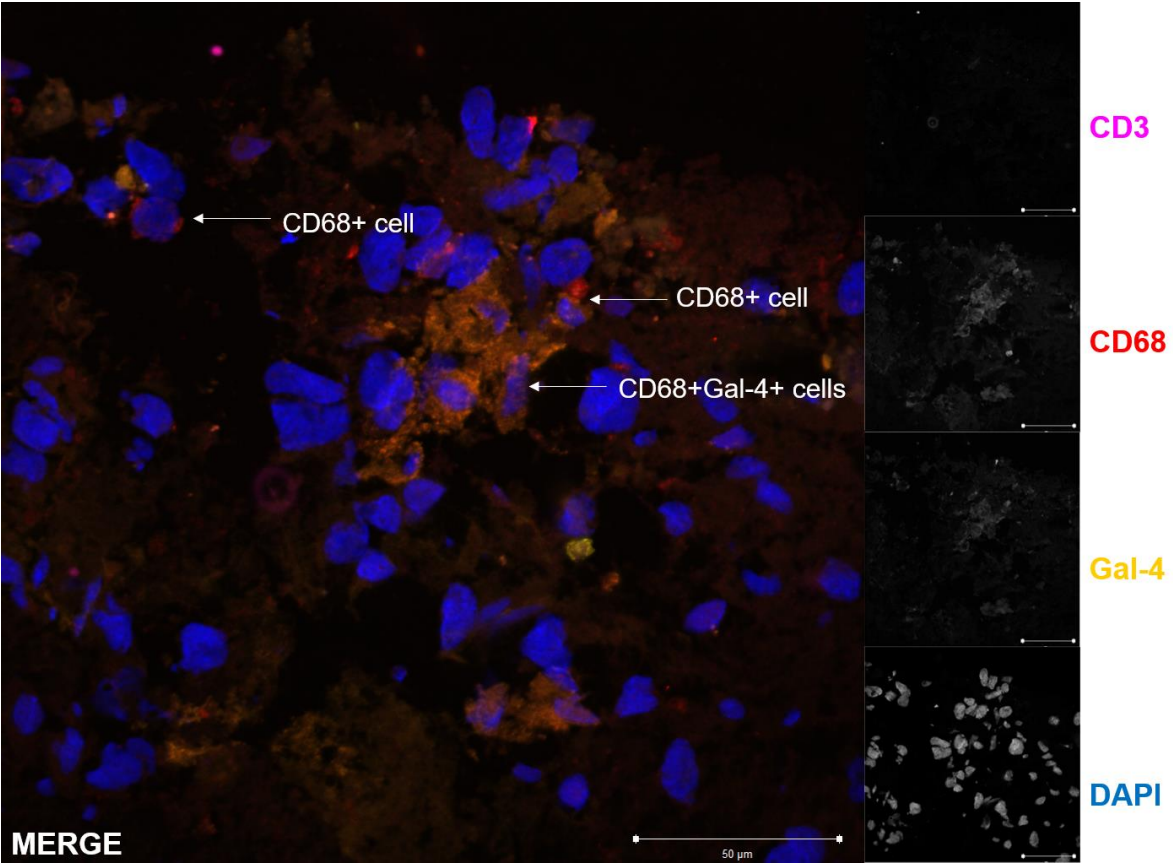


Figure 4.50. Intratumoural galectin-4+ cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-4, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm. Arrows=gal-4+CD68+ cells and single-positive TAMs.

The intratumoural location of the 336 galectin-4-expressing cells identified in these 11 GBM was then analysed. A majority of cells – 261, or 77.7% – were in the TB; the remaining 75 were PV. However, as shown in Figure 4.51, this distribution was not found to be significant ($p=0.1432$). It is possible that, with a greater sample size, this would have reached significance; however, this is not a certainty.

The size of gal-4+ cell clusters also did not show a significant difference between the PV and TB niches. In the PVN, average cell cluster size was 2.9, whereas TB cell clusters averaged 2.5 cells; this was not found to be significant ($p=0.5993$) by unpaired 2-tailed t-test with Welch's correction (Figure 4.52).

Based on the present data, it was concluded that gal-4 may be widely expressed in GBM, and that gal-4+ cells did not show a clear preference for different intratumoural niches.

Intratumoural location of galectin-4+ cells

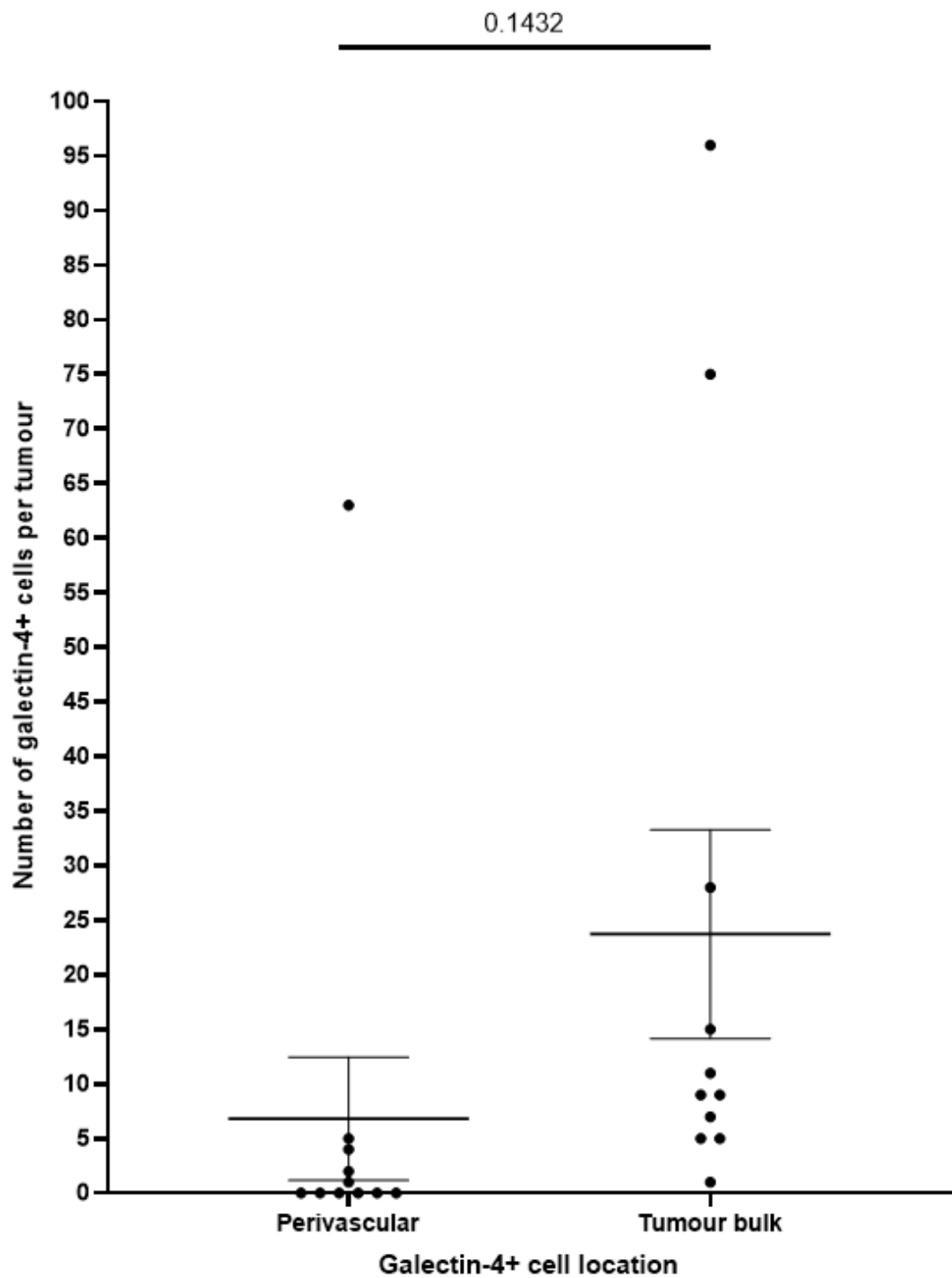


Figure 4.51. Intratumoural location of galectin-4-expressing cells. 336 cells were counted in 11 GBM stained with anti-galectin-4 and their location was categorised as perivascular (within 100µm of a vessel) or tumour bulk. Data were analysed using unpaired 2-tailed t-test with Welch's correction. Error bars=mean ± SEM.

4.2.2.3.4. *Galectin-7*

Galectin-7, like gal-4, is not well-characterised in the context of GBM. For this reason, it was considered worthwhile to perform an analysis of the intratumoural location and clustering of gal-7+ cells in a limited number of tumours.

To ascertain the intratumoural distribution of galectin-7+ cells, sections from 13 GBM were stained with anti-galectin-7. 10 of the 13 contained galectin-7+ cells, and six also contained extracellular gal-7. These data are summarised in Table 4.15, and a representative image of gal-7-expressing cells is shown in Figure 4.53.

Table 4.15. Summary of galectin-7 positivity in 13 GBM

Location of galectin-7	Number of positive tumours	% of positive tumours
Cellular	10/13	76.9
Extracellular	6/13	46.2

Table 4.15. Summary of galectin-7 positivity in 13 GBM. Tumour sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-galectin-7, FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Percentages are given to one decimal place.

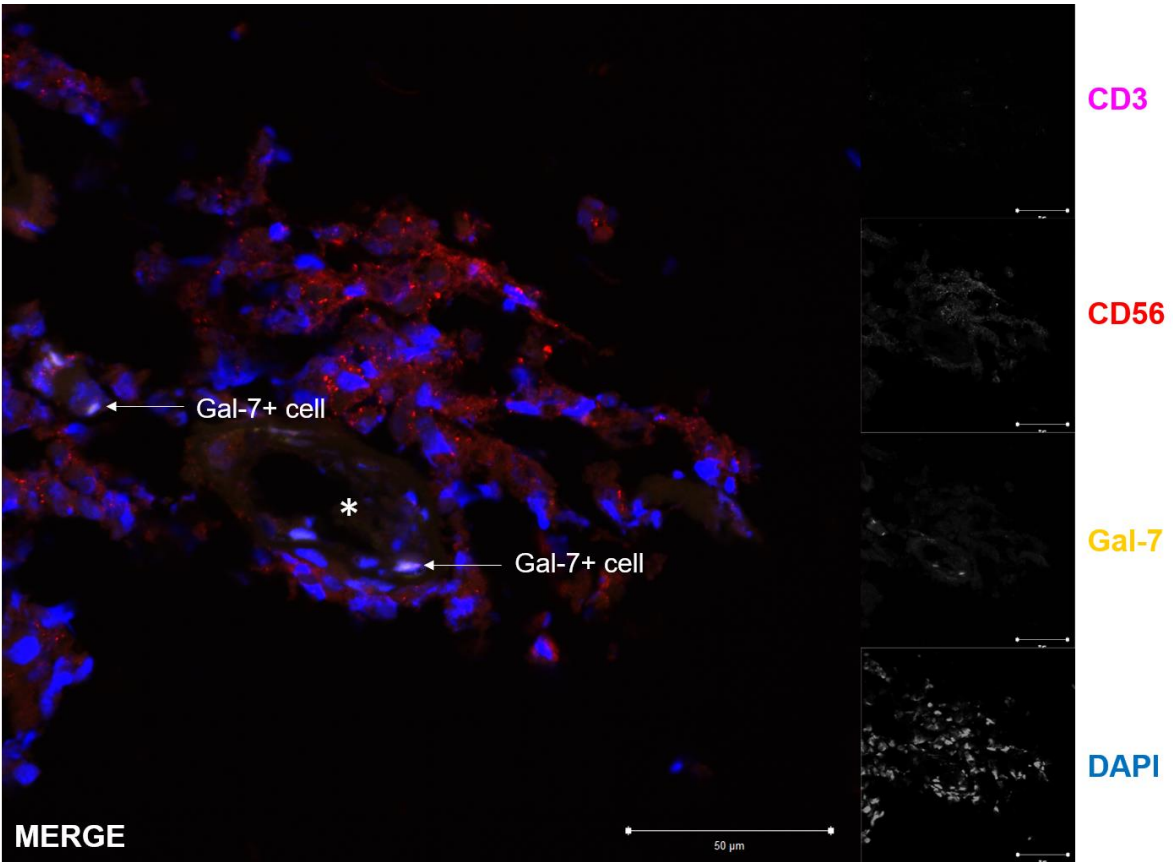


Figure 4.53. Intratumoural galectin-7+ cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-7, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm. Arrows=gal-7+ cells. Asterisk=vessel lumen.

Galectin-7-expressing cells in the 10 positive tumours were counted and their niche categorised as either PV or TB.

123 cells were observed in these 10 sections. 42, or 34.1%, were PV, and the remaining 81, or 65.9% were found in the TB. This was the most equitable distribution observed so far, and unsurprisingly, was not found to reach significance ($p=0.3149$) (Figure 4.54).

Gal-7+ cell clustering was also documented, and it was found that cell clusters showed very little variation: the mean average number of cells in PV clusters was 1.9, whereas in the TB it was 1.7. These data also did not reach significance ($p=0.7019$), and are shown in Figure 4.55.

In conclusion, galectin-7, like galectin-4, was relatively common in GBM, and did not show a clear presence in one niche over the other. Furthermore, there appeared to be no significant difference in gal-7+ cell cluster sizes between the TB and PVN.

Intratumoural location of galectin-7+ cells

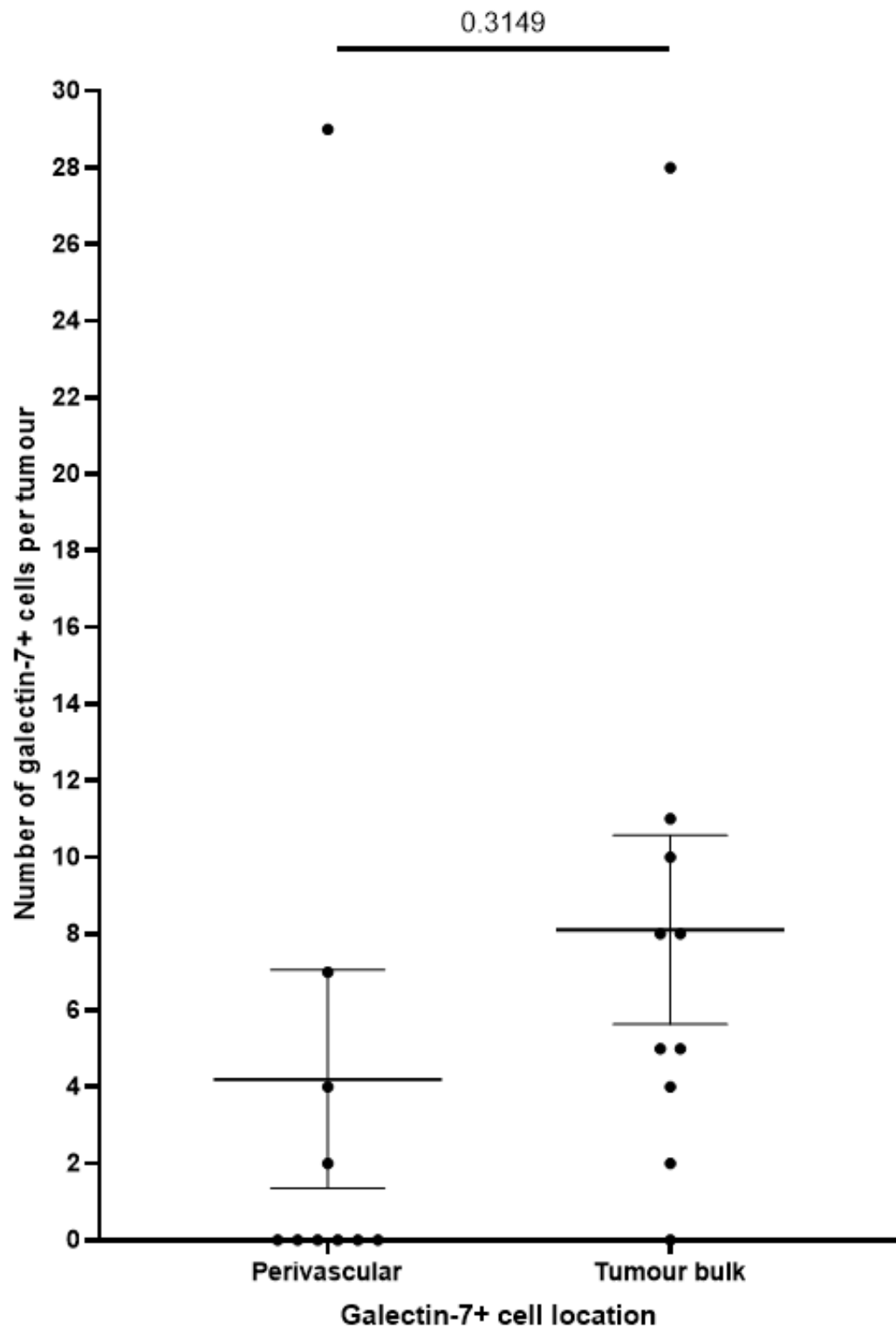


Figure 4.54. Intratumoural location of galectin-7-expressing cells. 123 cells were counted in 10 GBM stained with anti-galectin-7 and their location was categorised as perivascular (within 100µm of a vessel) or tumour bulk. Data were analysed using unpaired 2-tailed t-test with Welch's correction. Error bars=mean ± SEM.

4.2.2.3.5. CTGF

Finally, the intratumoural distribution of CTGF was determined. As with gal-1 and gal-3, this protein has been observed to have immunosuppressive properties.

Sections from 14 GBM were stained with anti-CTGF to determine the intratumoural distribution of this protein. Seven of the 14 tumours expressed CTGF both intra- and extracellularly, and a further two contained cellular CTGF, which is summarised in Table 4.16; Figure 4.56 is a representative image of CTGF+ cells.

Table 4.16. Summary of CTGF positivity in 14 GBM.

Location of CTGF	Number of positive tumours	% of positive tumours
Cellular	9/14	64.3
Extracellular	7/14	50.0

Table 4.16. Summary of CTGF positivity in 18 GBM. Tumour sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CTGF, FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Percentages are given to one decimal place.

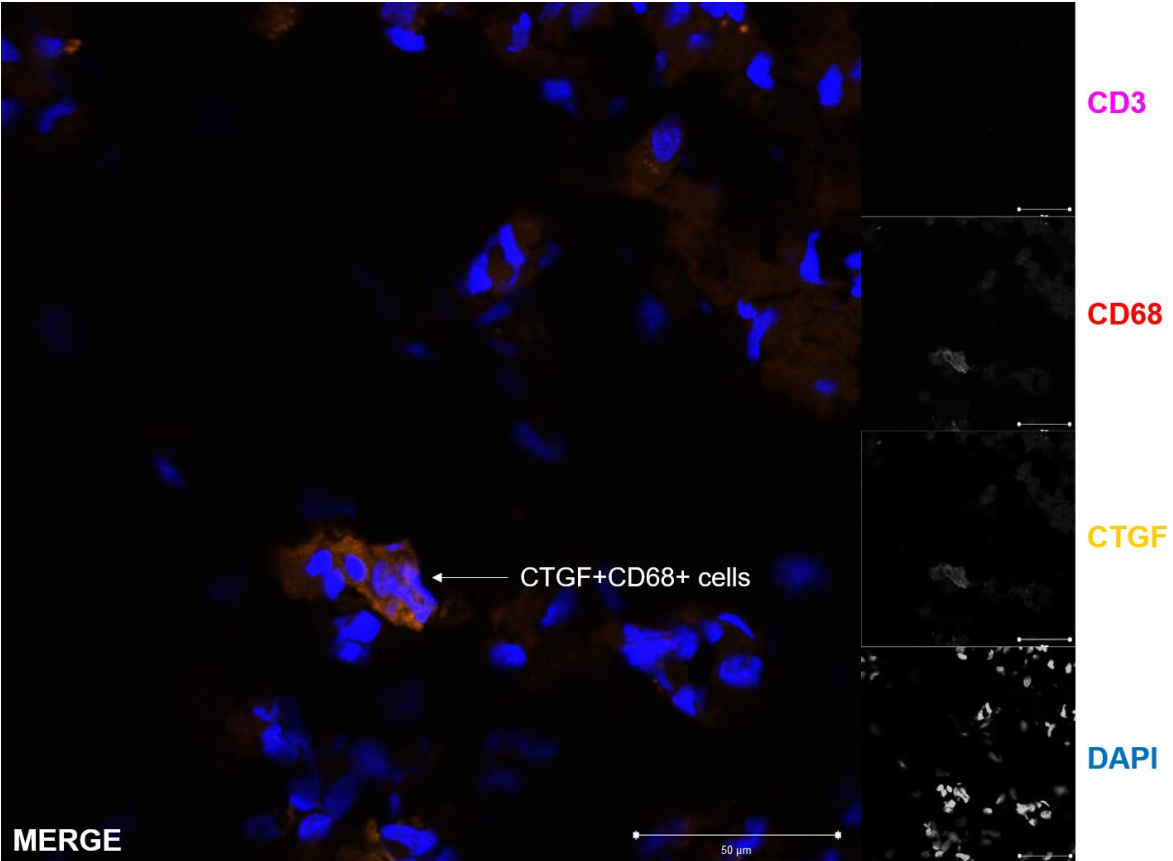


Figure 4.56. Intratumoural CTGF+ cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-CTGF, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50µm. Arrow=CTGF+ cells.

CTGF+ cells in the nine positive tumours were assayed for their intratumoural distribution; all 261 positive cells were observed to be in the TB. These data are shown in Figure 4.57; it is clear that CTGF appeared specifically restricted to the TB, but this did not reach significance by unpaired 2-tailed t-test with Welch's correction ($p=0.1113$), which may be due to limited sample size.

The mean average size of CTGF+ cell clusters was 2.9 cells, as shown in Figure 4.58; no analysis could be performed as there were no PV clusters to compare these data to.

It was clear from these data that CTGF, like the other target proteins, was expressed in the majority of GBM assayed. However, unlike every other protein, CTGF appeared heavily spatially restricted, as CTGF+ cells were only found in the TB.

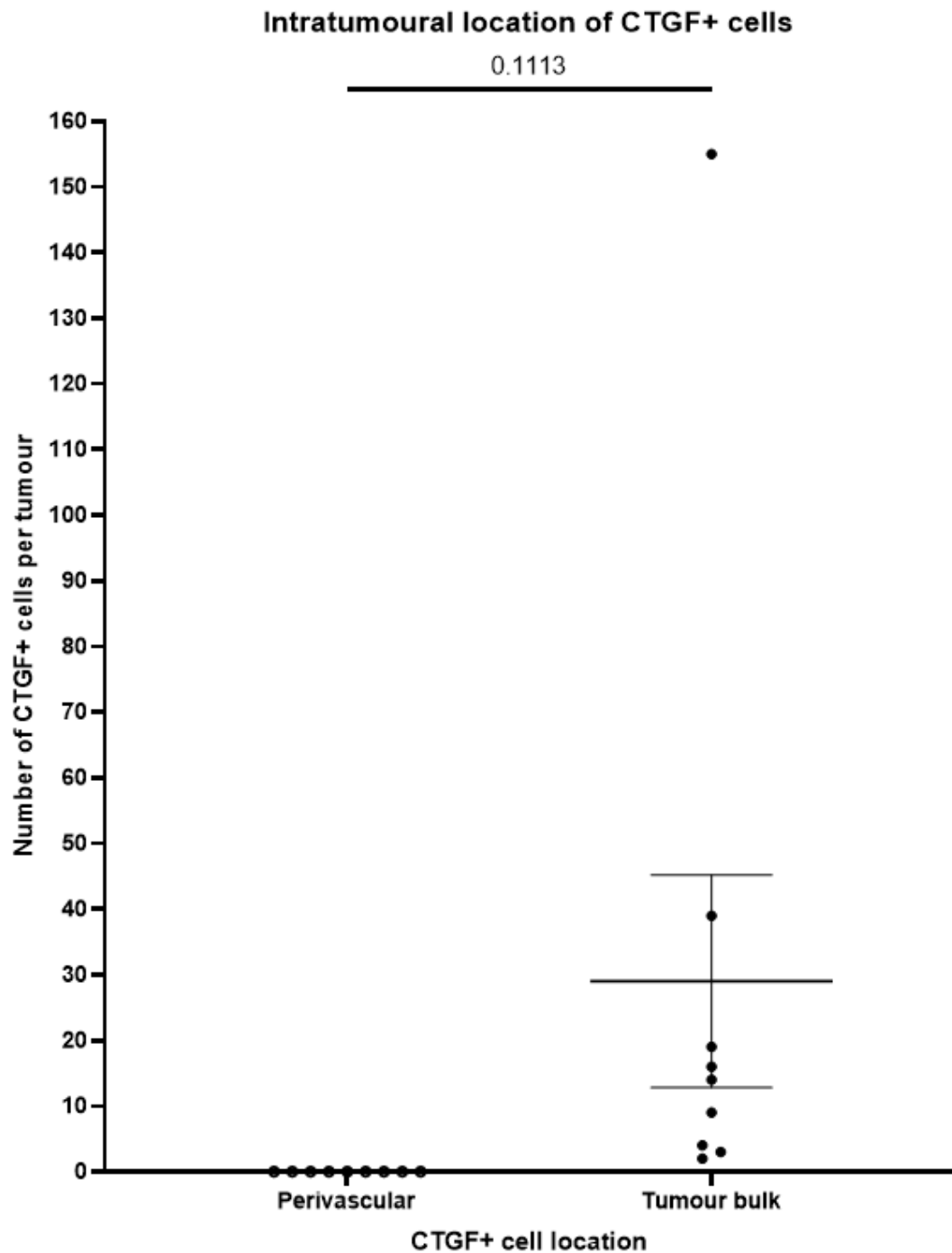


Figure 4.57. Intratumoural location of CTGF-expressing cells. 261 cells were counted in nine GBM stained with anti-CTGF and their location was categorised as perivascular (within 100 μ m of a vessel) or tumour bulk. Error bars=mean $\pm$ SEM.

4.2.2.3.6. *Relative prevalence of target proteins*

Having identified the presence of all five target proteins within GBM and described their intratumoural distribution, the relative prevalence of each was then considered.

Overall, galectins 1 and 3 were the most commonly observed, with 2956 and 1204 positive cells respectively. Galectins 4 and 7 and CTGF were, while still present, considerably rarer, with 336, 123 and 261 positive cells each respectively. However, as there was some variation in the number of GBM stained for each protein, the average number of positive cells per positive tumour was determined to examine the prevalence of different proteins. These data are shown in Table 4.17.

It transpired that gal-1 was most highly represented, with a mean average of 164.2 positive cells per tumour; all other proteins had fewer than 100 cells per tumour, with galectin-3 the next highest at 80.3 and galectin-7 the lowest at 12.3 cells per tumour. When these data were examined by one-way ANOVA with Welch's correction, it was found that at least one pair showed a significant difference in prevalence ($p=0.0144$). Dunnett's T3 multiple comparisons test revealed that the mean average number of gal-1+ cells and gal-7+ cells per positive tumour were significantly different ($p=0.0412$), and the averages for gal-1+ and gal-4+ cells approached significance ($p=0.0966$); all other pairs did not. These data are shown in Figure 4.59.

It was concluded from this that only gal-1, the most prevalent, and gal-7, the least prevalent, showed a significant difference in occurrence in these GBM samples.

Table 4.17. Average target protein positivity per tumour

Protein	Number of positive cells	Number of positive GBM	Average positive cells per tumour
Galectin-1	2956	18	164.2
Galectin-3	1204	15	80.3
Galectin-4	336	11	30.5
Galectin-7	123	10	12.3
CTGF	261	7	37.3

Table 4.17. Average target protein positivity per tumour. GBM sections were stained for each target protein. Positive cells were counted and the total number of positive cells was divided by the number of tumours positive for each protein. Averages are given to one decimal place.

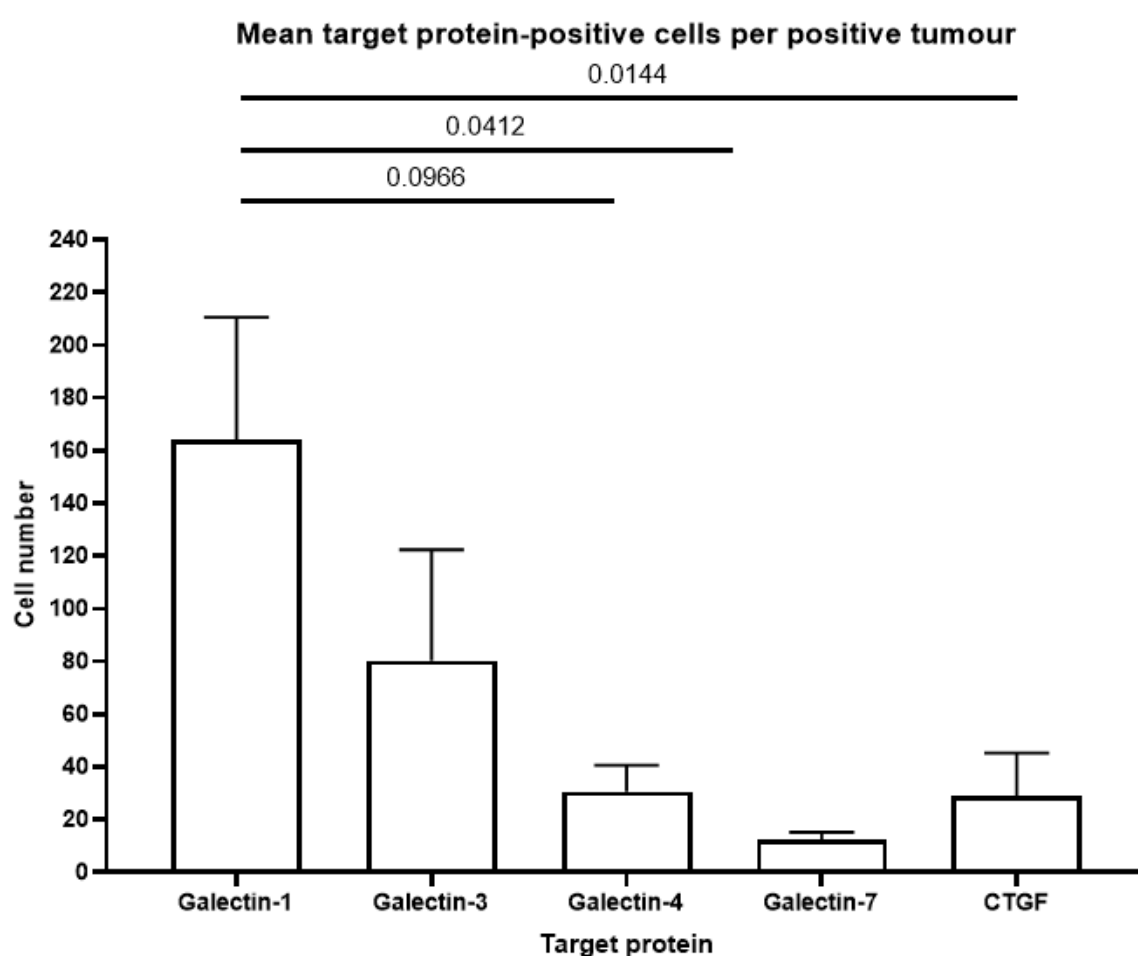


Figure 4.59. Mean target protein-positive cells per positive tumour. The total number of target protein-positive cells was counted in sections stained for these populations and divided by the number of positive tumours. Data were analysed by one-way ANOVA with Welch's correction (upper line) with Dunnett's T3 multiple comparisons test (lower lines). Error bars=SEM.

Next, Fisher's exact test was used to ascertain whether the presence of one target protein informed the presence of any others. This required each examined tumour to have been stained for at least two of the proteins across separate sections, resulting in reduced sample numbers. No galectin-3-negative tumours were stained for galectin-4, and so this pair could not be studied.

The results are shown in Table 4.18. The presence of one protein of interest did not inform the presence of any other to a degree that approached significance; for this reason, no post-hoc corrections for multiple comparisons were performed. It seemed from these data that, although the proteins of interest appeared to be commonly expressed in GBM, they may not have been co-regulated.

Table 4.18. Significance of target protein co-occurrence in GBM

Protein pair	N	P-value (Fisher's exact test)
Galectin-1 and galectin-3	10	>0.9999
Galectin-1 and galectin-4	9	0.2222
Galectin-1 and galectin-7	4	0.2500
Galectin-1 and CTGF	6	>0.9999
Galectin-3 and galectin-7	9	0.3333
Galectin-3 and CTGF	9	0.3333
Galectin-4 and galectin-7	8	0.2500
Galectin-4 and CTGF	5	0.4000
Galectin-7 and CTGF	8	0.4643

Table 4.18. Significance of target protein co-occurrence in GBM. Tumours where sections had been stained for both target proteins in a pair were scored according to the presence of each protein and results were analysed by Fisher's exact test.

4.2.2.4. Cellular sources of target proteins

Having ascertained the relative prevalence of target proteins, their likely cellular sources were then considered. Many of the tumour sections used in 4.2.2.3. were also stained with anti-CD3, -CD68 and -CD56; thus, approximate percentages of T-cells, TAMs or tumour cells expressing target proteins could be determined.

Additionally, it was considered that some pericytes may express gal-1, due to its presence in the PVN and the pericyte-like morphology of some gal-1+ cells – namely, their extensions around a vessel and “bump-on-a-log” nuclei. Some slides were co-stained with anti-galectin-1, an unconjugated antibody against PDGFR β (anti-PDGFR β) to detect pericytes (Smyth et al., 2018), and an AlexaFluor594-conjugated anti-mouse secondary antibody. Pericytes form part of the blood-brain barrier (BBB) alongside ECs, astrocytes, vasculature-adjacent neurons and the basement membrane. Details of the anti-PDGFR β stain are in Table 4.19; negative controls for anti-PDGFR β and the secondary antibody alone are shown in Figures 4.60A and 4.60B respectively. Figure 4.61 shows intratumoural pericytes.

Table 4.19. Antibody panel for detection of pericytes

Specificity	Cell type	Fluorophore	Host	Dilution or concentration ($\mu\text{g/mL}$)	Clone
PDGFR β	Pericyte	N/A	Mouse	10	PR7212
Mouse	N/A	AlexaFluor 594	Donkey	1:200	Polyclonal

Table 4.19. Antibody panel for detection of pericytes.

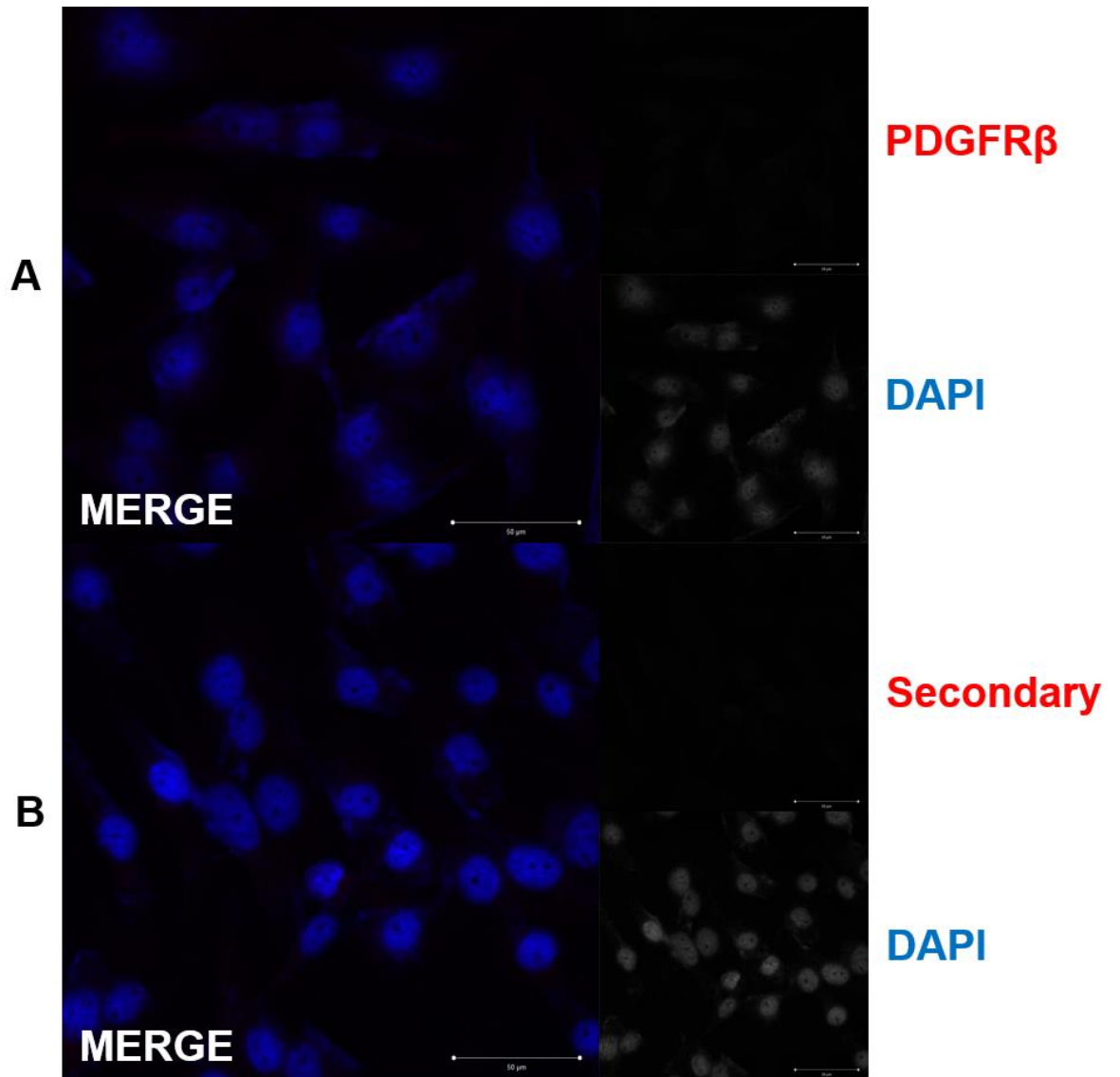


Figure 4.60. PDGFR β and AlexaFluor 594-conjugated secondary antibody negative controls. U251 cells were grown on glass slides and fixed in cold methanol for ten minutes. Cells were permeabilised with 0.05% PBS-Tween 20 and blocked with PBS 1% BSA. Cells were stained with anti-PDGFR β and AlexaFluor 594-conjugated secondary antibody (A) or secondary antibody alone (B) and DAPI. Cells were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50 μ m.

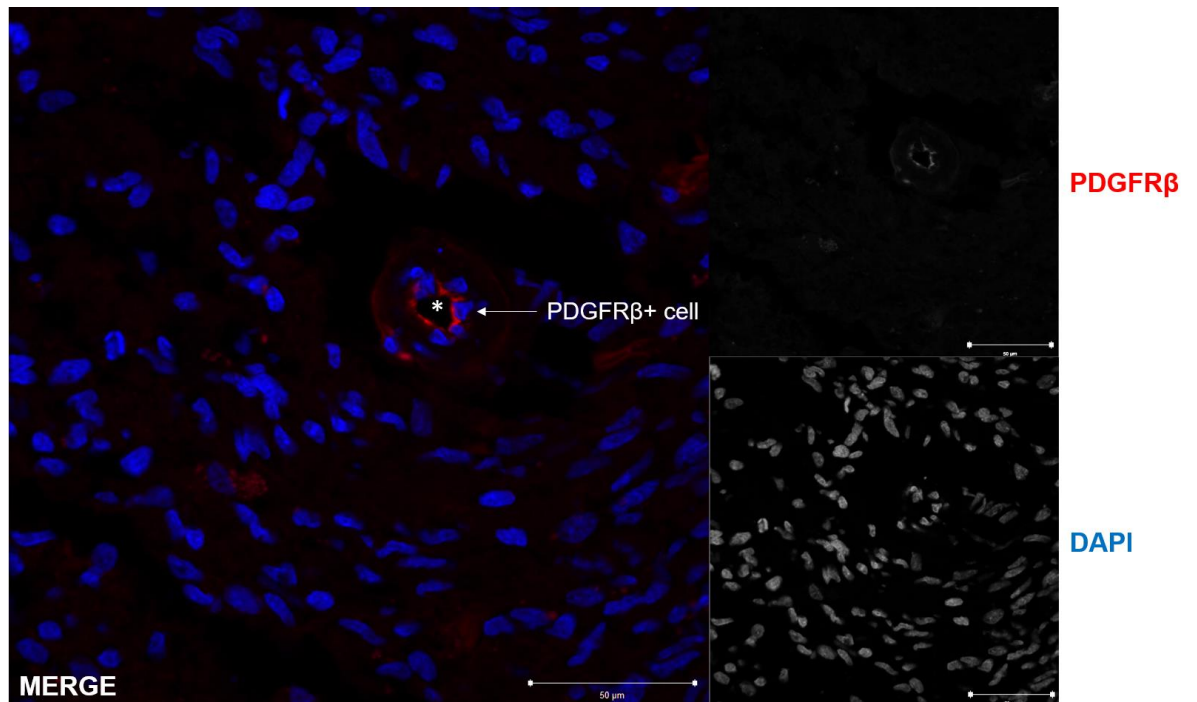


Figure 4.61. Pericytes in GBM vasculature. GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-PDGFR β and AlexaFluor 594-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50 μ m. Arrow=PDGFR β + cell. Asterisk=vessel lumen.

4.2.2.4.1. *Galectin-1*

Tumour sections were examined to identify likely cellular sources of galectin-1, which is produced by a variety of cell and tissue types. PV populations such as ECs are often gal-1+; however, populations outside the PVN were also of interest in this analysis. It was hoped that this examination would shed light on intratumoural heterogeneity and provide insight into likely interactions between this protein and TILs.

To ascertain cellular sources of galectin-1, sections from the 18 gal-1+ GBM were grouped according to the stains performed; one tumour was omitted at this stage as no co-stains were performed. The total number of galectin-1-expressing cells in co-stained sections was determined, as was the total number of gal-1+ cells that expressed additional markers. This allowed calculation of the percentage of gal-1+ cells that belonged to specific cellular populations. Data are shown in Table 4.20; the total numbers of costained cells per tumour are shown in Figure 4.62. CD3+ and CD68+ cells accounted for tiny percentages of total galectin-1+ cells, and thus TILs were likely not the primary intratumoural source of this protein. Galectin-1+CD3+ cells were identified in Figure 4.44: it is likely that these CD3+ cells lacked gal-1 but appeared double-positive because they were positioned over extracellular gal-1 sheets. As this could not be conclusively proven to be the case, these cells were recorded as gal-1+CD3+. Gal-1+CD56+ cells comprised only 5.3% of gal-1+ cells in co-stained slides. Images of gal-1+CD68+ and gal-1+CD56+ cells are shown in Figures 4.63 and 4.64 respectively. By contrast, 22.0% of gal-1+ cells also expressed PDGFR β in sections where both stains were performed (Figure 4.65). Based on other images, it appeared that some intratumoural ECs also express gal-

1 (Figure 4.66). However, these data were insufficient to conclude that non-pericyte vascular populations were the primary source or sources of gal-1 in GBM.

In conclusion, in this dataset, vascular cells may well have been a primary source of galectin-1 that potentially inhibited T-cell infiltration into the tumour. However – assuming the percentages of different galectin-expressing cell types were broadly representative of the TME – T-cells, macrophages, tumour cells and pericytes together only made up approximately 30% of gal-1+ cells. Clearly, other populations also produced intratumoural gal-1.

Table 4.20: Analysis of galectin-1 cellular sources

Co-stain	Number of GBM	Number of gal-1+ cells	Number of double-positive cells	Double-positive cells as a % of gal- 1+ cells
CD3	17	2208	14	0.6
CD56	6	677	36	5.3
CD68	5	59	1	1.7
PDGFR β	5	731	161	22.0

Table 4.20. Analysis of galectin-1 cellular sources. Sections from 18 GBM were stained with anti-galectin-1. Some sections were also stained with anti-CD3, anti-CD68, anti-CD56 and/or anti-PDGFR β . Sections were imaged on a Zeiss LSM 880 confocal microscope. Total numbers of galectin-1+ cells in slides co-stained for each marker were counted and these cells were assessed for co-expression of other markers. Percentages are given to one decimal place.

Galectin-1+ cell costaining

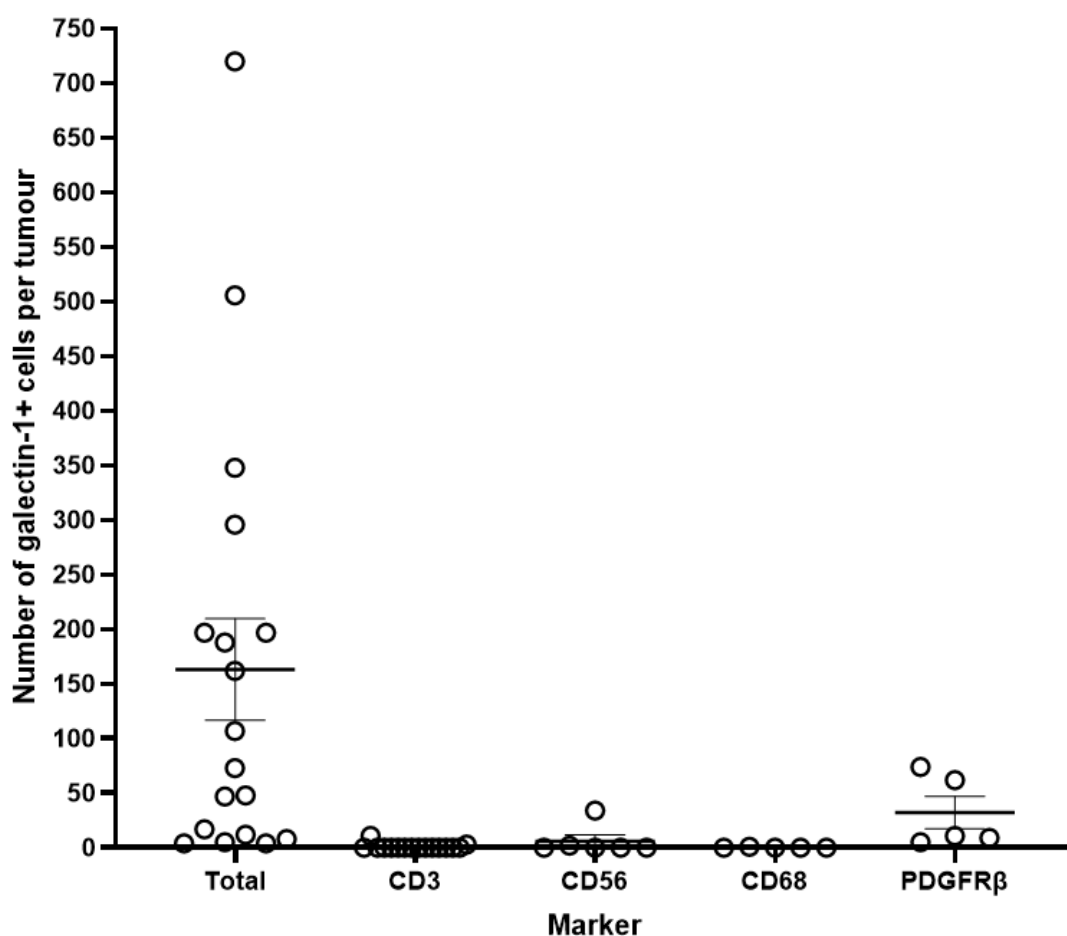


Figure 4.62. Galectin-1 costaining. 18 GBM were stained for galectin-1 alongside CD3 (N=17), CD56 (N=6), CD68 (N=5) and PDGFR β (N=5) and imaged on a Zeiss LSM confocal microscope. The total number of cells coexpressing gal-1 and another marker in each tumour was recorded. Each data point represents one tumour. Error bars=mean+SEM.

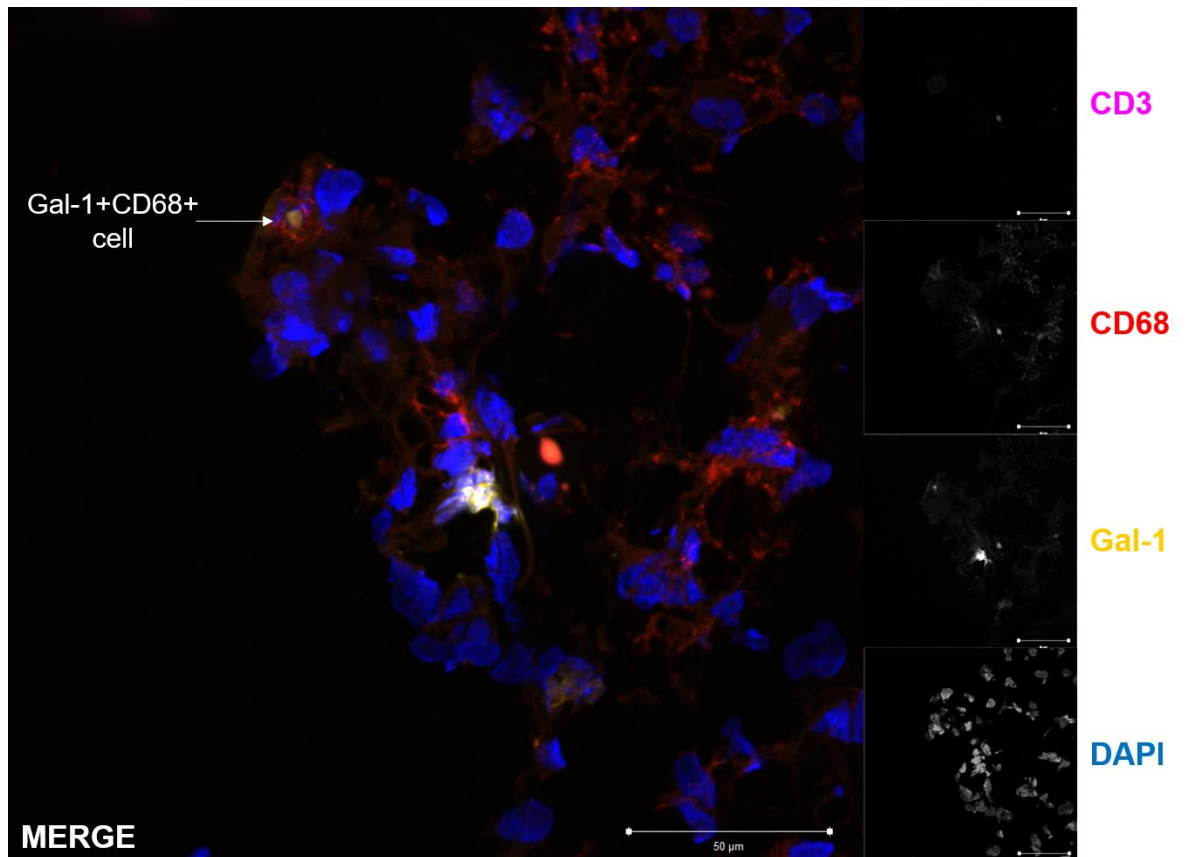


Figure 4.63. Galectin-1 and CD68 double-positive cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD68, anti-galectin-1, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=gal-1+CD68+ cell.

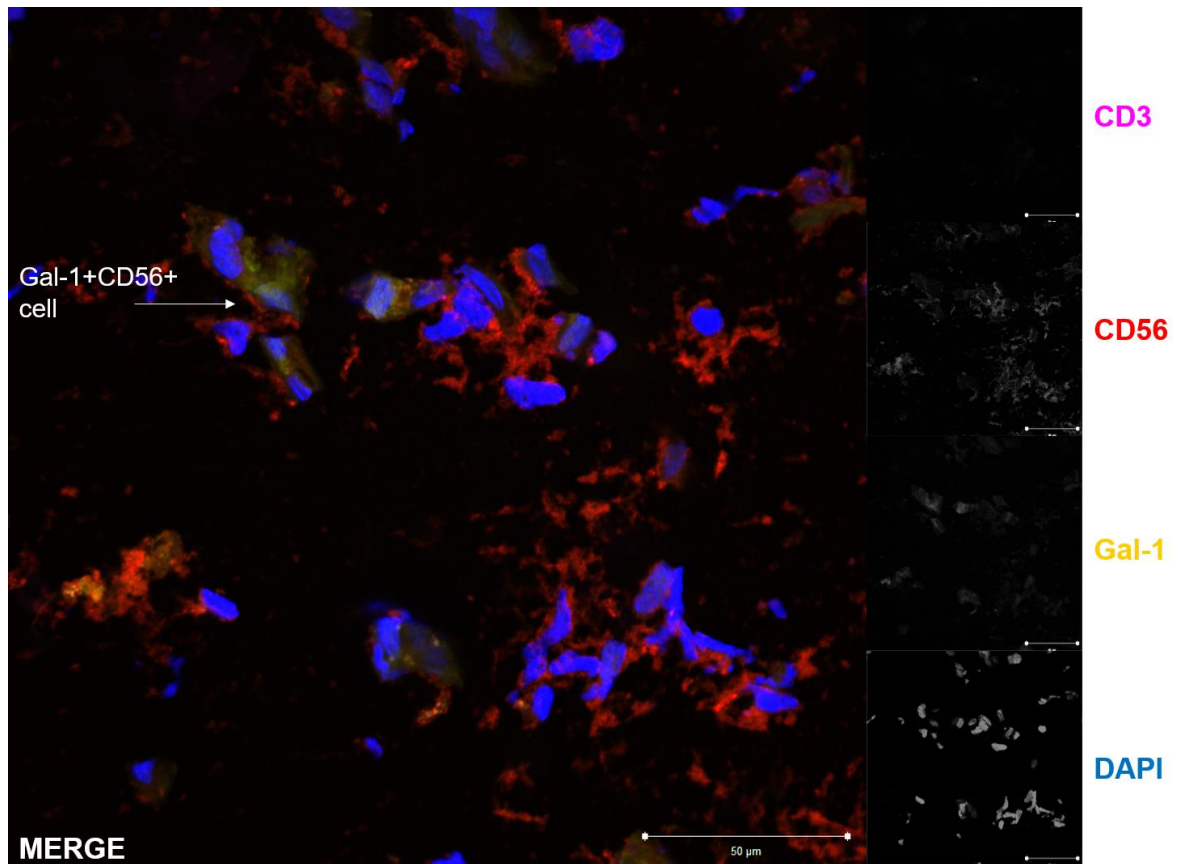


Figure 4.64. Galectin-1 and CD56 double-positive cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-1, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=gal-1+CD56+ cell.

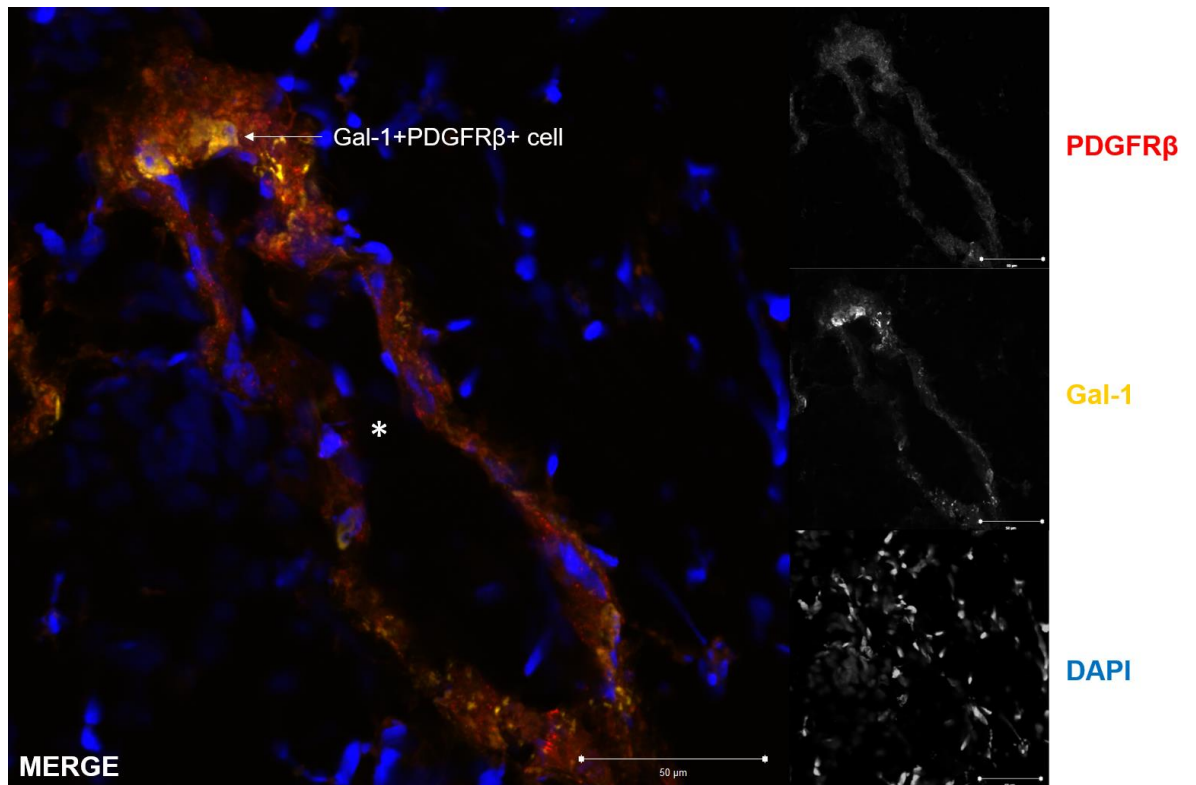


Figure 4.65. Galectin-1 and PDGFR β double-positive cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-PDGFR β , anti-galectin-1, FITC- and AlexaFluor 594-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50 μ m. Arrows=gal-1+ PDGFR β + cells. Asterisk=vessel lumen.

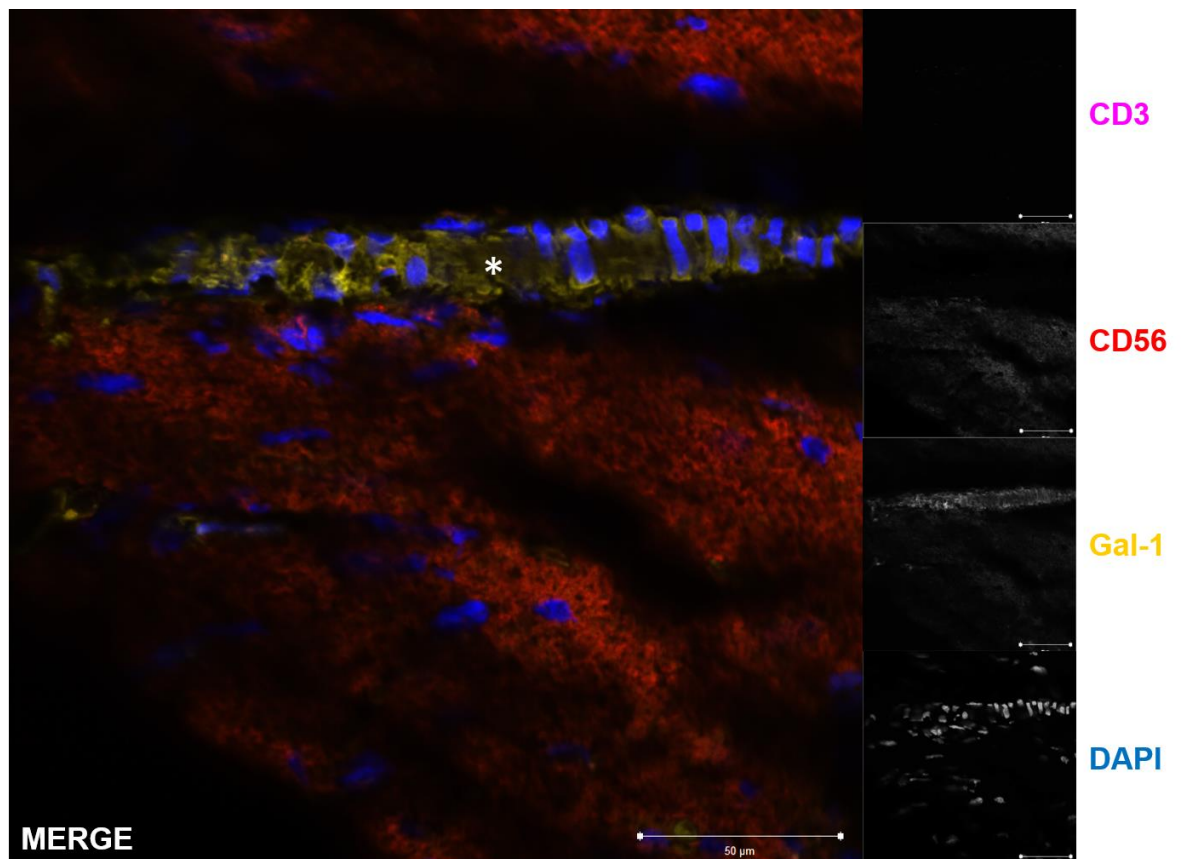


Figure 4.66. Suspected galectin-1-expression in intratumoural endothelial cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-1, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Asterisk=vessel lumen.

4.2.2.4.2. *Galectin-3*

Having identified that this analysis was suitable to give some insight into cellular sources of gal-1+ cells in section 4.2.2.4.1., it was then repeated for galectin-3+ cells. Gal-3 was found to be present in both the PVN and TB, and although most gal-3+ cells were found in the TB, this did not reach significance and was driven by four GBM with very high gal-3+ TB cell counts (Figure 4.47). While this was clearly reflective of intertumoural heterogeneity, these results could have been caused by either high native expression or an influx of gal-3+ TILs that only occurred in some GBM. Thus, examination of gal-3 cellular sources had the potential to reveal additional information about the TME. A summary of co-stained slides is shown in Table 4.21; the total numbers of costained cells per tumour are shown in Figure 4.67.

No gal-3+CD3+ cells were observed. However, CD68+ cells comprised almost 10% of gal-3-expressing cells in tumours where both antibodies were used (Figure 4.68). Caution was warranted when interpreting these results, as opposed to stating that gal-3 positivity in CD68+ cells was definitive proof of either expression or phagocytosis. The primary source of gal-3, though, appeared to be CD56+ cells: 48% of gal-3+ cells in tumours stained with anti-CD56 also expressed CD56. An example is shown in Figure 4.69.

The analysis described here found that, of the 1204 gal-3+ cells examined, 57.6% costained with either CD68, CD56 or CD3. This indicated that like gal-1, this protein was produced by varied intratumoural sources, some of which could not be identified using these markers.

Table 4.21: Analysis of galectin-3 cellular sources

Co-stain	Number of GBM	Number of gal-3+ cells	Number of double-positive cells	Double-positive cells as a % of gal- 3+ cells
CD3	15	1204	0	0.0
CD56	10	180	87	48.3
CD68	5	1024	95	9.3

Table 4.21. Analysis of galectin-3 cellular sources. Sections from 15 GBM were stained with anti-galectin-3. Some sections were also stained with anti-CD3, anti-CD68 and anti-CD56. Sections were imaged on a Zeiss LSM 880 confocal microscope. Total numbers of galectin-3+ cells in slides co-stained for each marker were counted and these cells were assessed for co-expression of other markers. Percentages are given to one decimal place.

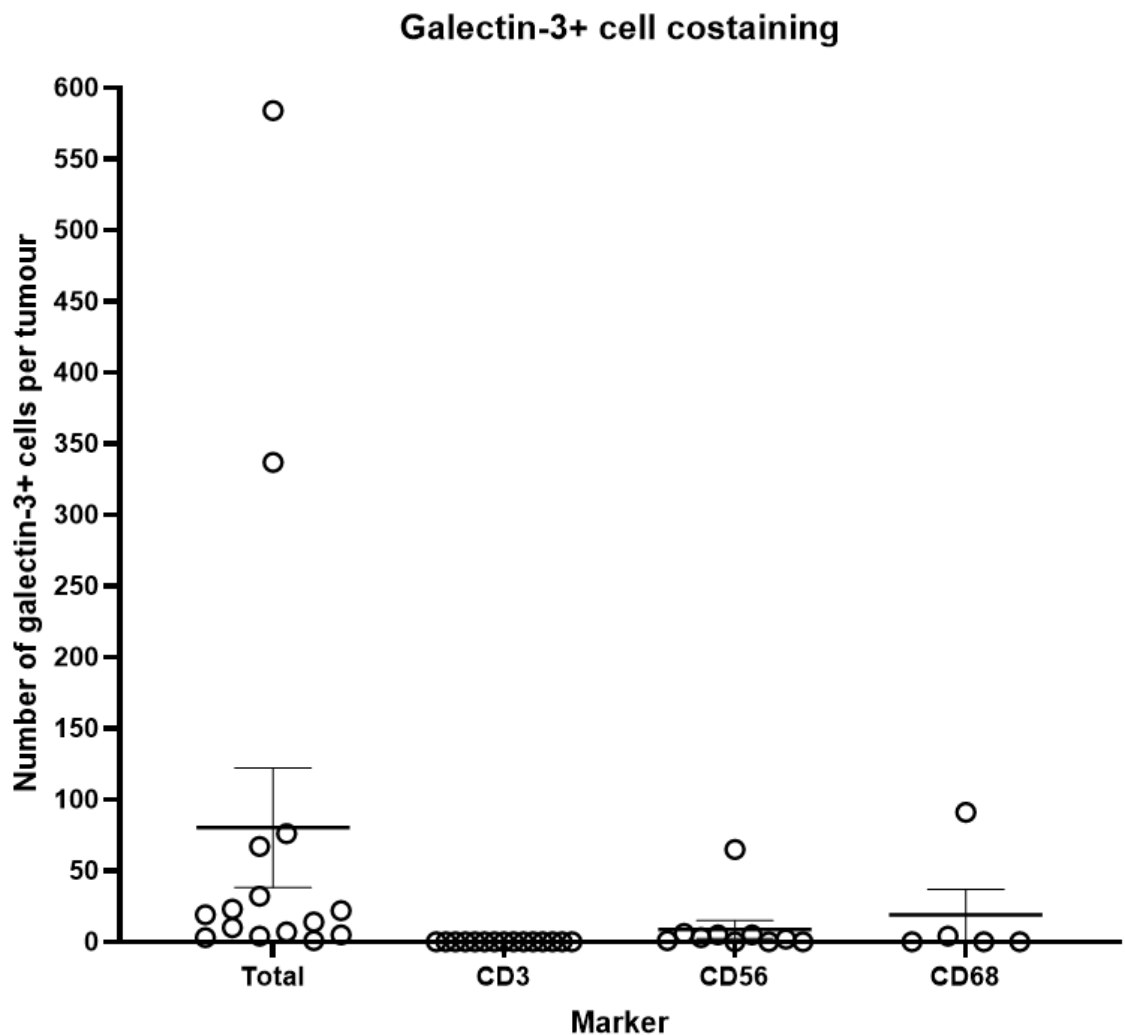


Figure 4.67. Galectin-3 costaining. 15 GBM were stained for galectin-3 alongside CD3 (N=15), CD56 (N=10) and CD68 (N=5) and imaged on a Zeiss LSM confocal microscope. The total number of cells coexpressing gal-3 and another marker in each tumour was recorded. Each data point represents one tumour. Error bars=mean+SEM.

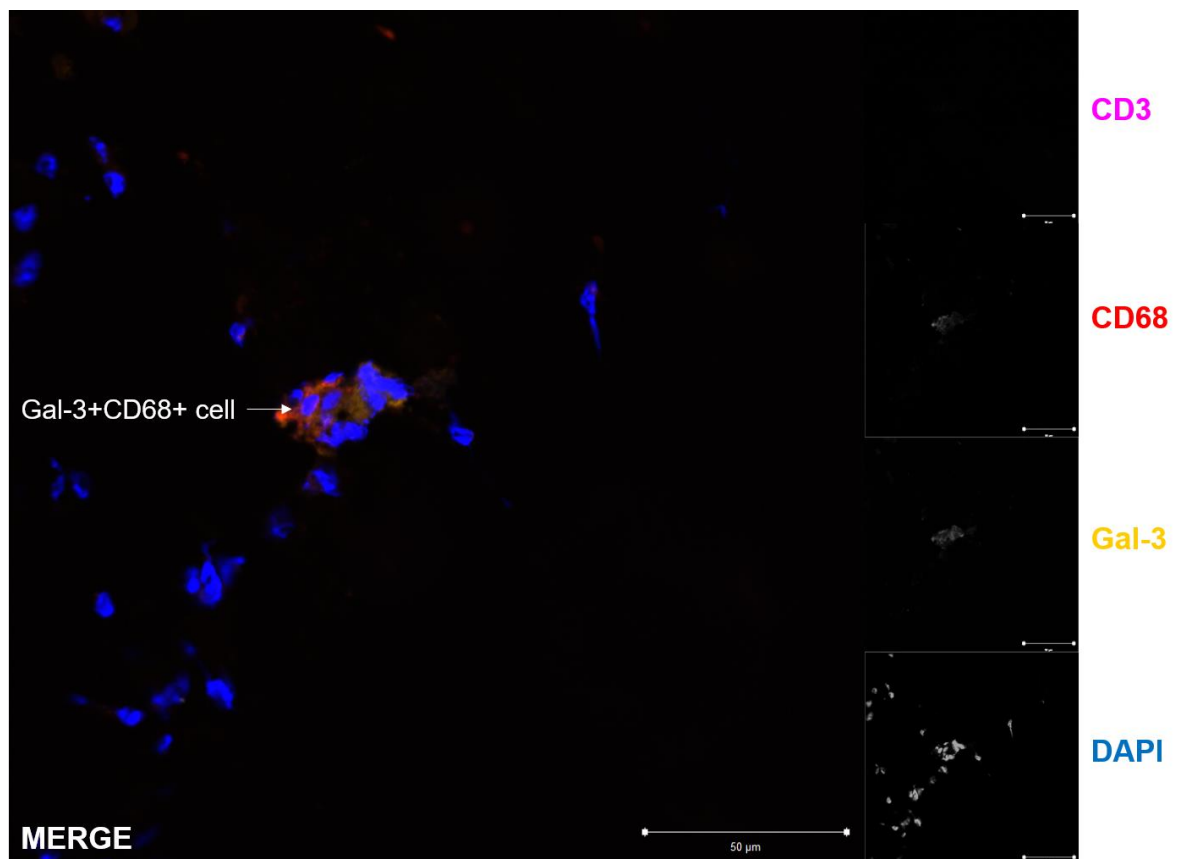


Figure 4.68. Galectin-3 and CD68 double-positive cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD68, anti-galectin-3, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=gal-3+CD68+ cell.

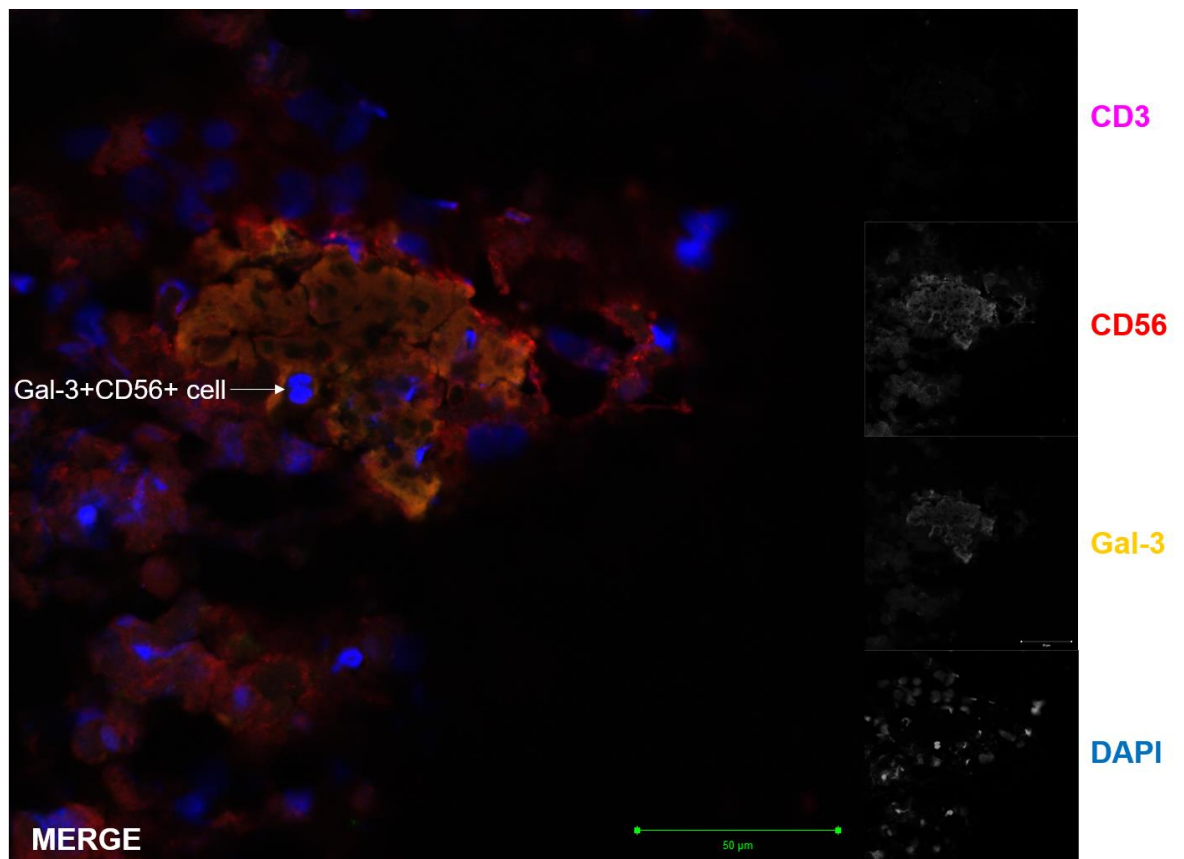


Figure 4.69. Galectin-3 and CD56 double-positive cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-3, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=gal-3+CD56+ cell.

4.2.2.4.3. *Galectin-4*

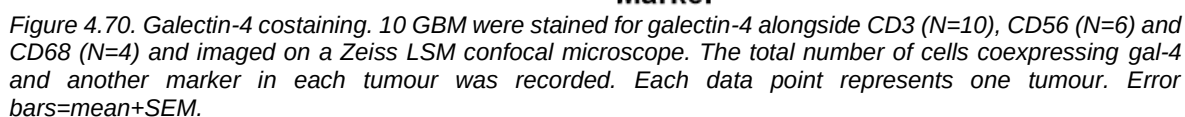
Next, co-staining in slides stained with anti-galectin-4 was analysed. Gal-4 was identified in 4.2.2.3.3. to be expressed in many GBM by small numbers of cells, and was equally distributed between the PV and TB niches; it was possible that gal-4 is expressed by a specific, small population found in both locations, or that it was heterogeneously expressed by more than one population. To ascertain likely cellular sources, the tissue sections used in 4.2.2.3.3. were re-examined for gal-4 and CD3, CD56 and CD68 co-staining. One tumour was omitted as no co-stains were performed, so a total of 10 GBM were included in this analysis.

Of the 10 tumours, all were stained for CD3, four for CD68 and six for CD56. In tumours stained for CD3, there were 327 gal-4+ cells, of which 12, or 3.7%, also expressed CD3, suggesting that a minority of T-cells may have produced gal-4. However, over 40% of gal-4+ cells also co-stained with anti-CD68 in sections where this antibody was used. An example is shown in Figure 4.50, alongside two TAMs lacking gal-4, indicating that gal-4 positivity was heterogenous even among closely colocalised macrophages. It is possible that gal-4 presence in TAMs was sometimes due to phagocytosis as well as innate expression. In sections co-stained with anti-CD56, a further 52.1% of gal-4+ cells were double-positive. These data are summarised in Table 4.22, and the total numbers of costained cells per tumour are shown in Figure 4.70. An example of Gal-4+CD56+ cells is shown in Figure 4.71.

In this analysis, approximately 99.4% of gal-4+ cells also costained with either CD56 or CD68, indicating that tumour cells and TAMs were amongst the primary producers of this protein.

Co-stain	Number of GBM	Number of gal-4+ cells	Number of double-positive cells	Double-positive cells as a % of gal-4+ cells
CD3	10	327	12	3.7
CD56	6	288	150	52.1
CD68	4	39	17	43.6

Galectin-4+ cell costaining



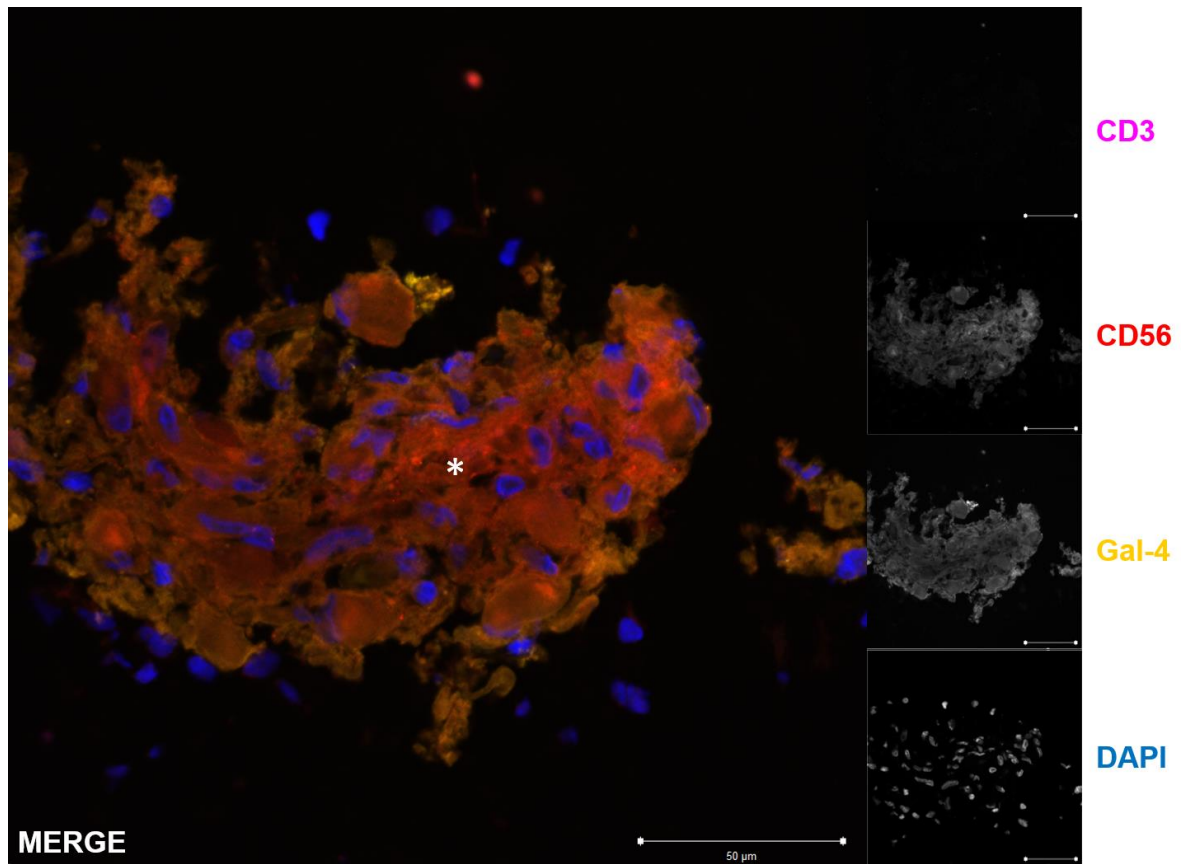


Figure 4.71. Galectin-4 and CD56 double-positive tissue. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-4, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Asterisk=gal-4+CD56+ tissue.

4.2.2.4.4. *Galectin-7*

Next, galectin-7 co-staining with CD3, CD56 and CD68 was analysed to ascertain potential cellular sources of this protein. Much like galectin-4, small numbers of gal-7+ cells were found in many GBM, with no significant prevalence in either the TB or PVN. An analysis of the cellular source or sources had potential to reveal further insight into heterogenous phenotypes of intratumoural populations; to this end, images of the sections used in 4.2.2.3.4 were re-examined. One tumour was omitted as no co-stains were performed, so a total of nine GBM were included in this analysis.

Of the included sections, nine were stained with anti-CD3, six with anti-CD56 and three with anti-CD68. 115 gal-7+ cells were observed in sections stained with anti-CD3, of which four, or 3.5%, were double-positive, indicating that T-cells were a minor source of gal-7. 22 gal-7+ cells were seen in sections stained with anti-CD68, of which 15, or 68.2%, were double-positive. Finally, eight of 93 gal-7+ cells expressed CD56 in sections where anti-CD56 was used, representing 8.6% of gal-7+ cells observed (summarised in Table 4.23 and Figure 4.72). Examples of gal-7+CD3+, gal-7+CD68+ and gal-7+CD56+ cells are shown in Figures 4.73, 4.74 and 4.75 respectively.

This data demonstrated that T-cells and tumour cells were likely a minor source of gal-7, in contrast to TAMs; though it is worth noting here that the sample size was limited, and gal-7 may be a result of phagocytosis rather than an innately expressed protein. This analysis also accounted for 80.3% of gal-7+ cells, and thus implied that further populations of gal-7-producing cells may exist.

Table 4.23: Analysis of galectin-7 cellular sources

Co-stain	Number of GBM	Number of gal-7+ cells	Number of double-positive cells	Double-positive cells as a % of gal- 7+ cells
CD3	9	115	4	3.5
CD56	6	93	8	8.6
CD68	3	22	15	68.2

Table 4.23. Analysis of galectin-7 cellular sources. Sections from nine GBM were stained with anti-galectin-7. Some sections were also stained with anti-CD3, anti-CD68 and anti-CD56. Sections were imaged on a Zeiss LSM 880 confocal microscope. Total numbers of galectin-7+ cells in slides co-stained for each marker were counted and these cells were assessed for co-expression of other markers. Percentages are given to one decimal place.

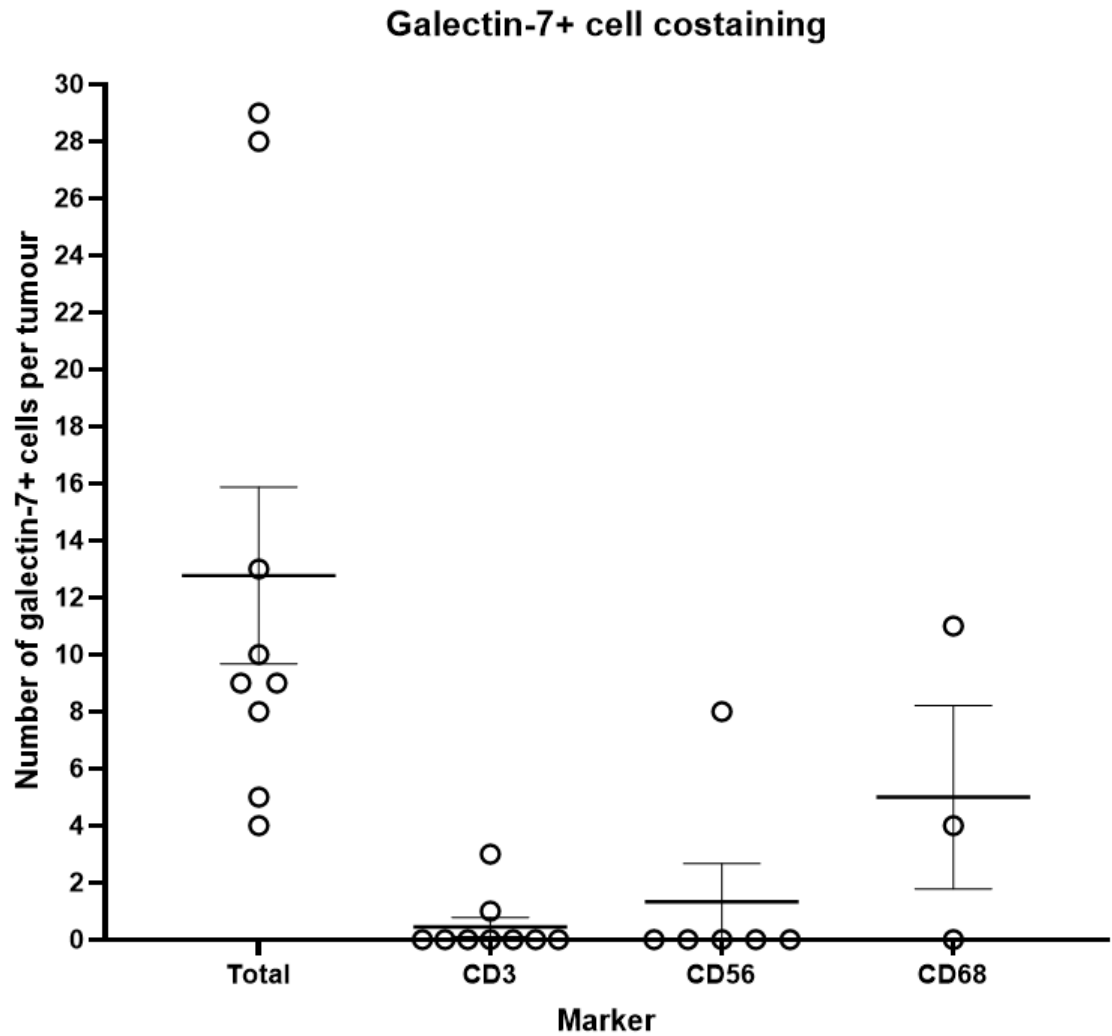


Figure 4.72. Galectin-7 costaining. 9 GBM were stained for galectin-4 alongside CD3 (N=9), CD56 (N=6) and CD68 (N=3) and imaged on a Zeiss LSM confocal microscope. The total number of cells coexpressing gal-7 and another marker in each tumour was recorded. Each data point represents one tumour. Error bars=mean+SEM.

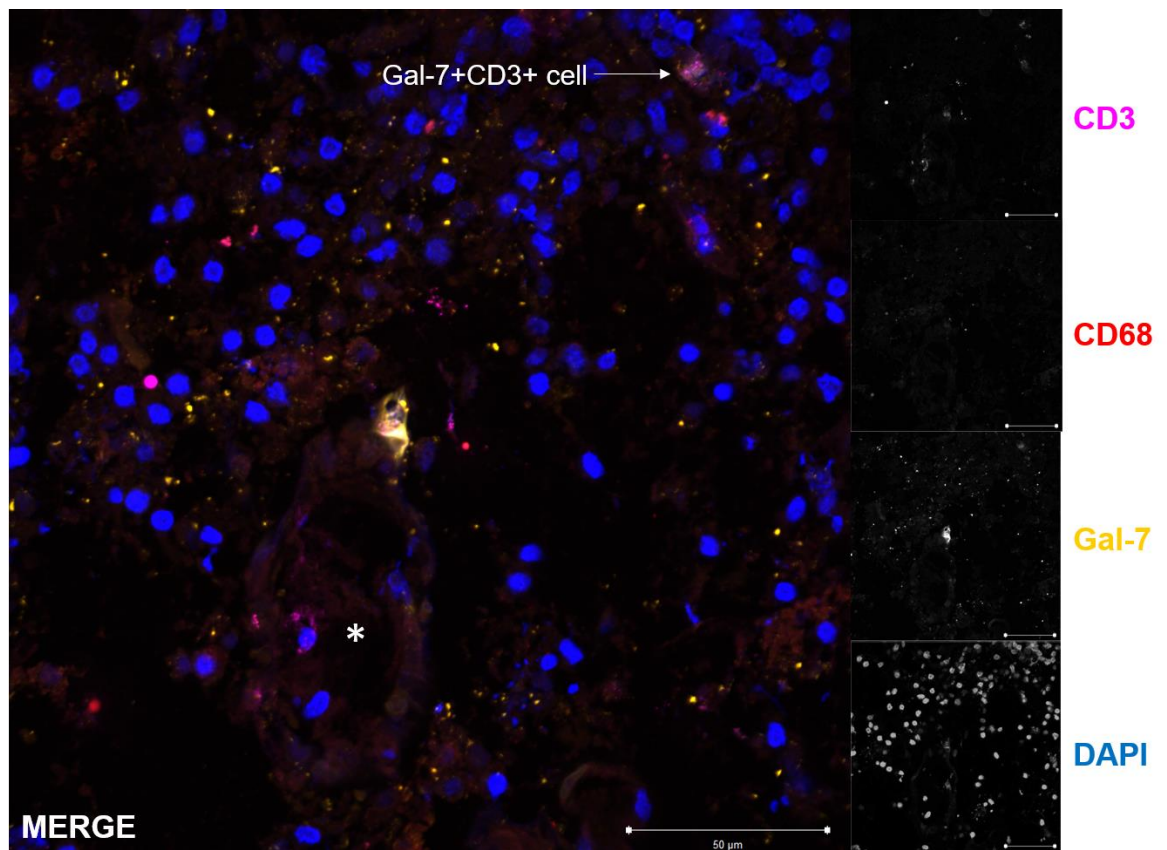


Figure 4.73. Galectin-7 and CD3 double-positive cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD68, anti-galectin-7, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=gal-7+CD3+ cell.

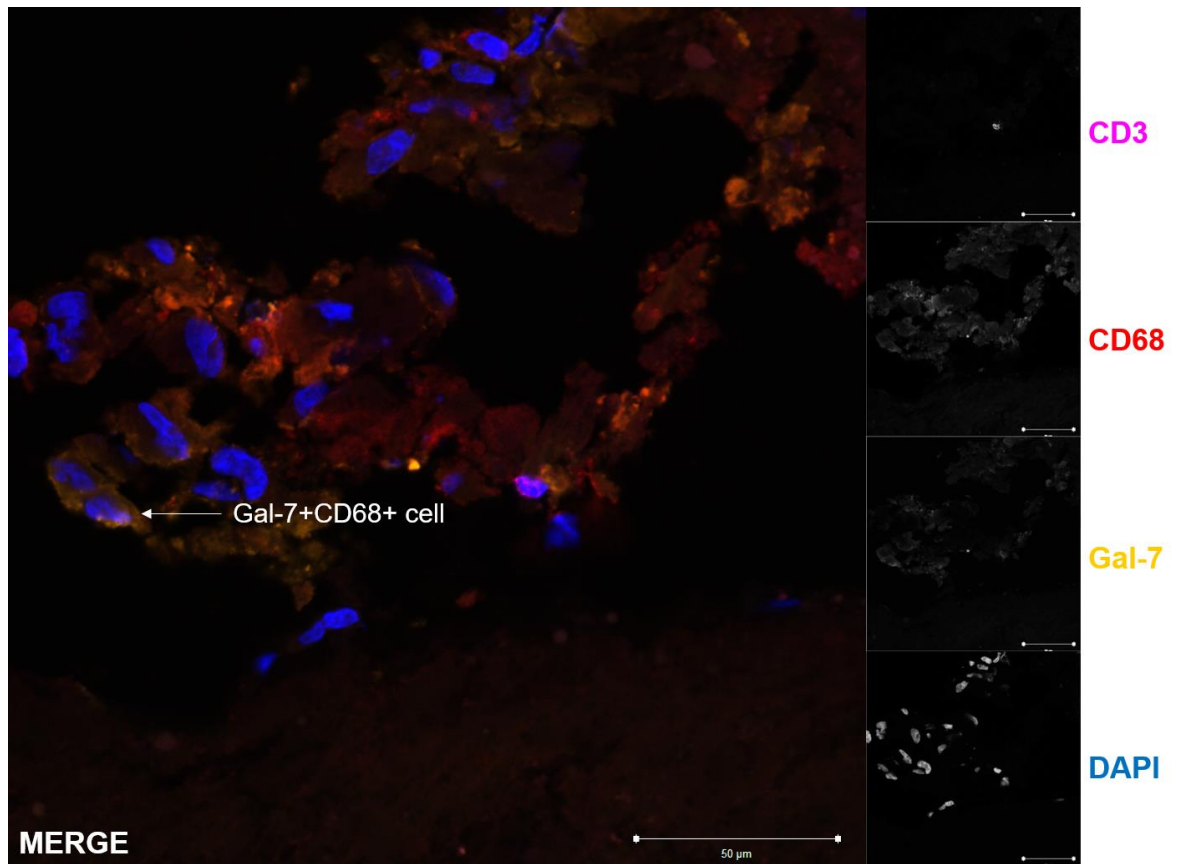


Figure 4.74. Galectin-7 and CD68 double-positive cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD68, anti-galectin-7, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=gal-7+CD68+ cell.

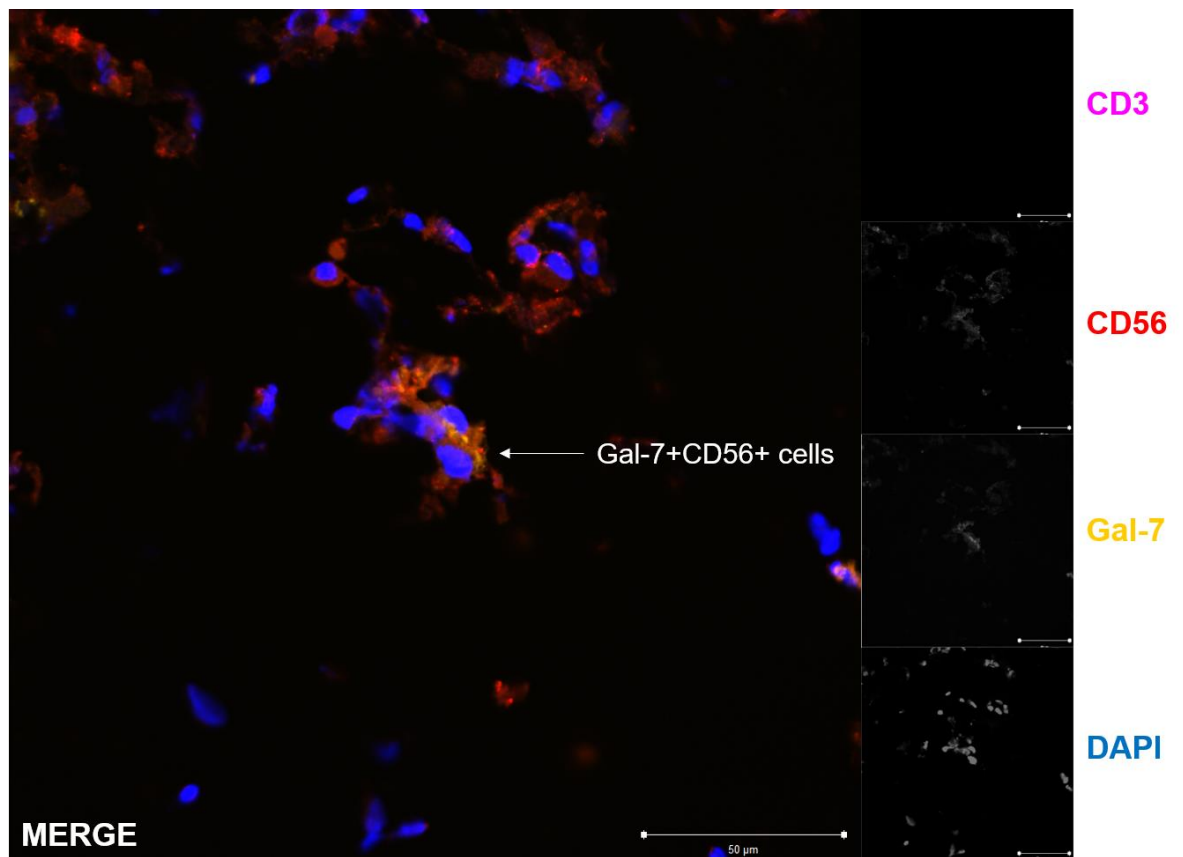


Figure 4.75. Galectin-7 and CD56 double-positive cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-galectin-7, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=gal-7+CD56+ cell.

4.2.2.4.5. CTGF

The analysis of cellular sources of target proteins was concluded by examining sections stained for CTGF. In 4.2.2.3.5., CTGF was found to be exclusively present in the TB. To examine whether it was restricted by cell type as well as spatially, the expression of CD3, CD56 and CD68 in CTGF+ cells was studied. One tumour was omitted as no co-stains were performed, so a total of eight GBM were analysed.

Of the eight sections, all were co-stained for CD3, five for CD56 and three for CD68. Data are summarised in Table 4.24 and total numbers of costained cells per GBM are shown in Figure 4.76. Only two of the 245 CTGF+ cells identified in sections co-stained with CD3 were CD3+, an example of which is shown in Figure 4.77. However, 164 of 208 CTGF+ cells co-stained with CD68 across the three sections where both antibodies were used, representing 78.9% of CTGF+ cells present in these sections; an example is seen in Figure 4.56. 16.2% of CTGF+ cells were also CD56+ in sections where this stain was performed (Figure 4.78).

It can be concluded from this that the majority of intratumoural CTGF may have been produced by TAMs; a significant minority of CTGF-expressing cells costained with CD56. As with other target proteins, co-staining with CD3 was very rare. In total, CTGF+ cells that co-stained with either CD3, CD56 or CD68 accounted for 95.9% of the total CTGF+ cells in these sections. It appeared that another limited population of populations of CTGF+ cells may exist, but TAMs were likely the primary source.

Table 4.24: Analysis of CTGF cellular sources

Co-stain	Number of GBM	Number of CTGF+ cells	Number of double-positive cells	Double-positive cells as a % of CTGF+ cells
CD3	8	245	2	0.8
CD56	5	37	6	16.2
CD68	3	208	164	78.9

Table 4.24. Analysis of CTGF cellular sources. Sections from nine GBM were stained with anti-CTGF. Some sections were also stained with anti-CD3, anti-CD68 and anti-CD56. Sections were imaged on a Zeiss LSM 880 confocal microscope. Total numbers of CTGF+ cells in slides co-stained for each marker were counted and these cells were assessed for co-expression of other markers. Percentages are given to one decimal place.

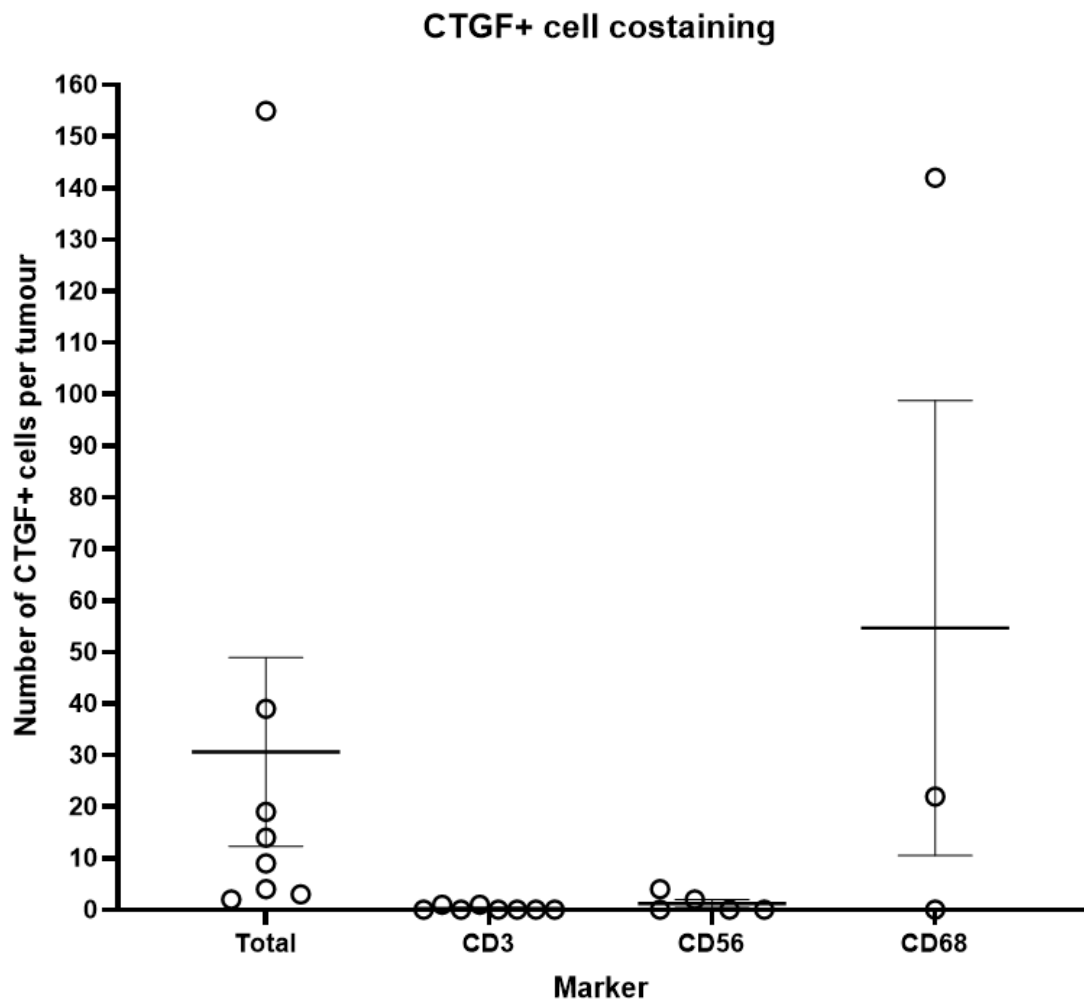


Figure 4.76. CTGF costaining. 8 GBM were stained for CTGF alongside CD3 (N=9), CD56 (N=5) and CD68 (N=3) and imaged on a Zeiss LSM confocal microscope. The total number of cells coexpressing CTGF and another marker in each tumour was recorded. Each data point represents one tumour. Error bars=mean+SEM.

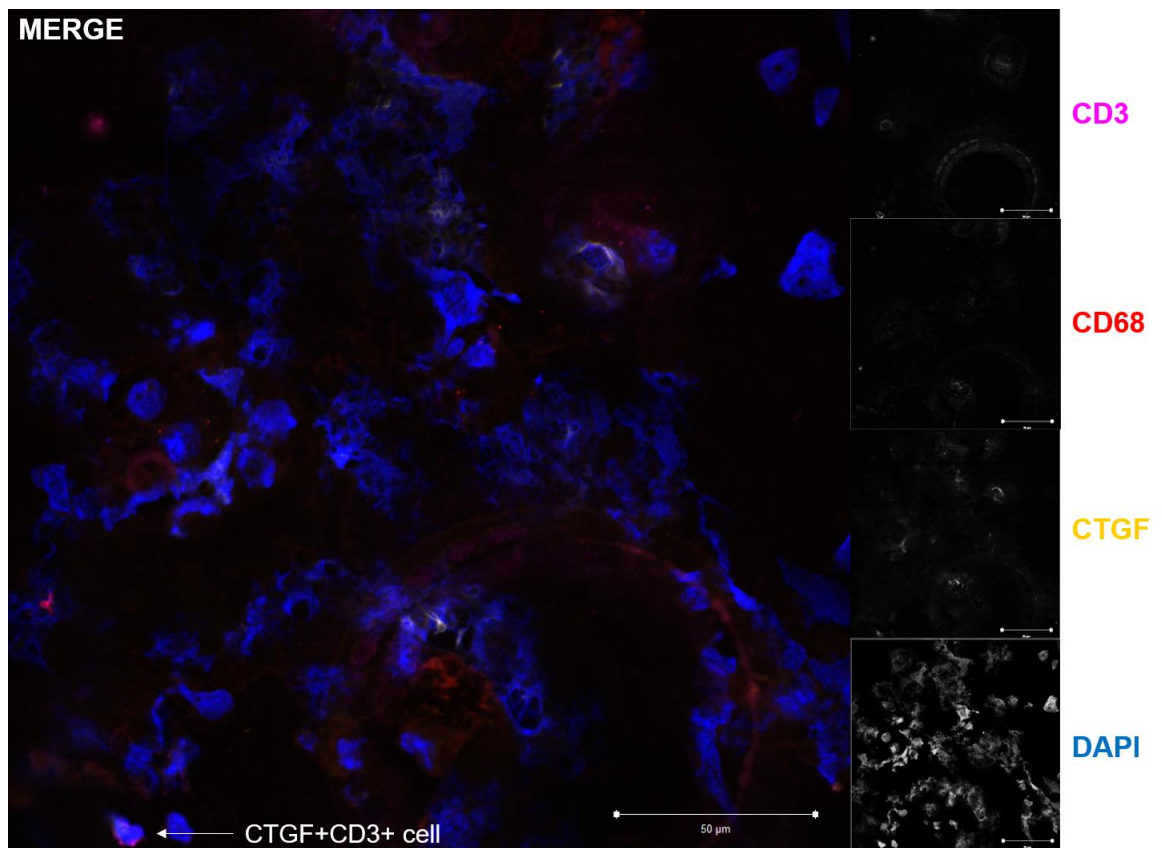


Figure 4.77. CTGF and CD3 double-positive cell. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD68, anti-CTGF, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50 μ m. Arrow=CTGF+CD3+ cell.

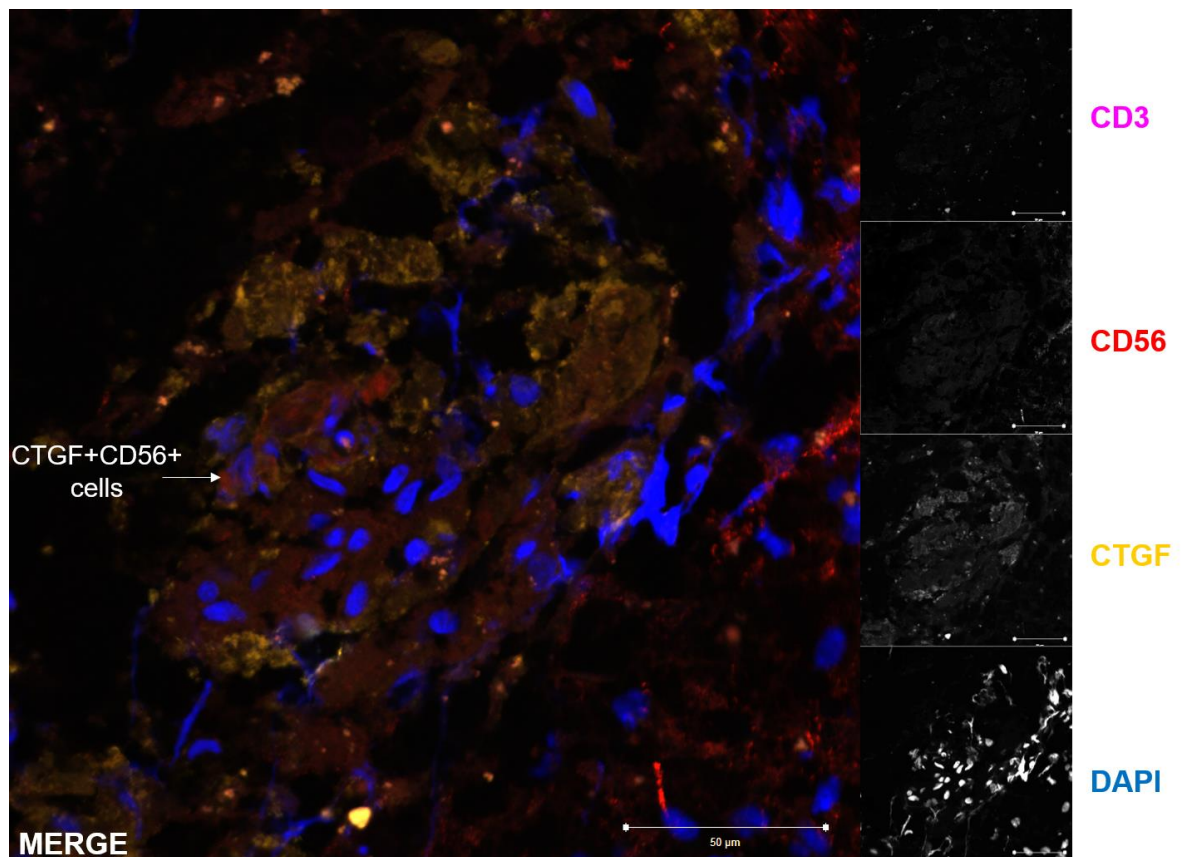


Figure 4.78. CTGF and CD56 double-positive cells. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-CD56, anti-CTGF, FITC- and RRX-conjugated secondary antibodies and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrow=CTGF+CD56+ cells.

4.2.2.5. Cell-protein interactions

In the above sections, the presence of multiple intratumoural effector populations and their distribution within GBM was identified. The presence of all five target proteins was confirmed in at least half of GBM assayed; furthermore, likely cellular sources for each protein were identified by using slides stained with anti-CD3 and either anti-CD56 or anti-CD68.

Next, the same dataset was used to examine interactions between TILs and target proteins. As with the cell-cell interaction analysis in 4.2.1.4., positive staining for both proteins, a cell nucleus, and no intervening cell bodies or extracellular space was required to positively identify an interaction.

Interactions between T-cells or TAMs and target proteins were easily identifiable using the previously-stained slides. However, as both anti-CD19 and the anti-goat secondary antibody used to identify galectins were FITC-conjugated, no analysis of B-cell interactions with target proteins could be performed.

4.2.2.5.1. T-cell and protein interactions

First, interactions between T-cells and target proteins were examined. T-cell/gal-1 interactions were of particular interest, given the trends for both to localise in the PVN (4.2.1.3.2. and 4.2.2.3.1.), the presence of T-cell aggregations, and gal-1's known T-cell-suppressive effects. Results such as Figure 4.43, which showed intravascular T-cells that would contact gal-1+ cells during extravasation, suggested that T-cell/gal-1 interactions in the PVN may occur frequently – indeed, Figure 4.79 showed T-cells aggregating in the PVN in proximity to gal-1, further suggesting gal-1 may inhibit T-cell motility. However, interactions involving the other four proteins and T-cells were also of interest.

Sections positive for both T-cells and the protein of interest were examined. T-cells interacted more with galectin-1 than any other protein, with a mean average of 28.6 interactions per double-positive tumour; galectin-3 was tenfold lower at 2.6 interactions per tumour. All other proteins had a frequency of 1 or fewer T-cell interactions per tumour. These data are summarised in Table 4.25.

The difference in means approached significance ($p=0.0753$) according to one-way ANOVA with Welch's correction (Figure 4.80). The tendency of T-cells to interact with gal-1+ cells over other target protein-expressing cells approached, but did not reach significance, in much the same way as the PV presence of T-cells and gal-1 ($p=0.0932$ and 0.0844 respectively). It is worth noting that this analysis was limited by small sample size, and observed T-cell and protein interactions may not equal the true number of interactions. However, the clear trend was towards preferential interaction of T-cells with intratumoural gal-1 over other proteins examined.

Table 4.25. Analysis of T-cell and target protein interactions

Target protein	Number of double-positive GBM	Number of interactions	Average interactions per double-positive tumour
Galectin-1	10	286	28.6
Galectin-3	11	29	2.6
Galectin-4	8	8	1.0
Galectin-7	7	6	0.9
CTGF	7	2	0.3

Table 4.25. Analysis of T-cell and target protein interactions. Averages are given to one decimal place.

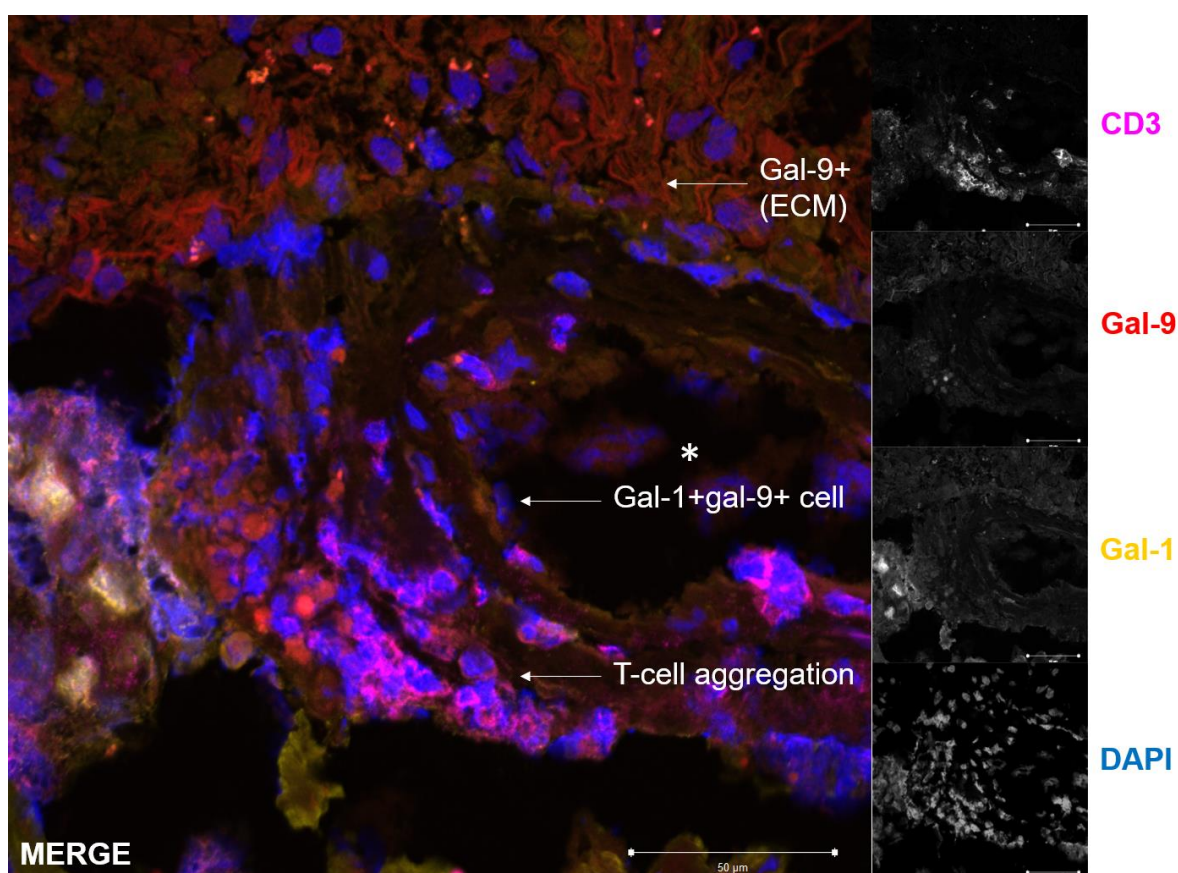


Figure 4.79. T-cell aggregation in proximity to gal-1 and gal-9. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-galectin-9, anti-galectin-1, FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrows indicate T-cell, gal-1+gal-9+ cell and ECM-bound gal-9. Asterisk=vessel lumen.

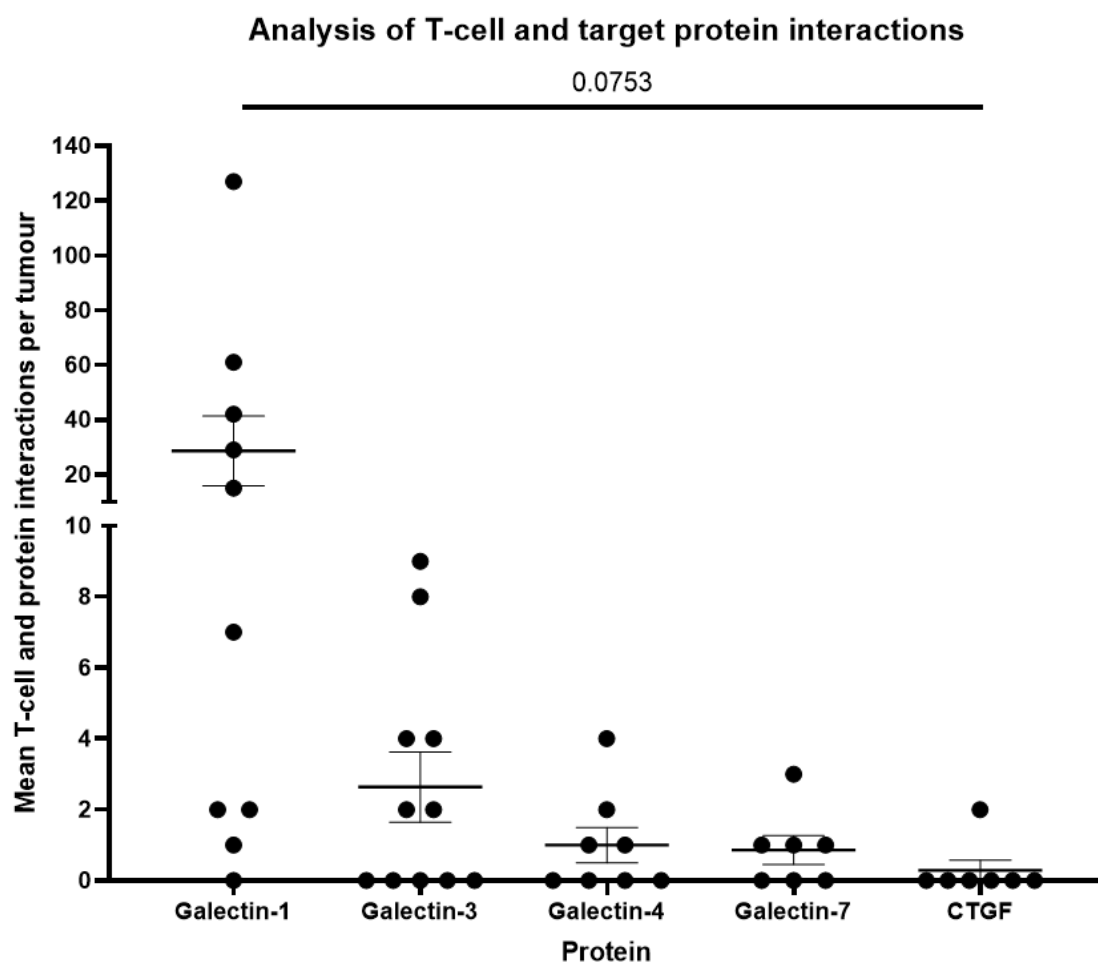


Figure 4.80. Analysis of T-cell and target protein interactions. Direct interactions between T-cells and proteins in double-positive GBM were counted. N=286 (galectin-1), 29 (galectin-3), 8 (galectin-4), 7 (galectin-7) and 2 (CTGF). Data were analysed by one-way ANOVA with Welch's correction. Error bars=mean $\pm$ SEM. Each data point represents one tumour.

4.2.2.5.2. Macrophage and protein interactions

Having completed analysis of interactions between T-cells and target proteins, interactions between TAMs and target proteins were studied, again using the same dataset.

TAMs were most likely to interact with gal-3, with an average of 5.25 interactions per tumour; gal-1 was the second most common, at 4.5 interactions per tumour. Interactions with gal-4 were more rare, and gal-7 or CTGF interactions were completely absent (Table 4.26). When these data were analysed by Kruskal-Wallis test, a significant difference was identified ($p=0.0017$, Figure 4.81). However, Dunn's multiple comparisons test did not return a significantly differing pair, although the difference between gal-3 and gal-7 or CTGF interactions approached significance ($p=0.0941$, both).

Overall, it was clear that TILs were most likely to interact with gal-1 or gal-3, dovetailing with the finding that these proteins were most abundant. Additionally, these data were reflective of intratumoural location: T-cells primarily interacted with gal-1, and both populations trended towards a significant PVN location, whereas TAMs – which were preferentially located in TB – interacted more with gal-3.

These analyses had clear limitations: for both cell types, the sample size was small, and it was necessary to evaluate whether close localisation of CD3/CD68 and target proteins was an interaction or co-expression, which may have lowered the numbers of documented cell-protein interactions. This was particularly the case with TAMs. Finally, this analysis could identify paracrine effects.

Table 4.26. Analysis of macrophage and target protein interactions

Target protein	Number of double-positive GBM	Number of interactions	Average interactions per double-positive tumour
Galectin-1	4	18	4.5
Galectin-3	4	21	5.25
Galectin-4	2	3	1.5
Galectin-7	3	0	0.0
CTGF	3	0	0.0

Table 4.26. Analysis of macrophage and target protein interactions. Mean averages are given to one decimal place.

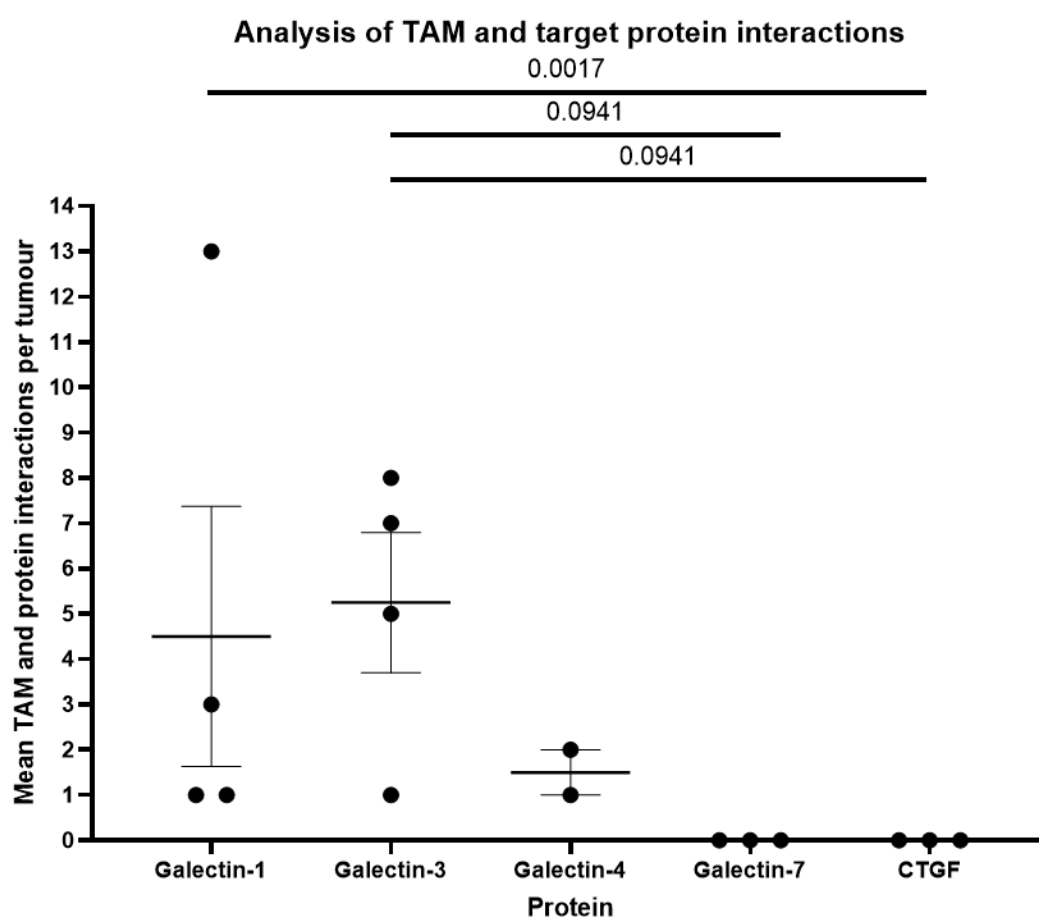


Figure 4.81. Analysis of TAM and target protein interactions. Direct interactions between macrophages and proteins in double-positive GBM were counted. $N=18$ (gal-1), 21 (gal-3), 3 (gal-4), 7 and 0 (gal-7, CTGF). Data were analysed by Kruskal-Wallis test (upper line) and Dunn's multiple comparisons test (lower lines). Error bars=mean $\pm$ SEM. Each data point represents one tumour.

4.2.3. Immune checkpoints

4.2.3.1. Preliminary work

Next, immune checkpoints (ICs) were considered. Preceding sections illustrated the presence, distribution and interactions of TILs and soluble proteins of interest. All three TIL populations and all five target proteins were frequently present, and T-cells and macrophages directly interacted with proteins in some GBM.

Given that potential suppression of T-cells was of particular interest, GBM sections were stained for two well-known immune checkpoints: galectin-9 (gal-9) and PD-L1 (for outline see 1.3.2.1.4.). Both have been widely studied in solid tumours; while they were absent in Gkanatsiou's preliminary mass spectrometry dataset (2022), their absence from tumour samples cannot be inferred from this; indeed, their absence could be due to sequence similarity with related proteins – especially in the case of gal-9 – or protein instability. GBM sections were assayed for the presence of these two molecules and their interactions with T-cells. Cell counts in these experiments were not verified by a second operator; the number of GBM used for each stain was limited by their use in other panels.

AlexaFluor594-conjugated antibodies against both proteins were obtained (anti-galectin-9 and anti-PD-L1). Details are given in Table 4.27; negative controls are shown in Figures 4.82A and B respectively. Human tonsil was used as a negative control for PD-L1. While this protein is expressed in the tonsil, it is localised to the crypt; thus, the follicle was imaged and no PD-L1 was observed. This was considered to be a reasonable negative control. HUVECs were used as the gal-9

negative control. Gal-1 was added to the panel to ascertain whether gal-1+PD-L1+ or gal-1+gal-9+ cells were apparent.

Table 4.27. Antibody panels for detection of immune checkpoint molecules

Specificity	Cell type	Fluorophore	Host	Dilution or concentration ($\mu\text{g/mL}$)	Clone
CD3	T-cell	AlexaFluor 647	Mouse	1:20	UCHT8
Galectin-1	N/A	N/A	Goat	10	Polyclonal
Galectin-9	N/A	AlexaFluor 594	Mouse	7.5	9M1-3
PD-L1	N/A	AlexaFluor 594	Mouse	7.5	29E.2A3
Goat	N/A	FITC	Donkey	1:100	Polyclonal

Table 4.27. Antibody panels for detection of immune checkpoint molecules.

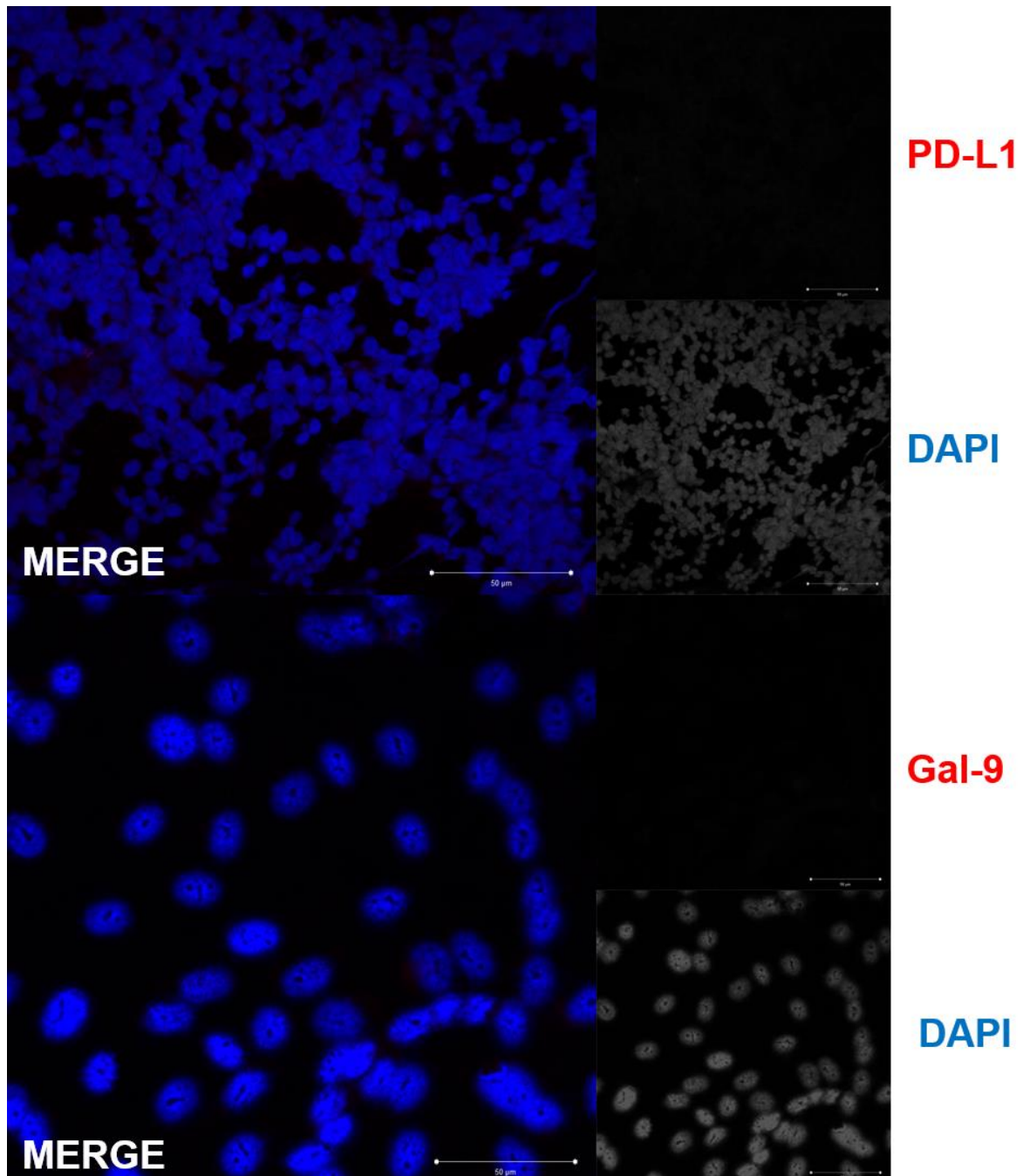


Figure 4.82. PD-L1 and galectin-9 negative controls. A: An FFPE human tonsil section was dewaxed in xylene and antigen retrieval was carried out using five rounds of heating in citrate buffer. Section was permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Tonsil was stained with anti-PD-L1 and DAPI. B: HUVECs were grown on a glass slide and fixed in cold methanol for ten minutes. Cells were permeabilised with PBS 0.05% Tween-20 and blocked with PBS 1% BSA. Cells were stained with anti-gal-9. Slides were imaged on a Zeiss LSM 880 confocal microscope. Scale bars=50μm.

4.2.3.2. Prevalence of galectin-9 and PD-L1

Paired sections from eight GBM were stained with anti-PD-L1 and anti-gal-9 alongside anti-CD3. Some sections were also stained with anti-galectin-1. This allowed for both ascertainment of the presence of these immune checkpoint molecules, and their interactions with T-cells.

Of the eight GBM examined, all expressed gal-9 and half expressed PD-L1. 75.0% of gal-9+ tumours contained extracellular gal-9, which appeared to be bound to the extracellular matrix (ECM). 50.0% of PD-L1+ tumours also contained extracellular PD-L1. These data are summarised in Table 4.28. Representative images are shown in Figure 4.83 (gal-9) and 4.84 (PD-L1).

Gal-1 and gal-9 colocalised frequently in the seven GBM stained for both, though no significant correlation between the presence of these two proteins was observed ($p=0.9999$, 2-tailed Fisher's exact test). Six sections were co-stained for gal-1 and PD-L1, which also did not correlate ($p=0.9999$, 2-tailed Fisher's exact test). Additionally, gal-9 and PD-L1 themselves did not occur together significantly ($p=0.9999$, 2-tailed Fisher's exact test). These data are shown in Table 4.29.

It was clear from these data that galectin-9 and PD-L1, two of the best-characterised immune checkpoint molecules, were present within GBM tumours; while much attention in the literature is given to PD-L1, it was the less widely represented of the two. While this experiment used a small dataset of only eight tumours and thus should be regarded as preliminary work, the possibility of widespread galectin-9-mediated immunosuppression merits further study.

Table 4.28: Summary of PD-L1 and galectin-9 positivity in 8 GBM

Location	Number of gal-9 positive tumours	% of gal-9 positive tumours	Number of PD- L1 positive tumours	% of PD-L1 positive tumours
Cellular	8/8	100.0	4/8	50.0
Extracellular	6/8	75.0	2/8	25.0

Table 4.28. Summary of PD-L1 and galectin-9 positivity in eight GBM. Tumour sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-PD-L1 or anti-galectin-9, anti-CD3, anti-galectin-1, a FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Percentages are given to one decimal place.

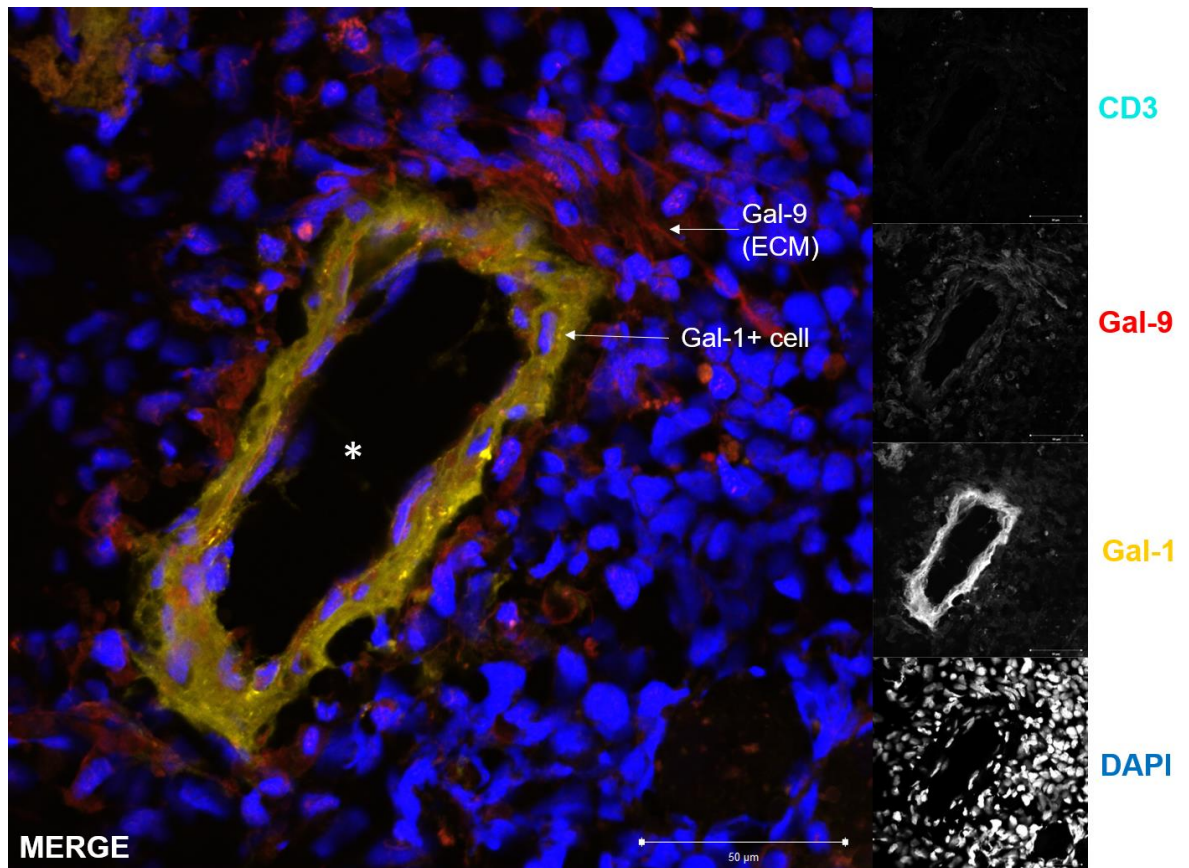


Figure 4.83. Representative image of intratumoural galectin-9. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-galectin-9, anti-galectin-1, FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm. Arrows indicate gal-1+ cells and ECM-bound gal-9. Asterisk=vessel lumen.

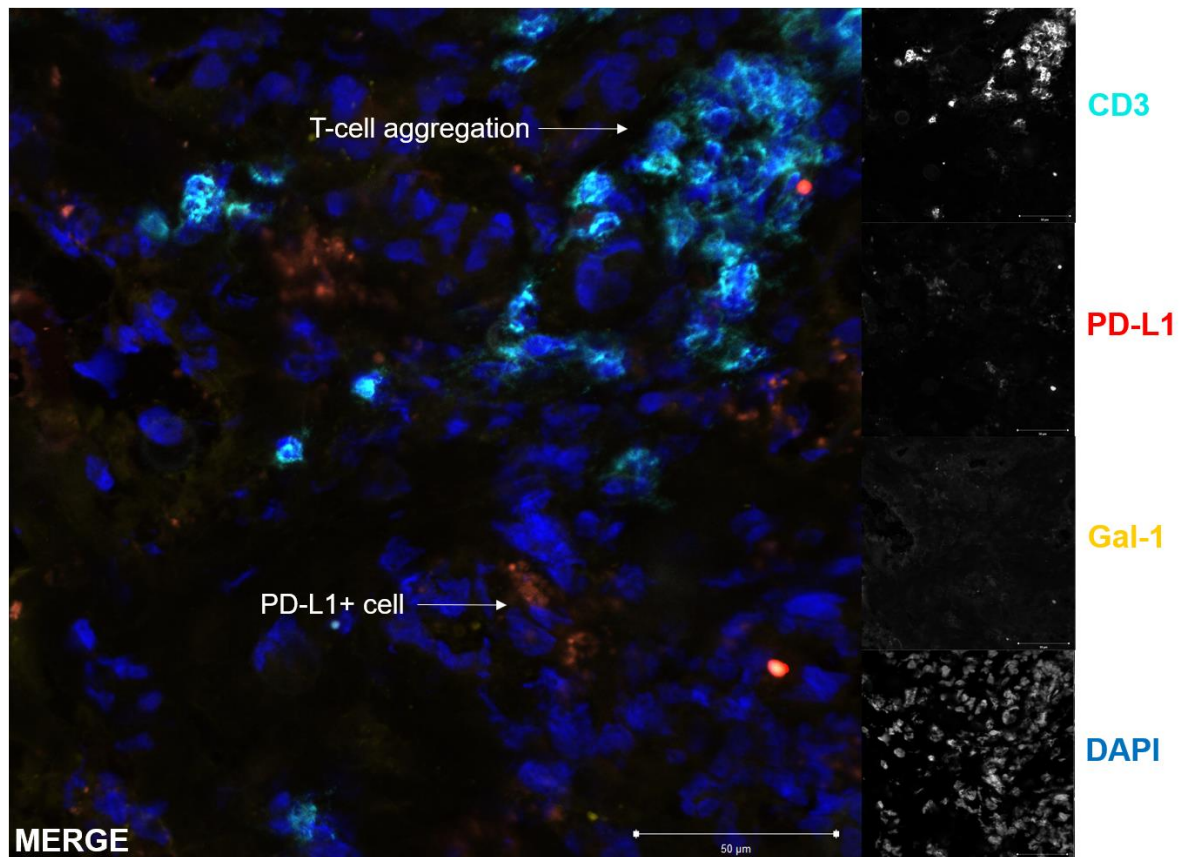


Figure 4.84. Representative image of intratumoural PD-L1. Frozen human GBM sections were permeabilised with 0.05% PBS-Tween-20 and blocked with PBS 1% BSA. Sections were stained with anti-CD3, anti-PD-L1, anti-galectin-1, FITC-conjugated secondary antibody and DAPI. Sections were imaged on a Zeiss LSM 880 confocal microscope. Scale bar=50μm.

Table 4.29. Significance of immune checkpoint and galectin-1 co-occurrence in GBM

Pair	N	P-value (Fisher's exact test)
Galectin-1 and galectin-9	7	>0.9999
Galectin-1 and PD-L1	6	>0.9999
Galectin-9 and PD-L1	8	>0.9999

Table 4.29. Significance of immune checkpoint and galectin-1 co-occurrence in GBM. Tumours where sections had been stained for both proteins in a pair were scored according to the presence of each protein and results were analysed by Fisher's exact test.

After confirmation that both PD-L1 and gal-9 were detectable in this dataset, the relative prevalence and intratumoural distribution of each was examined.

562 gal-9+ cells were noted in the eight gal-9+ GBM, and 14 PD-L1+ cells in the four PD-L1+ GBM. This gave mean averages of 70.3 and 3.5 positive cells per positive tumour, respectively (Table 4.30). This difference in means was found to be statistically significant ($p=0.0050$) by unpaired 2-tailed t-test (Figure 4.85), indicating that gal-9+ cells were significantly more common than PD-L1+ cells within GBM.

Table 4.30. Average immune checkpoint protein positivity per tumour

Protein	Number of positive cells	Number of positive tumours	Average positive cells per tumour
Galectin-9	562	8	70.3
PD-L1	14	4	3.5

Table 4.30. Average immune checkpoint protein positivity per tumour. Sections from eight GBM were stained for each molecule. Positive cells were counted and the total number of positive cells was divided by the number of positive tumours for each protein. Averages are given to one decimal place.

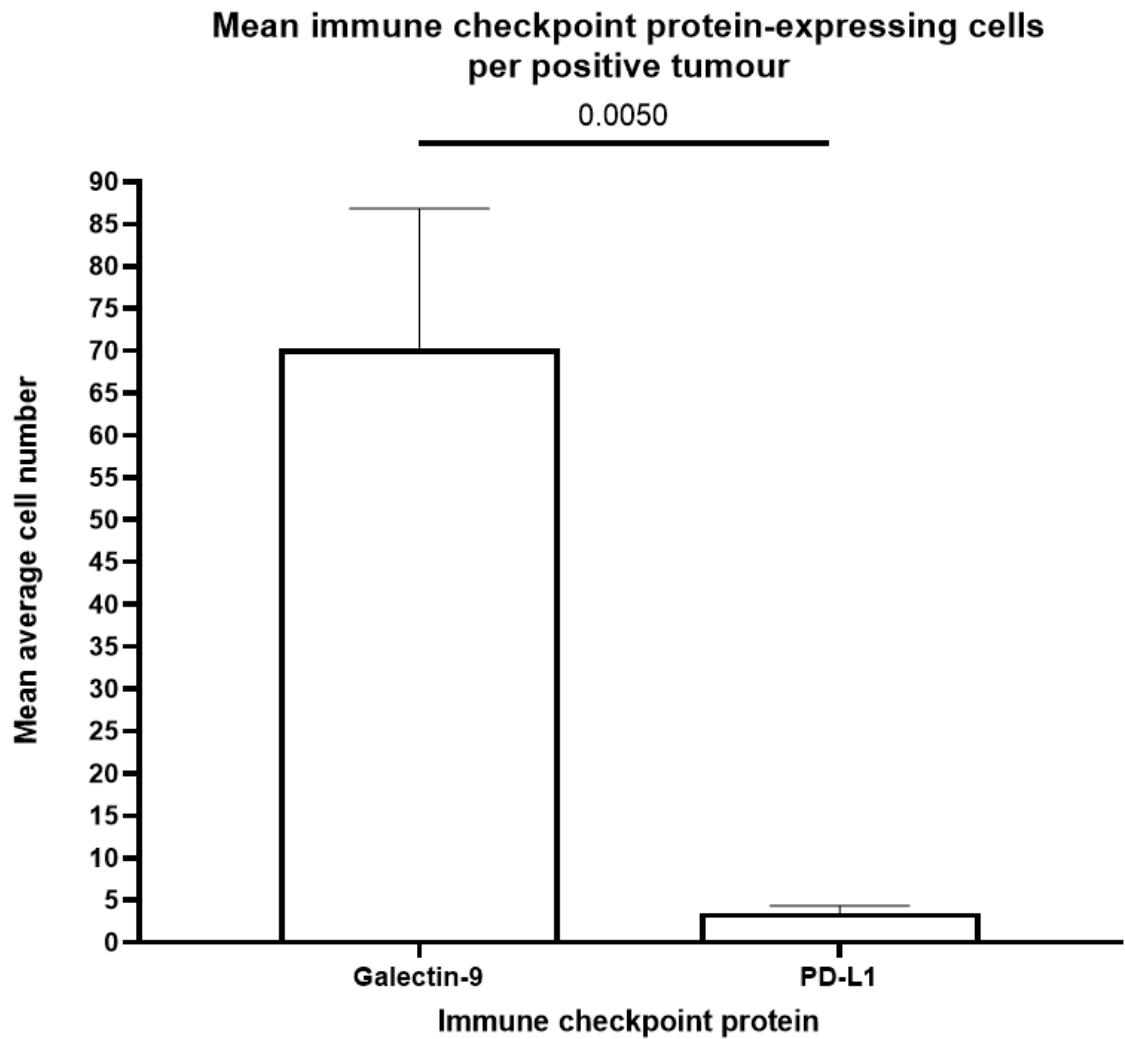


Figure 4.85. Mean immune checkpoint protein-expressing cells per positive tumour. The total number of immune checkpoint protein-positive cells was counted in sections stained for these populations and divided by the number of positive tumours. Data were analysed by unpaired 2-tailed t-test with Welch's correction. Error bars= SEM.

4.2.3.3. Distribution of galectin-9 and PD-L1

As gal-9+ and PD-L1+ cells were counted, their intratumoural niche was also recorded. As with TILs and target protein-expressing cells, PV cells were defined as those within 100µm of a vessel.

Of the 562 gal-9+ cells, 357, or 63.5%, were PV; the remaining 205, or 36.5%, were found in TB. Across the eight gal-9+ GBM, this gave a mean average of 44.6 and 25.6 gal-9+ cells per tumour in the PV and TB regions, respectively. For PD-L1+ cells, 3, or 21.4%, were PV, and 11, or 78.6%, were in TB; as only four GBM contained PD-L1+ cells, this gave a mean average of 0.8 PD-L1+ PV cells and 2.8 TB PD-L1+ cells per GBM. These data are summarised in Table 4.31. As gal-9 was frequently observed bound to the ECM, there were some difficulties in distinguishing gal-9+ cells and cells embedded in gal-9+ ECM.

When the mean number of cells per GBM in each niche was compared, no significant difference was found by unpaired 2-tailed t-test for either gal-9+ cells ($p=0.2747$) or PD-L1+ cells ($p=0.2192$) (Figure 4.86).

To conclude, while gal-9+ cells were more frequent in the PVN and PD-L1+ cells in the TB, these trends did not approach significance. However, it was possible that, with greater GBM numbers, a significant distribution would have been observed. It was also of interest that, while both immune checkpoint ligands were present, gal-9 was more prevalent and each molecule trended towards a different intratumoural localisation. This may be an indication that different immunosuppressive mechanisms were relevant in the PV and TB niches.

Table 4.31. Intratumoural distribution of galectin-9+ and PD-L1+ cells

Protein	Number of positive GBM	Number of positive PV cells	Mean positive PV cells per tumour	Number of positive TB cells	Mean positive TB cells per tumour
Galectin-9	8	357	44.6	205	25.6
PD-L1	4	3	0.8	11	2.8

Table 4.31. Intratumoural distribution of galectin-9+ and PD-L1+ cells. Gal-9+ and PD-L1+ cells in positive GBM were counted and the mean average number of cells per tumour was determined. Averages are given to one decimal place.

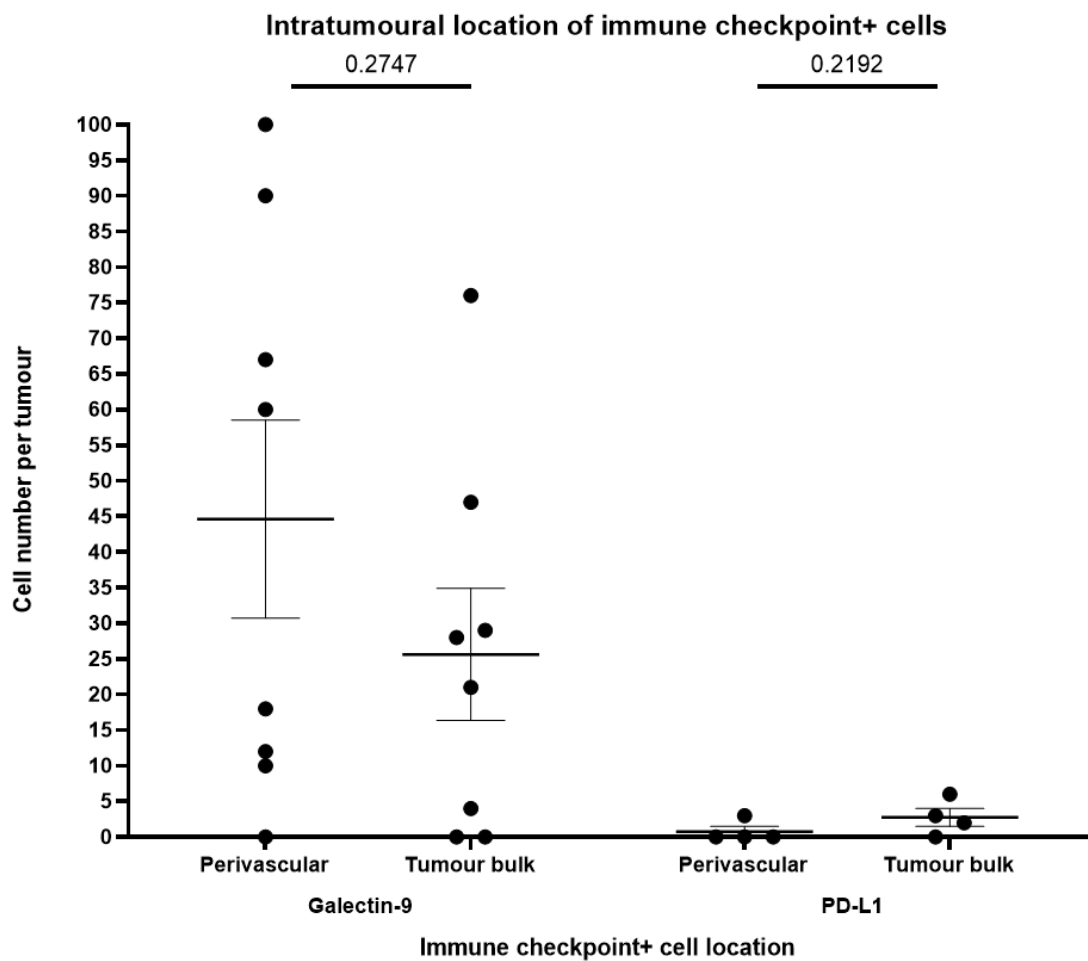


Figure 4.86. Mean galectin-9- and PD-L1-expressing cells in different intratumoural niches. 562 gal-9+ cells from eight GBM and 11 PD-L1+ cells from four GBM were counted and their location was categorised as perivascular (within 100µm of a vessel) or tumour bulk. Data were analysed by unpaired 2-tailed t-test. Error bars=mean ± SEM.

4.2.3.4. T-cell and immune checkpoint interactions

In the above sections, sections from eight GBM were used to outline the presence of the IC molecules galectin-9 and PD-L1 in the intratumoural environment. All GBM examined in this dataset contained gal-9 and half contained PD-L1; gal-9+ cells were significantly ($p=0.0050$) more abundant than PD-L1+ cells. Although gal-9+ cells were more represented in the PVN and PD-L1+ cells in the TB, there was no statistically significant compartmentalisation of these molecules. Finally, direct interactions between these IC molecules and T-cells were quantified.

Seven GBM were both gal-9 and T-cell positive; from these tumours, five sections contained both populations of interest, within which 39 instances of T-cell/gal-9 interactions were counted. Of the four PD-L1+ sections, three also contained T-cells; eight interactions were counted, all of which occurred in one section. This gave mean averages of 5.6 and 2.0 interactions per double-positive tumour for gal-9 and PD-L1 respectively (summarised in Table 4.32). Analysis by unpaired two-tailed t-test did not reveal any significant tendency for T-cells to interact with one checkpoint molecule over the other ($p=0.3538$, Figure 4.87).

It was concluded that, despite the significantly higher presence of gal-9 than PD-L1 in GBM, T-cells may have been equally likely to interact with either IC. However, of particular interest was Figure 4.74, which showed a PV T-cell aggregation in proximity to gal-1 and gal-9 positivity. While T-cells did not show preferential interactions with different IC molecules in this preliminary dataset, gal-1 and potentially gal-9 may facilitate reduced T-cell migration.

Table 4.32. Analysis of T-cell and immune checkpoint interactions

Protein	Number of double-positive GBM	Number of interactions	Mean T-cell and protein interactions per double-positive tumour
Galectin-9	7	39	5.6
PD-L1	4	8	2.0

Table 4.32. Analysis of T-cell and immune checkpoint protein interactions. Averages are given to one decimal place.

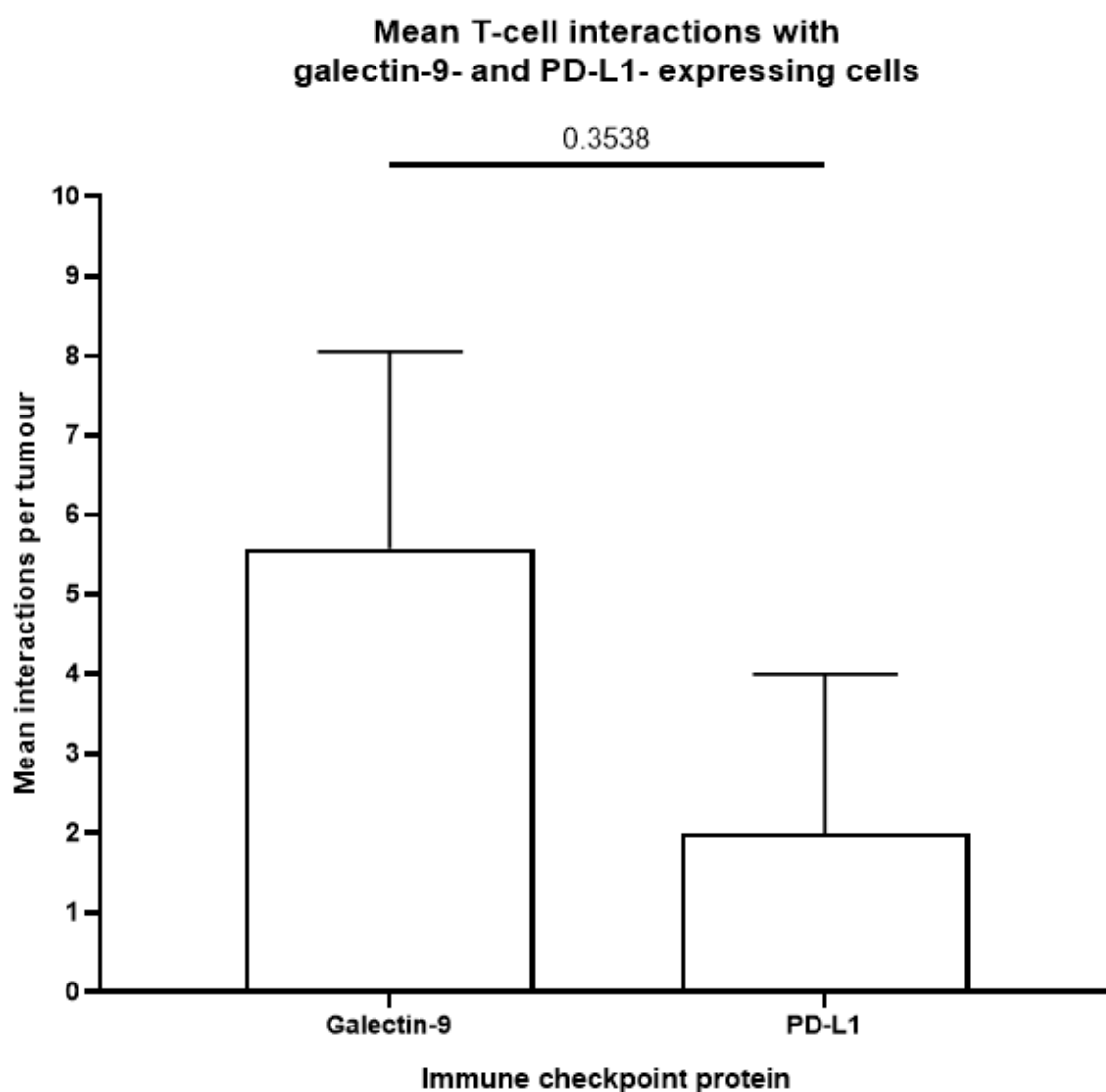


Figure 4.87. Mean T-cell interactions with galectin-9- and PD-L1-expressing cells. 39 interactions across seven gal-9+ T-cell+ tumours and eight interactions across four PD-L1+ tumours were observed. Data were analysed by unpaired 2-tailed t-test without Welch's correction. Error bars=SEM.

4.3. Discussion

4.3.1. Characterisation of intratumoural immune populations

In 4.2.1., the presence and distribution of T-cells, B-cells and macrophages in 50 GBM (48 for B-cells) was outlined. Intratumoural TILs were common: 84.0% of tumours were T-cell+, 72.0% contained macrophages and 45.8% were B-cell+. However, while both B-cells and macrophages were significantly more common in the TB ($p=0.0053$ and 0.0087 respectively), T-cells were predominantly located in the PVN ($p=0.0932$), suggesting that T-cells infiltrated into the tumour less effectively. T-cells were the most common population observed in terms of percentage positivity and overall cell number (2235 compared to 171 B-cells and 1318 TAMs), but in terms of mean cells per triple-stained tumour, TAMs were most prevalent ($p=0.0114$ compared to B-cells, 0.0646 compared to T-cells).

Compared to other authors (1.4.4.2.), this work described a reduced incidence of TAMs: these cells are widely held to be present in the majority of GBM and to comprise anywhere from 50-90% of cells within the tumour (Kmiecik et al., 2013; Berghoff et al., 2015; Fu et al., 2020). TAMs in the analysis herein accounted for a lower proportion of intratumoural cells. Several possible explanations were apparent: many papers asserting a high prevalence of TAMs assessed whole tumours with flow cytometry (FC), whereas this work used IF to assess GBM sections. These sections may not have been representative of the whole tumour from which they derived, and while IF and immunohistochemistry (IHC) provide spatial information that FC cannot, standard IF is less well-suited to providing a proportional overview of cell types. Additionally, multiple markers can be used to identify TAMs. When the above results were compared to those of Orrega et al.,

who in 2018 used CD68 IHC to identify 9% of cells within GBM as TAMs, the impact of experimental technique and marker selection was immediately clear. As this work used CD68 IF to detect TAMs, a reduced prevalence may be expected. As discussed in 1.4.4.1, figures for T-cell positivity in GBM are also varied. Authors have described T-cell positivity in anywhere from 18% (Lee et al., 2018) to 100% (Heimberger et al., 2008); most research indicated a high prevalence of T-cells (Leung et al., 1997; Berghoff et al., 2015) and thus the figure of 84.0% in this work was in line with other results.

Despite the commonality of TILs, cell-cell interactions were not commonly observed: 36 T-cell/TAM interactions were documented, most of which were ambiguous in nature, and no interactions of B-cells with either other phenotype. However, these data did not account for intratumoural paracrine signalling. The lack of observed T-cell interactions with astrocytes or B-cells, both of which also act as APCs, suggested that intratumoural antigen presentation may be impaired, resulting in T-cell exit and reduced levels of anti-tumoural immunity.

4.3.2. Immunosuppression in the GBM TME

4.3.2.1. The PVN versus the TB

Gal-1 and CTGF were the main differentially expressed proteins between the TB and PVN. The PV distribution of gal-1 approached significance ($p=0.0844$) and CTGF was exclusively present in the TB. Gal-3, -4, -7 and PD-L1 prevalence trended towards the TB and gal-9 in favour of the PV, but in no case did these data reach significance ($p=0.1069$, 0.1432 , 0.3149 , 0.2192 and 0.2747 respectively). These data suggested a spatial compartmentalisation of certain immunosuppressive proteins. A broader characterisation of potential immunosuppressive mechanisms operating in differential intratumoural niches may have benefit in alleviating immunosuppression and boosting the efficacy of novel immunotherapies. While theoretically either CTGF or gal-1 could suppress T-cell-mediated immunity through different means, the PV localisation of T-cells approached significance ($p=0.0932$) and so they were more likely to be suppressed by gal-1 than CTGF.

4.3.2.2. Effects on B-cells

B-cells were significantly under-represented compared to TAMs ($p=0.0114$) but were significantly more common in the TB than PVN ($p=0.0053$), showing that those few cells that entered the tumour infiltrated effectively. At which stage might B-cell suppression have been relevant: inhibited extravasation, death in the TB, or both? This may be partially answered by the significant prevalence of both B-cells and TAMs ($p=0.0087$) in the TB in combination with observations of CD19/CD68 co-localisation, which potentially indicated macrophage internalisation of B-cell-derived material. Whether this was due to direct phagocytosis of B-cells or scavenging of material from apoptotic B-cells could not be answered in the course of this work, but the role of GBM TAMs in B-cell suppression merits further investigation.

Currently, no literature appears to examine gal-1 effects on B-cell motility, and reports on how it affects B-cell function are conflicting: some authors suggest gal-1 promotes B-cell activity and others have found it to be inhibitory or pro-apoptotic (for review see Giovannone et al., 2018).

The same review notes inhibitory effects of gal-3 and gal-9 on B-cell function. B-cells would encounter gal-1 in the PVN followed by gal-3 and -9 in the TB; it is possible that this sequential exposure inhibited their function. Gal-9 expression on HUVECs has been documented to promote B-cell adhesion while inhibiting trans-endothelial migration, which may have contributed to lower numbers of infiltrating B-cells relative to other TILs. It is possible that only a highly motile subset of B-cells was able to transmigrate and infiltrate the TB. In these experiments, gal-9 exposure increased expression of the B-cell regulatory molecule SLAMF7; however, these

cells were stimulated with a high concentration of recombinant gal-9 (4µg/mL) (Chakraborty et al., 2021), which may not be relevant in the context of GBM.

Given the preferential location of both in the TB, CTGF could be suggested to suppress B-cell function. However, this theory did not appear to be supported by the literature. No clear evidence of a suppressive effect of CTGF on B-cells could be discerned; indeed, it has been found to support B-cell development and proliferation in murine bone marrow (Cheung et al., 2014). Regardless, it is likely that the cocktail of known immunosuppressive proteins B-cells would be exposed to during and following extravasation would decrease their ability to act against GBM.

4.3.2.3. Effects on T-cells

Inhibited T-cell migration offered an explanation for the T-cell aggregations observed in 22.0% of GBM: if motility were sufficiently impeded, T-cells could be retained in the tissue regardless of encountering their cognate antigen. It is also possible that these were tertiary lymphoid structures (TLS), which have been noted in GBM by other authors based on the presence of both B- and T-cells (Mauldin et al., 2021). TLS have been documented in other solid cancers: these structures permit B-cell maturation outside of the LN and spleen, and have often been regarded as beneficial in tumours (for review, see Jacquelot et al., 2021). However, B-cells were not observed in any aggregations, so the former explanation may be more relevant to this dataset.

Gal-1 suppression of T-cell motility has been well-documented in the literature. This protein has been found to inhibit extravasation of peripheral mononuclear cells (La et al., 2003) and inhibit T-cell binding to HUVECs; conversely, its downregulation promoted T-cell adhesion *in vitro* and *in vivo* (Norling et al., 2008). Conditioned media from prostate cancer cell lines stimulated HUVEC gal-1 production *in vitro*, resulting in inhibited T-cell trans-endothelial migration; ECM-bound gal-1 can inhibit T-cell migration by limiting CD43 redistribution (He and Baum, 2006). In the work described herein, gal-1 expression was noted in what appeared to be vascular ECs in several GBM (Figures 4.65 and 4.78) as well as extracellular deposits that appeared to inhibit T-cell migration (Figure 4.44). In light of these results, it appeared very likely that gal-1 was a key mechanism of GBM T-cell suppression.

Additionally, gal-3 has been recently described to play a role in reducing T-cell infiltration into solid cancer. *In vitro*, matrix-associated gal-3 removed cytokines such as IFN γ and IL-12 from solution, resulting in reduced expression of CXCL9, a chemokine that promotes T-cell infiltration into solid tumours. Gal-3 inhibition promoted T-cell influx into an *in vivo* model of human melanoma (Gordon-Alonso et al., 2017). Thus, gal-1 and gal-3 may work in concert in GBM to limit T-cell infiltration. Both proteins have also been described to promote T-cell apoptosis through distinct mechanisms (Stillman et al., 2006), but given that the T-cells observed in this work generally appeared intact and CD3 $^{+}$ remnants were not noted inside TAMs, induction of apoptosis was considered to be a less likely method of immunosuppression in this dataset.

The other studied proteins may further immunosuppression. Gal-4 has been described as pro-apoptotic and migration-inhibitory, though this paper used 100 μ g/mL of gal-4 (Paclik et al., 2008). Gal-4 may promote T-cell apoptosis and has been associated with reduced TIL presence in a murine pancreatic cancer model, which was alleviated in gal-4-null transplants, resulting in improved survival (Lidström et al., 2023). Conversely, high concentrations of gal-7 promoted pro-inflammatory cytokine secretion and proliferation of activated CD4 $^{+}$ T-cells (Luo et al., 2018). Very little research appears to exist on the effects of CTGF on T-cells. However, one paper has suggested CTGF does not influence proliferation or viability of CD4 $^{+}$ T-cells (Kunzmann et al., 2008). Additionally, the presence of the immune checkpoint molecules PD-L1 and gal-9 would also result in a reduction in T-cell function.

4.3.2.4. Effects on TAMs

The presence of gal-1 in GBM may also be indirectly harmful by facilitating TAM functions. Gal-1 has been found to function as a monocyte chemoattractant at high concentrations, both *in vitro* and *in vivo* (Malik et al., 2009; Gil, Gullo and Oliani, 2010); thus, gal-1 may promote monocyte recruitment. Transcriptomic analysis has shown lower (300ng/mL) concentrations of gal-1 to induce a specific expression program, particularly in M2- but also in M1-polarised macrophages, which resulted in a TAM phenotype and PD-L1 upregulation (Rudjord-Levann et al., 2023), and thus widespread gal-1 expression in GBM may well support TAM development.

Gal-3 has also been described as a chemoattractant *in vitro* and at low concentrations, promoted chemokinetic, or non-directed, monocyte migration; it also induced macrophage migration (Sano et al., 2000), whereas gal-1 was monocyte-specific (Malik et al., 2009). Their binding to laminin and fibronectin promoted migration of monocytes, and for the latter, macrophages, further (Danella Polli et al., 2013). In murine melanoma and lung cancer models, steeper gal-3 concentration gradients promoted M2 macrophage infiltration, though these results only used one M2-associated marker (Jia et al., 2013). Work by other authors has suggested that exposure to gal-1, -3 and -4 resulted in inhibited monocyte migration and increased apoptosis – however, these authors used 50µg/mL galectin, which is not at all relevant to the GBM TME (Paclik et al., 2011). Thus, intratumoural gal-1 and gal-3 may draw monocytes into the tumour, where they are able to further GBM growth by suppressing the activity of other infiltrating effectors.

4.3.2.5. Intratumoural immune checkpoints

Concurrently, a preliminary dataset of eight GBM was examined for the presence of IC molecules galectin-9 and PD-L1, which were present in 50% and 100% of tumours surveyed, respectively. Gal-9+ cells were significantly more common ($p=0.0050$) than PD-L1+ cells and showed a greater frequency in the PV niche than the TB niche; conversely, PD-L1-expressing cells were mostly present in the TB, although the differential distribution of each protein did not approach significance.

Other authors have found PD-L1 and gal-9 to be widely present in GBM (for details see 1.4.5.4.1.). Many of these papers identified PD-L1 in the majority of GBM assayed (Berghoff et al., 2015; Nduom et al., 2016; Knudsen et al., 2021); Lee et al., conversely, found 32% of GBM to be PD-L1+ in their 2018 paper. These analyses all used large datasets of 99 or more tumours, and as this dataset was far smaller, PD-L1 tumours may have been over-represented. Regardless, the observed 50% positivity mark was in the range described by previous authors. Unlike some of the above authors, CD3+PD-L1+ cells were not identified in sections where both markers were present. The high levels of intratumoural heterogeneity within GBM and the small sample size likely contributed to the observed difference between these and other authors' results. Conversely, the finding that gal-9 was universally detectable by IF in an initial dataset of eight GBM appeared to be in line with the results of other authors. Knudsen et al. found gal-9 to be universally present in 163 GBM in their 2021 paper, with high co-expression of gal-9 and PD-L1. They also suggested that gal-9 is widely expressed by TAMs and tumour cells, which was not confirmed in the work described here.

The widespread presence of both ICs, alongside the known immunosuppressive proteins gal-1 and gal-3, lent weight to the idea of using checkpoint blockade of widely expressed molecules to alleviate intratumoural suppression. As outlined in 1.4.6.2., blockade of the PD-1/PD-L1 signalling axis alone has not been effective in human clinical trials, but may synergise well with other therapies in murine pre-clinical models (1.4.6.1.), leading to new clinical trials utilising combined therapies such as anti-PD-1 alongside anti-CTLA-4 (Wen, 2021). Combined blockade of galectins may be of use, and merits further study in pre-clinical models.

4.3.2.6. Vessel structures and formation

In 4.2.1.2., the presence of intratumoural CD34-LYVE1+ lymph vessels and CD34+LYVE1+ mixed-phenotype vessels was described. LVs have recently been documented in the mammalian CNS (Aspelund et al., 2015; Louveau et al., 2015). LYVE1+ cells were first described in glioma in 2020 by Meng et al., who used a panel of seven patients, three of whom were diagnosed with GBM; they also observed intratumoural CD34+LYVE1+ structures, and suggested that tumour cells directly differentiate into lymphatic structures. Their results were not stratified by diagnosis, likely due to limited sample size.

If intratumoural LVs originated from ingrowth of meningeal LVs and were thus connected to the wider lymphatic system, they may represent an efficient mechanism for TIL infiltration; if the vessels truly originated from tumour cells, this would be unlikely to be relevant. Not all GBM in this dataset examined herein were found to contain CD34-LYVE1+ vessels. However, if they originated from the meningeal lymphatic system, LV presence would be affected by tumour location: tumours distant from meningeal lymphatics would by necessity be less likely to promote successful infiltration by new LVs.

Additionally, the prevalence of CD34+LYVE1+ vessels was of interest. Due to clear EC expression of CD34 and vessel-like morphology, it was concluded that the CD34+LYVE1+ structures were likely blood vessels, though LYVE1 positivity in non-lymphatic vessels was not expected. However, LYVE1 has been detected in mouse yolk sac and embryonic blood vessels (Gordon, Gale and Harvey, 2008) and in human liver sinusoidal capillaries (Mouta Carreira et al., 2001). It was therefore

possible that human CNS capillaries in healthy tissue also display both markers, which comparison could not be performed here. T-cells were significantly more likely to be associated with mixed-phenotype vessels than others, which may indicate impaired infiltration by TILs carried in mixed-phenotype vessels as compared to physiologically normal blood or lymph vessels.

4.3.3. Secretome differences between *in vitro* cultures and intratumoural populations

Gal-1, gal-3 and CTGF were all reliably detected in U251 and U373 supernatants by ELISA (3.2.2.2. and subsections therein) and cells of the same lines by IF. Some heterogeneity was noted in that gal-1 and CTGF appeared to be produced by subpopulations of U251 and U373 cells; the same was observed for gal-3 in U373, but not U251, cells. By contrast, gal-4 was absent in both cell lines, and gal-7 was present in a subset of U373 cells by IF but undetectable in supernatant. It was clear from these data that, in *in vitro* cultures, galectin secretion may be more tightly regulated than synthesis. Conversely, it was possible that the IF antibodies had a lower detection threshold than the ELISA antibodies. Finally, all proteins except gal-1 were undetectable by IF in THP1- and Monomac-6-derived macrophages. Thus, it was considered likely that all proteins may be detected in intratumoural tumour cells and that gal-1 would additionally be present in TAMs.

However, TAMs accounted for greater proportions of gal-3, -4, -7 and CTGF+ cells (9.3%, 43.6%, 68.2% and 78.9% respectively) than gal-1+ cells (1.7%) in slides co-stained with anti-CD68. Human macrophages have been described to upregulate gal-3 during M1/M2 polarisation (Novak, Dabelic and Dumic, 2012), which process did not affect their intracellular gal-1 pools (Krautter et al., 2020). As discussed in 1.4.5.1., the M1/M2 dichotomy likely does not replicate the TAM phenotype well and thus these sets of results may not be directly comparable. It is worth bearing in mind that IF could not distinguish between native expression of a protein and its intracellular presence as a result of phagocytosis.

For gal-1, -3, -4, -7 and CTGF, tumour cells accounted for 5.3%, 48.3%, 52.1%, 8.6% and 16.2% of cells in slides co-stained with anti-CD56, respectively. As with macrophages, these results did not show high concordance between U251 and U373 cultures – which lacked gal-4 and gal-7 – although only subpopulations were gal-1+ or CTGF+. Work in 3.2.2.2. already identified differences between the secretome of GBM TM samples and culture-derived supernatants.

These results underscored the importance of not relying on simplistic *in vitro* models to recapitulate a highly complex intratumoural environment, especially in relation to such a plastic cell type. This research highlights the need for use of patient-derived samples and great caution when extrapolating results from *in vitro* cultures to *in vivo* tumours. Along these lines, spheroid culture models utilising multiple cell types to replicate the TME more accurately may have great potential in future solid tumour secretome studies.

Additionally, it was not possible to confirm if gal-1 and CTGF were expressed by the same subpopulations within GBM cell lines, or if a subpopulation of tumour cells or TAMs existed that expressed more than one target protein, as all stains used the same secondary antibody.

4.3.4. Conclusions and limitations

This series of experiments set out to identify the prevalence and distribution of T-cells, B-cells and TAMs, as well as immunosuppressive target proteins, in sections from 50 human GBM tumours. This dataset was then used to ascertain cellular sources of target proteins and document cell-cell and cell-protein interactions, with a view to elucidating the intratumoural relevance of these proteins and characterising any potential effects on immune effector behaviour.

All three populations of interest were represented, but B-cells were significantly rarer than T-cells and TAMs. While both B-cells and TAMs showed a significant preference for the TB, T-cells were often found in the PVN, and 22% of GBM contained T-cell aggregations, which did not appear to be TLS. Analysis of immunosuppressive proteins revealed that only gal-1 and CTGF showed preferences for either niche: the PVN and TB, respectively.

Altogether, these data raised the possibility that immunosuppressive environments in the TB and PVN were different in terms of both protein and effector cell presence. This was further confirmed by cell-protein interactions: while T-cells were more likely to interact with gal-1, TAMs preferentially interacted with gal-3. Additionally, contacts between T-cells and APCs were rare: one potentially stimulatory T-cell/TAM interaction was observed, and no T-cell/B-cell or T-cell/astrocyte contacts. Furthering the theory of a uniquely suppressive environment in the PVN was the observation that immune checkpoint molecule gal-9 was widespread in GBM, and was often found surrounding vessels and in proximity to gal-1.

In conclusion, the PVN immunosuppressive environment appeared to be particularly geared towards inhibition of T-cell mediated immunity, which may occur through multiple complementary mechanisms. First, PV gal-1 likely inhibits T-cell extravasation and migration into the TB. Antigen-specific T-cells that avoid migration arrest may return to the vasculature, as the paucity of APCs in the PVN renders encounter of their cognate antigen less likely. Of the T-cells that did enter TB, only a minority were observed making contact with CD56+ cells or tissue, suggesting limited direct cytolytic activity.

Additionally, B-cells may be uniquely suppressed through different mechanisms to T-cells, as well as being comparatively understudied in GBM. B-cells were by far the rarest effector population in this dataset, and despite their observed ability to infiltrate GBM, most B-cells appeared unhealthy. Their extravasation, but not migration, may have been inhibited by PV gal-1. As B-cells move through tumour tissue, they would be likely to come into contact with other known suppressive molecules, such as gal-3, potentially resulting in their demise and ingestion – either through phagocytosis or post-death scavenging – by TAMs. Very little literature about the roles, prognostic effect or suppression of B-cells in GBM is apparent. However, the recent observation of potential TLS in GBM (Mauldin et al., 2021) and the presence of multiple class-switched antibody fragments in whole-GBM-derived mass spectrometry data suggests that this population is worthy of more attention. It is hoped that future researchers will build upon these results to build a more comprehensive model of the GBM TME.

It must be noted that this work had several limitations, one of which was sample size and another of which was diagnosis. As with the TM samples examined in Chapter 3, the majority of these samples were obtained prior to 2021: before the requirement for GBM to be IDH-wild type came into effect. Thus, some IDH-mutant samples that would today be diagnosed as high-grade glioma were likely included in the dataset, and so inferences made about the intratumoural immune environment and its implications for immunotherapy on the basis of this dataset may not be readily extrapolated to post-2021 GBM patients.

Additionally, because whole-tumour sections were not available, these results could not be stratified by intratumoural location beyond PV or TB niches. Notably, the presence of TILs or target proteins across tumour core, infiltrating margin and adjacent brain tissue could not be compared. Further, results could not be stratified by patient characteristics such as age, treatment, molecular subtype or survival time, all of which could potentially offer further insight. In particular, comparisons of immune effector and target presence with diagnostic and prognostic data could position certain target proteins as potential therapeutic targets in subsets of patients. It is hoped that later work will remedy this.

Finally, many analyses identified clear trends without reaching statistical significance. The most relevant examples included the PV presence of T-cells ($p=0.0932$) and gal-1 ($p=0.0844$), and thus the tendency of T-cells to interact with gal-1 over every other target protein ($p=0.0753$). These data may have been affected by small sample size: many studies examining cell or protein populations

of interest in GBM use datasets of 100 or more tumours. Regardless, the near-significance of the interaction implied that the location trend is genuine.

There were also financial limitations at play. Examination of cell-protein interactions was made more difficult by target protein expression in some cell types, as well as the difficulties inherent to interpreting these highly necrotic tumours. Furthermore, as the use of a specialist IF platform allowing analysis of a wide variety of markers was not possible, this work was limited to two to three non-DAPI markers per section, which increased the likelihood of over- or under-estimating the presence of different populations. TAMs in particular have been documented to return different numbers depending on the marker used.

While this work had some clear drawbacks, it nevertheless offered valuable insight into the immunosuppressive GBM TME. Herein, the presence of gal-4 and -7 – two proteins generally regarded as absent from the intracranial environment – has been described. It was identified that PV gal-1 may inhibit both T- and B-cell extravasation as well as T-cell migration, and that B-cells may be subject to scavenging or phagocytosis by TAMs in the TB. Finally, the presence of multiple galectins, at least three of which – gal-1, -3 and -9 – are known to be immunosuppressive, opened the possibility of galectin synergy that may be a potent inhibitor of immune effector function. Further study into the clinical relevance of galectins in the GBM TME may offer new targets for blockade alongside existing therapies.

5: EFFECTS OF RECOMBINANT HUMAN PROTEINS ON MACROPHAGE FUNCTION AT GBM-RELEVANT CONCENTRATIONS

5.1. Introduction

In the previous chapters of this thesis, immunomodulatory proteins were identified in GBM mass spectrometry data (3.2.1.2.1.). The presence of these proteins was confirmed in paired GBM transport media (TM) and culture supernatant (CS), and GBM cell line supernatant using enzyme-linked immunosorbent assay (ELISA) (3.2.2.2. and subsections therein). A subset of these proteins – four galectins and CTGF – were then examined in GBM sections using immunofluorescence (IF) staining and confocal microscopy (4.2.2.3. and subsections therein). These results allowed characterisation of the intratumoural distribution and potential cellular sources of these proteins. In this chapter, the functional effects of these proteins on immune cells *in vitro* – both individually and pooled – were examined. In comparison with glioma cell line CS, these experiments would illustrate whether any observed changes in behaviour were primarily mediated through these immunomodulatory proteins or other soluble factors.

While experiments were originally intended to use donor-derived T-cells, it became necessary to find a replacement. Thus, experiments proceeded to use the monocyte cell lines THP1 and Monomac-6 to study the effects of proteins of interest on phagocytosis and antigen presentation-associated molecules.

5.2. Results

5.2.1. Preliminary work

5.2.1.1. Recombinant human proteins

Mean average target protein concentrations in 23 GBM TM as determined by ELISA were compiled from 3.2.2.2. and subsections therein for use in *in vitro* assays. These data are given in Table 5.01. Protein concentrations used in cell media were made up to the nearest whole number. It must be noted here that, for CTGF, one GBM datapoint was erroneously replaced with data from a meningioma sample, which contained a far higher concentration of CTGF than any assayed GBM. For this reason, the mean average was incorrectly taken to be 925.7pg/mL; as stated in 3.2.2.2.6, where this mistake was not made, the true mean concentration across the 23 assayed GBM was 246.0pg/mL.

Table 5.01. Mean average soluble protein concentrations in 23 GBM TM as determined by ELISA

Protein	Range	Mean average	Experimental concentration
Galectin-1 (ng/ml)	0-118.2	11.2	11.0
Galectin-3 (ng/mL)	0-3.7	1.8	2.0
Galectin-4 (pg/mL)	0-765.3	33.7	34.0
Galectin-7 (pg/ml)	0-848.2	36.9	37.0
CTGF (pg/ml)	0-15627.0	925.7	926.0

Table 5.01. Mean average soluble protein concentrations in 23 GBM TM as determined by ELISA. Values are given to one decimal place.

Since galectin-1 in particular is vulnerable to oxidation, which renders the carbohydrate recognition domain (CRD) non-functional (Tracey et al., 1992), the reducing agent dithiothreitol (DTT) was added to media at 1mM to maintain galectin activity. A blocking experiment using 0.1M lactose was also performed. Lactose preferentially binds the CRD and thus should prevent active galectins from exerting biological effects, by rendering them unable to bind glycosylated proteins. In addition, a third experimental group of cells treated with proteins alone was added, as well as an untreated control. Experimental details are shown in Table 5.02.

Table 5.02. Flow cytometry and phagocytosis experimental groups

Group	Recombinant protein	DTT (mM)	Lactose (M)
Untreated control	No	0	0
Protein only	Yes	0	0
Active protein	Yes	1	0
Blocked protein	Yes	1	0.1

Table 5.02. Experimental groups for flow cytometry experiments.

5.2.1.2. Phagocytosis assay controls

Differentiated monocyte-derived macrophages were treated with either phagocytosis assay reagent or phosphate-buffered saline (PBS) as a negative control. Control cells are shown in Figure 5.01A (THP1) and B (Monomac-6), where no red fluorescence is visible. Although background red fluorescence in treated wells was high, marked fluorescence inside some cells could be easily visualised (shown in panel C for THP1- and panel D for Monomac-6-derived cells, respectively). Examples of cells containing red fluorescence above background levels, which were counted as phagocytic, are indicated by red-filled arrowheads; cells that were considered to not be phagocytic are indicated by unfilled arrowheads.

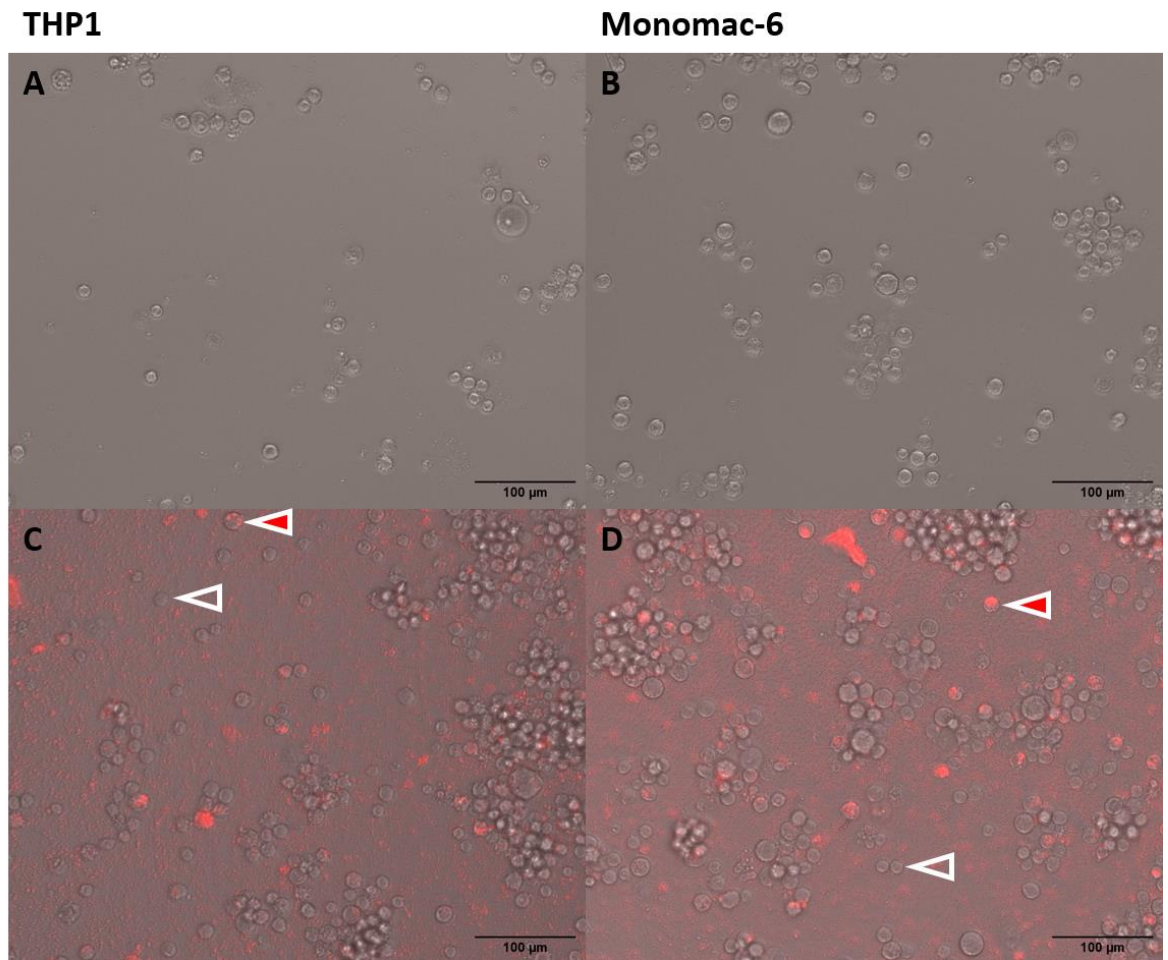


Figure 5.01. Phagocytosis assay controls. Cells were seeded in 20ng/mL PMA for 24 hours, washed and grown in fresh media for a further 48 hours. Cells were incubated with phagocytosis assay reagent in a non-CO₂ incubator for two hours and imaged on an Evos FL Auto 2 using transmitted light and a red fluorescent protein (RFP) filter. A: THP1-derived cells treated with PBS. B: Monomac-6-derived cells treated with PBS. C: THP1-derived cells treated with phagocytosis assay reagent. D: Monomac-6-derived cells treated with phagocytosis assay reagent. Exemplar phagocytic cells in C and D are indicated by red-filled arrowheads; non-phagocytic cells are indicated by unfilled arrowheads. Scale bars represent 100μm.

5.2.1.3. Antibodies, isotype controls and gating strategy

The gating strategy used to assess MHCII, CD80 and CD86 expression is shown in Figure 5.02. Populations were gated on forward and side scatter (FSC and SSC). Cells within the gate were analysed for CD86-FITC using the FL1 channel, MHCII-PE using FL2, and CD80-APC using FL4. Fluorescence gating was performed using a quadrant strategy such that the upper right quadrant contained only positive cells. Approximately 1-3% of unstained cells were placed within this quadrant to account for autofluorescence. Unstained Monomac-6 cells are shown in terms of FSC and SSC (A), CD86 (B), MHCII (C) and CD80 (D). Unstained THP1 cells are shown in terms of FSC and SSC (E), CD86 (F), MHCII (G) and CD80 (H). Large amounts of cell death were apparent, likely due to detachment using a cell scraper as opposed to trypsin.

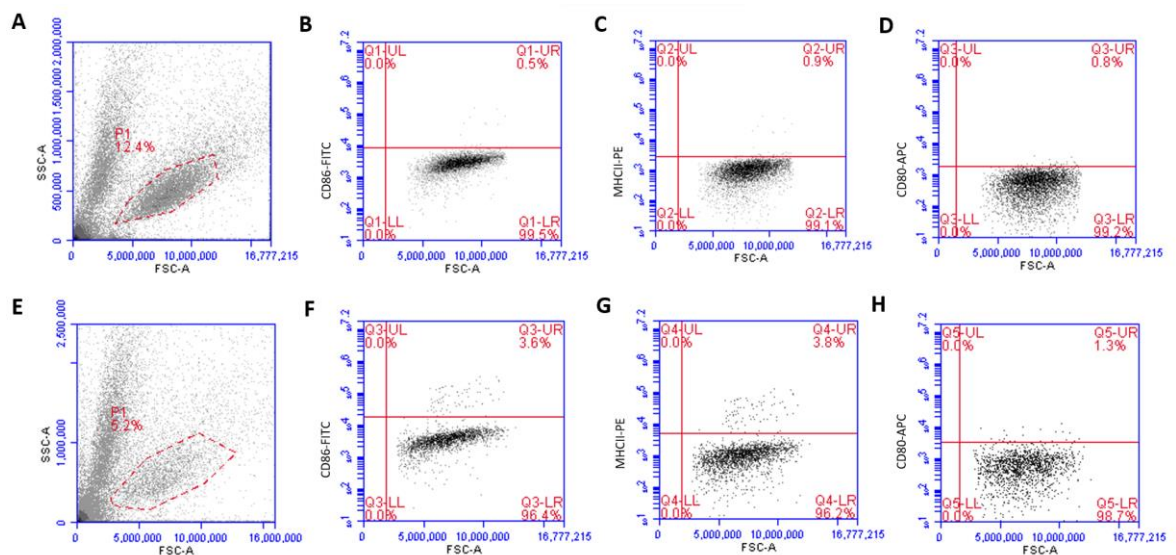


Figure 5.02. Gating strategy used for flow cytometry experiments. Cells were seeded in 20ng/mL PMA for 24 hours. Cells were collected and analysed by flow cytometry. A: Monomac-6-derived cells on FSC/SSC plot. B: CD86 gate with Monomac-6-derived cells. C: MHCII gate with Monomac-6-derived cells. D: CD80 gate with Monomac-6-derived cells. E: THP1-derived cells on FSC/SSC plot. F: CD86 gate with THP1-derived cells. G: MHCII gate with THP1-derived cells. H: CD80 gate with THP1-derived cells. N=1 for each cell line.

Cells were stained with differing volumes of antibody to ascertain a suitable concentration for flow cytometry experiments. These experiments used anti-CD86 conjugated to fluorescein isothiocyanate (CD86-FITC), anti-MHCII (major histocompatibility complex II) with phycoerythrin conjugate (MHCII-PE) and anti-CD80 conjugated to allophycocyanin (CD80-APC). For details of antibodies and isotype controls, see Table 5.03.

Table 5.03. Antibodies and isotype controls used in flow cytometry experiments

Type	Specificity	Fluorophore	Host	Clone	Isotype
Antibody	MHCII	Phycoerythrin	Mouse	Tü39	IgG2a, κ
Antibody	CD80	Allophycocyanin	Mouse	2D10	IgG1, κ
Antibody	CD86	Fluorescein isothiocyanate	Mouse	BU63	IgG1, κ
Isotype control	N/A	Phycoerythrin	Mouse	MOPC-173	IgG2a, κ
Isotype control	N/A	Allophycocyanin	Mouse	MOPC-21	IgG1, κ
Isotype control	N/A	Fluorescein isothiocyanate	Mouse	MOPC-21	IgG1, κ

Table 5.03. Antibodies and isotype controls used in flow cytometry experiments.

100 μ L cell suspensions were stained with 3 μ L and 1 μ L of each antibody. Figure 5.03 shows results for THP1-derived macrophages stained with CD86-FITC (panel A), MHCII-PE (B) and CD80-APC (C), and for Monomac-6-derived cells stained with anti-CD86-FITC (D), MHCII-PE (E) and CD80-APC (F). Based on these results, 3 μ L of each antibody was used to stain cells. However, cell number was not directly controlled, which likely affected the observed results. Additionally, poor results were obtained for CD80 staining in this experiment and it is likely that CD80 staining in subsequent experiments was also unreliable; thus, solid conclusions about CD80 regulation cannot be drawn from this work.

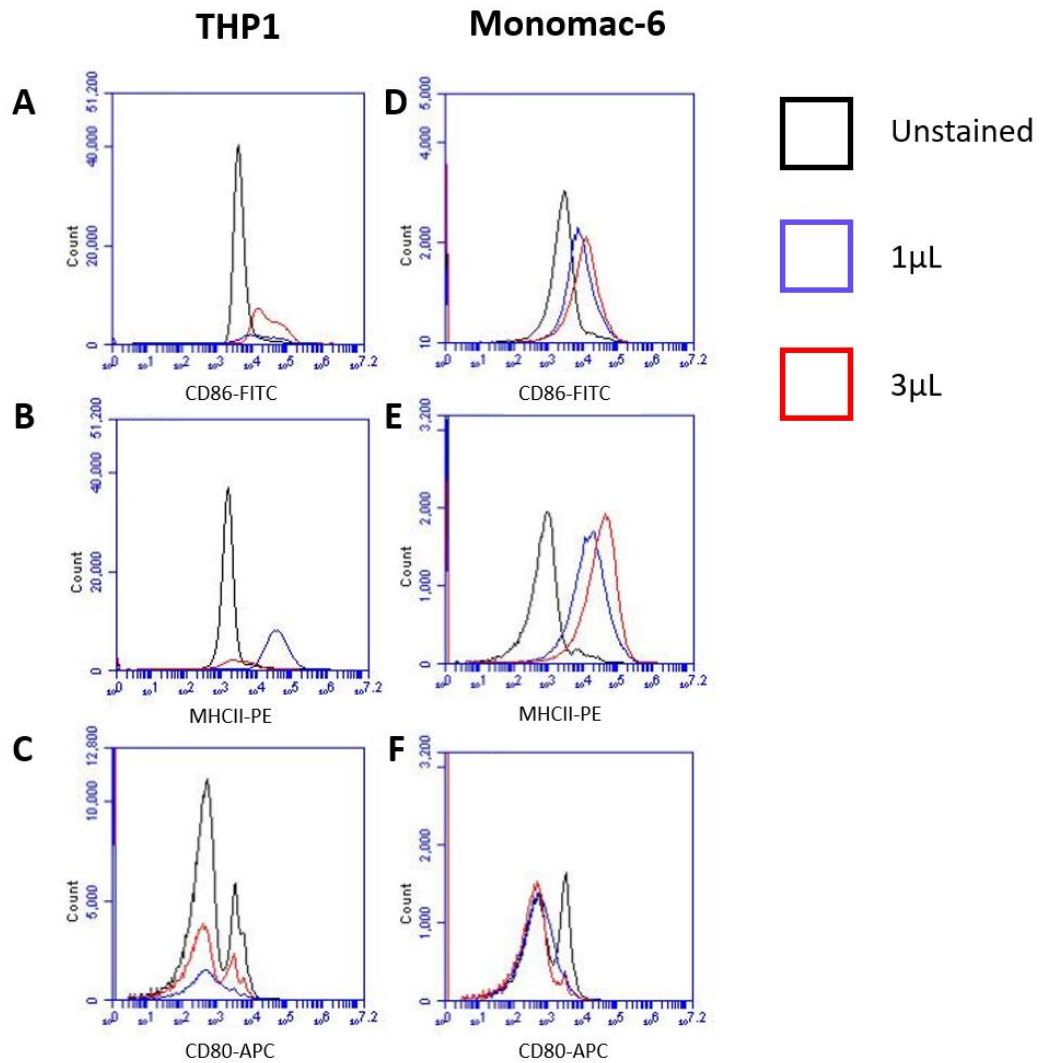


Figure 5.03. THP1- and Monomac-6-derived macrophages stained with differing volumes of antibody. Cells were seeded in media with 20ng/mL PMA for 24 hours, collected and stained with 1 or 3 μ L of antibody or left unstained, then analysed using flow cytometry. A: CD86-FITC titration on THP1-derived cells. B: MHCII-PE titration on THP1-derived cells. C: CD80-APC titration on THP1-derived cells. D: CD86-FITC titration on Monomac-6-derived cells. E: MHCII-PE titration on Monomac-6-derived cells. F: CD80-APC titration on Monomac-6-derived cells. N=1 for each condition.

Next, 100 μ L differentiated Monomac-6 cells were stained with 3 μ L of each antibody and corresponding isotype controls to confirm the observed staining was genuine. Figure 5.04 shows histograms of CD86-FITC and isotype control (panel A), MHCII-PE and isotype control (B) and CD80-APC and isotype control (C). In each case, separate peaks for antibody and isotype conditions were observed, demonstrating specific antibody binding.

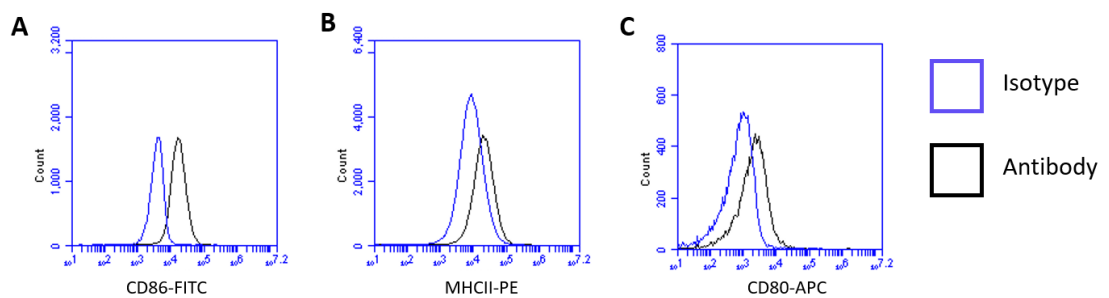


Figure 5.04. Antibody and isotype controls. Cells were seeded in 20ng/mL PMA for 24 hours. Cells were collected and stained with CD86-FITC, MHCII-PE or CD80-APC or isotype controls and analysed by flow cytometry. A: Cells stained for CD86-FITC and fluorescein isothiocyanate isotype. B: Cells stained for MHCII-PE and phycoerythrin isotype. C: Cells stained for CD80-APC and allophycocyanin isotype. N=1 for each condition.

5.2.2. Effects on phagocytosis

First, the effects of target immunomodulatory proteins on phagocytosis were examined. Impaired phagocytosis would necessarily reduce the ability of macrophages to present antigens and promote anti-tumoural immunity, even if MHCII and costimulatory molecules were unaffected. Additionally, phagocytosis is a primary mechanism through which macrophages kill transformed cells. Conversely, increased but misdirected phagocytosis could be a mechanism through which GBM protects itself from immune attack.

A series of phagocytosis assays utilising individual and pooled proteins of interest, as well as supernatant from glioma cell lines, were performed. These experiments aimed to permit identification of protein-specific effects on phagocytosis; pooled protein assays would allow identification of synergistic effects. Furthermore, galectin activity could be blocked by addition of lactose, thus allowing identification of CTGF-specific effects.

5.2.2.1. Untreated cells

GBM tumour-associated macrophages (TAMs) are generally regarded to be polarised towards an M2-like phenotype, with concurrent reductions in phagocytic and antigen-presentation activity. To assess how soluble immunomodulatory proteins may affect less strongly polarised macrophages, or macrophages that retain some pro-inflammatory behaviour, the behaviour of unpolarised and M1-polarised macrophages was studied. Cells were treated with and without 100ng/mL lipopolysaccharide (LPS) and the percentage of phagocytic cells in each condition was compared, to ascertain whether LPS-induced M1 polarisation significantly influenced phagocytic behaviour when compared to M0 cells (Figure 5.05).

For each cell line, phagocytosis was significantly increased following exposure to LPS. In THP1-derived macrophages, the mean percentage of phagocytic cells increased from 10.2% to 13.9% after exposure to LPS, which was found to be a significant difference ($p=0.0196$). In Monomac-6-derived macrophages, mean phagocytosis more than doubled from 10.6% to 24.4%, which was also significant ($p=0.0003$). From this, it was concluded that LPS-induced M1 polarisation significantly increased phagocytic behaviour in human macrophage cell lines *in vitro*.

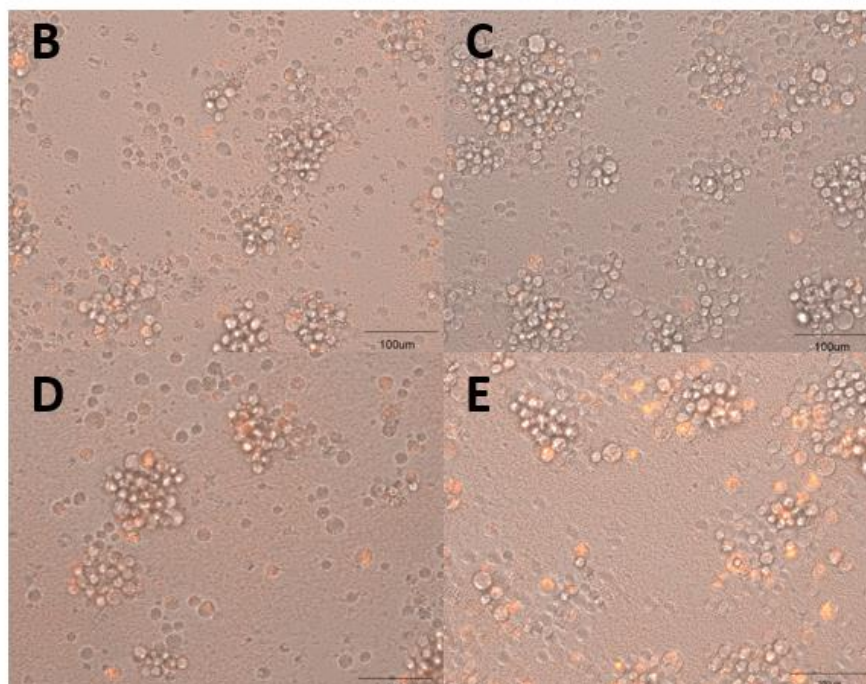
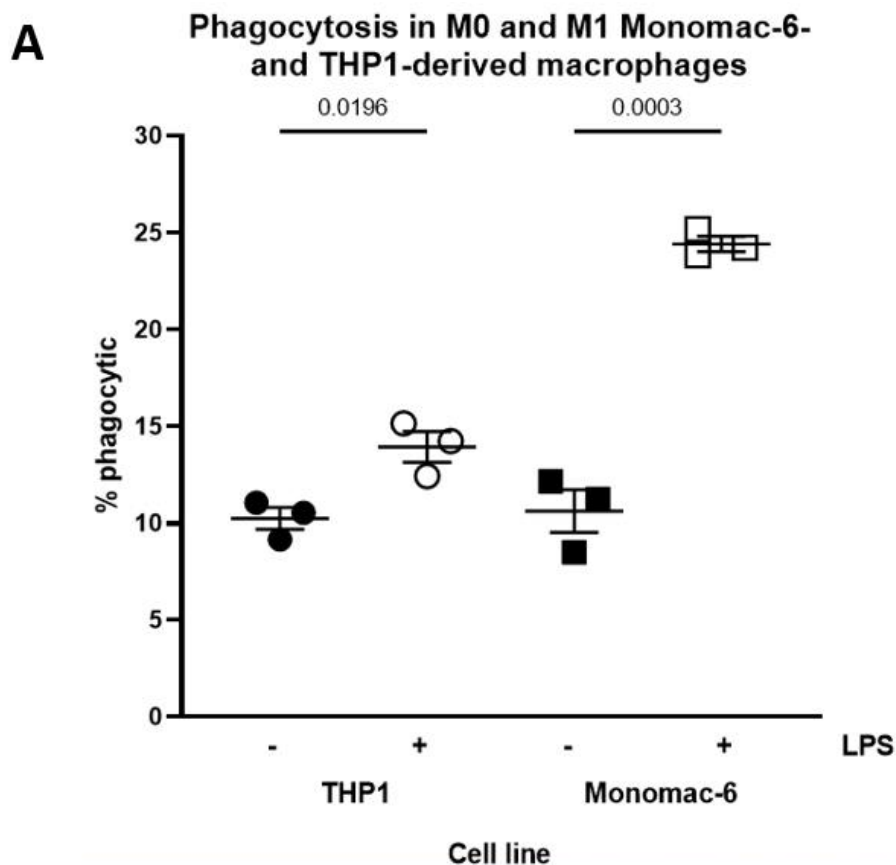


Figure 5.05. Percentage of phagocytic cells in M0 and M1 THP1- and Monomac-6-derived macrophages. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours (empty shapes) or rested for a further 24 hours (filled shapes). Cells were incubated with phagocytosis assay reagent for 2 hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. Data were analysed by two-tailed unpaired t-test. **A:** Percentage of phagocytic cells. $N=3$ for each experiment. Error bars=mean $\pm$ SEM (standard error of the mean). **B:** THP1-derived macrophages without LPS. **C:** Monomac-6-derived macrophages without LPS. **D:** THP1-derived macrophages with LPS. **E:** Monomac-6-derived macrophages with LPS.

5.2.2.2. Effects of galectin-1

In 4.2.2.3.2, gal-1 was identified in the majority of tumours assayed, and was found in the perivascular niche (PVN) to a nearly-significant degree ($p=0.0844$). Gal-1 is likely one of the first – and potentially one of the most frequent – immunosuppressive proteins encountered by tumour-infiltrating leukocytes (TILs). In addition, galectin-1 had the highest mean concentration of all assayed proteins secreted into GBM TM, at 11.2ng/mL (3.2.2.2.2). The percentage of phagocytic cells in THP1- and Monomac-6-derived M1 and M0 macrophages following exposure to physiologically relevant concentrations of active, inactive and blocked galectin-1 was examined.

Figure 5.06A shows that gal-1 non-significantly promoted phagocytosis in Monomac-6-derived M0 cells, from 10.6% in control to 14.3%, 15.8% and 9.1% following exposure to inactive, active and blocked protein respectively ($p=0.3577$, one-way analysis of variance (ANOVA)). The same trend was seen in THP1-derived counterparts (B, $p=0.5019$, ANOVA). Conversely, inactive, active and blocked gal-1 non-significantly suppressed M1 Monomac-6 mean phagocytosis from 24.4% to 15.6%, 12.6% and 14.6% (C, $p=0.5007$, ANOVA). These means were increased by one outlier in each experimental condition, without which the suppression may be significant. Mean phagocytosis in M1 THP1-derived macrophages was 10.2% in control cells and 12.4%, 18.3% and 12.3% following exposure to inactive, active and blocked gal-1 respectively (D, $p=0.3442$, ANOVA).

From these data, it appeared that active galectin-1 generally induced a slight increase in phagocytic behaviour, but specifically suppressed phagocytosis in pro-inflammatory Monomac-6-derived macrophages.

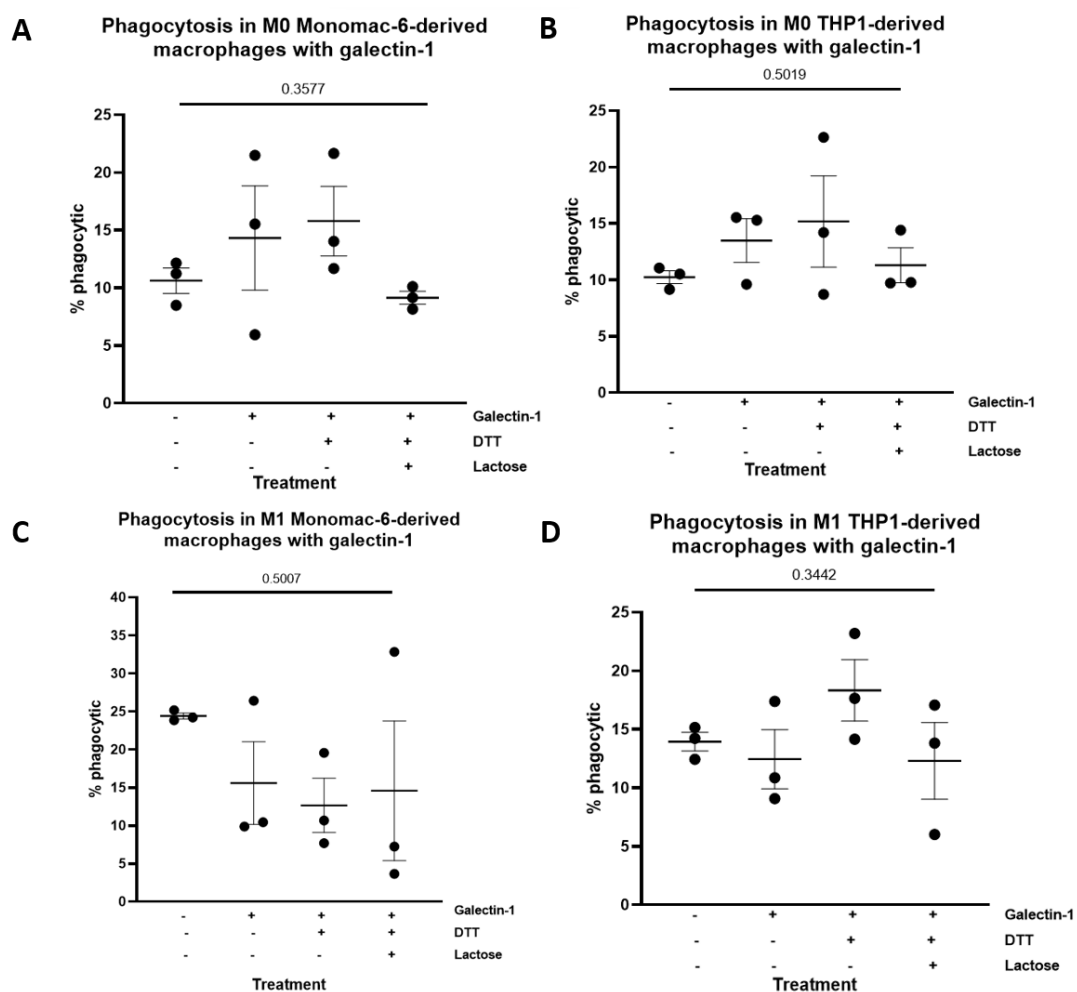


Figure 5.06. Percentage of phagocytic cells in THP1- and Monomac-6-derived macrophages treated with galectin-1. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-1 alone, galectin-1+DTT, galectin-1+DTT+lactose, or left untreated, for a further 24 hours. Cells were incubated with phagocytosis assay reagent for two hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. A: Monomac-6-derived M0 cells. B: THP1-derived M0 cells. C: Monomac-6-derived M1 cells. D: THP1-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.2.3. Effects of galectin-3

Experiments were then performed to examine the percentage of phagocytic cells in macrophages treated with inactive, active and blocked galectin-3 (gal-3), which was present in the majority of GBM TM and sections assayed (3.2.2.2.3. and 4.2.2.3.2.).

As shown in Figure 5.07, gal-3 did not have significant effects on phagocytic behaviour. M0 Monomac-6-derived cells appeared entirely unresponsive (A, $p=0.9834$, ANOVA). THP1-derived M0 cells showed a slight increase in mean phagocytosis from 10.2% in control cells to 13.9% with inactive galectin (B, $p=0.3667$, ANOVA). However, both M1 conditions showed a decrease in mean phagocytosis with galectin-3. Monomac-6-derived macrophage phagocytosis decreased from 24.4% (control) to 8.5%, 18.0% and 11.9% when cells were treated with inactive, active and blocked galectin-3 respectively (C, $p=0.2116$, ANOVA), which may have reached significance if the mean were not increased by outliers. For THP1-derived cells, active and blocked gal-3 reduced phagocytosis from 13.9% in control to 12.6 and 9.0% respectively (D, $p=0.4788$, ANOVA).

Galectin-3, at GBM-relevant concentrations, non-significantly promoted phagocytosis in THP1-derived M0 cells and suppressed phagocytosis in Monomac-6-derived M1 cells, but had little effect on other conditions.

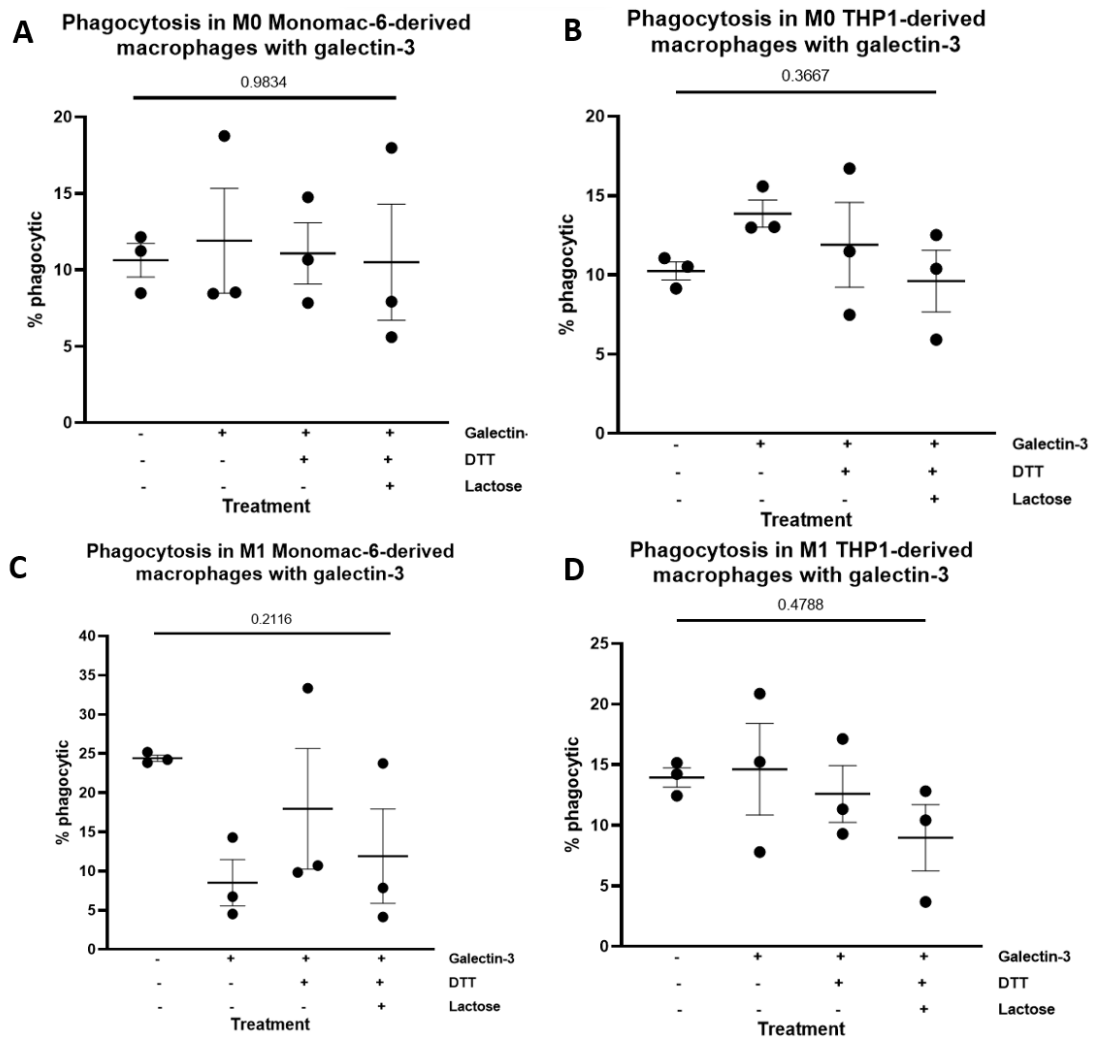


Figure 5.07. Percentage of phagocytic cells in THP1- and Monomac-6-derived macrophages treated with galectin-3. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-3 alone, galectin-3+DTT, galectin-3+DTT+lactose, or left untreated, for a further 24 hours. Cells were incubated with phagocytosis assay reagent for two hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. A: Monomac-6-derived M0 cells. B: THP1-derived M0 cells. C: Monomac-6-derived M1 cells. D: THP1-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.2.4. Effects of galectin-4

In 3.2.2.2.4., galectin-4 (gal-4) was found in a minority of the TM samples assayed but was present in the majority of GBM assayed by IF (4.2.2.3.3.). Galectin-4 has not been widely studied in the context of GBM, and for this reason it was considered worth studying its effects on phagocytosis.

Results are shown in Figure 5.08. A slight but insignificant ($p=0.7194$, ANOVA) reduction in mean average phagocytosis was seen in Monomac-6-derived M0 cells, from 10.6% to 7.3%, following treatment with active gal-4. The same effect was not seen in THP1-derived cells (B), where an average of 9.5% of active gal-4-treated cells were phagocytic compared to 10.2% of control cells ($p=0.8939$, ANOVA). M1 Monomac-6-derived cells were more suppressed, with 15.7%, 13.4% and 13.8% of cells showing phagocytic behaviour following treatment with inactive, active and blocked gal-4 respectively, compared to 24.4% of control cells; however, this result also did not reach significance (C, $p=0.5299$, ANOVA). As with the results for gal-1 and gal-3, the spread of the data in this experiment was comparatively large and may be raising the mean. In D, mean phagocytosis increased from 13.9 to 18.0 and 15.5% of cells following exposure to inactive or active gal-4; blocked gal-4 decreased phagocytosis to 12.2%, which was not found to be significant ($p=0.5116$, ANOVA) and also appeared outlier-driven.

From these data, it appeared that physiologically relevant concentrations of gal-4 suppressed phagocytic behaviour in M1 Monomac-6-derived macrophages, while promoting the same in M1 THP1-derived macrophages, though not to any significant degree.

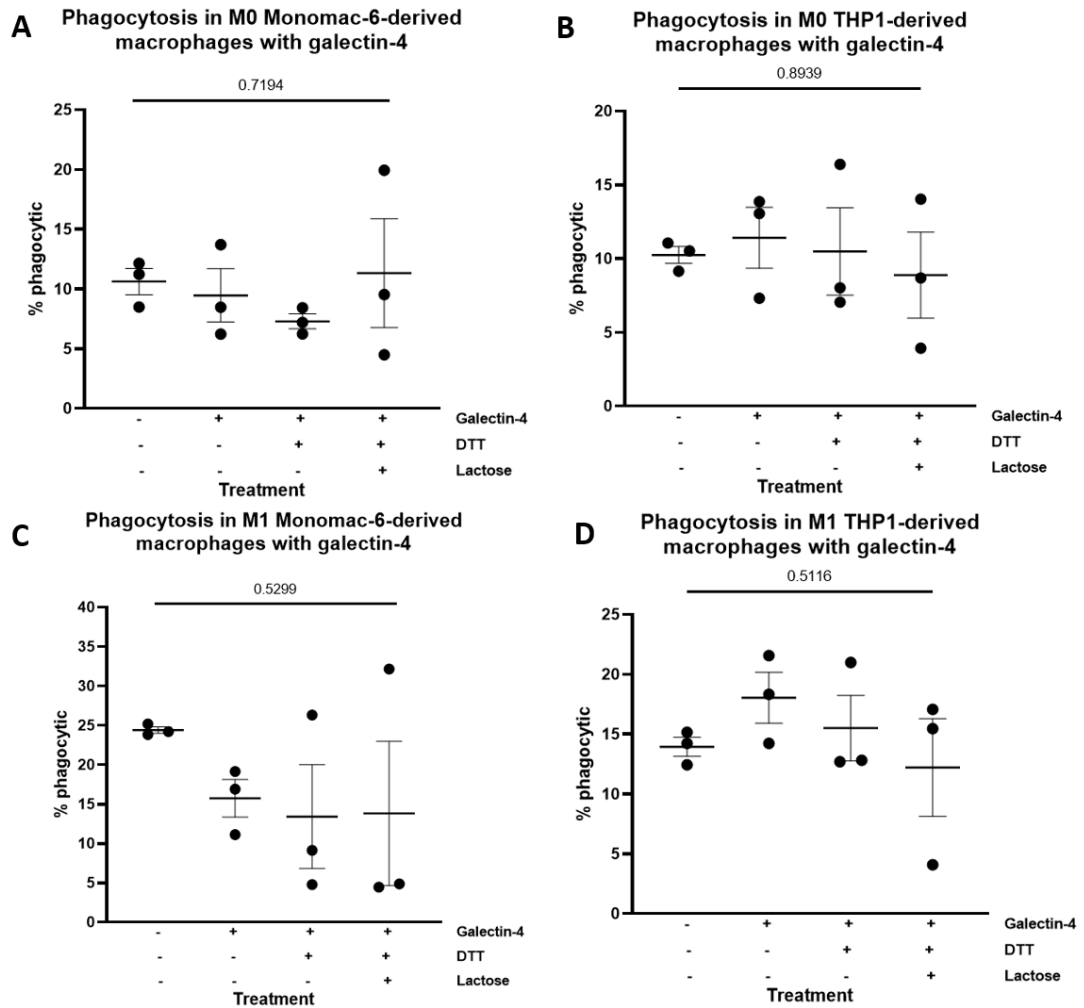


Figure 5.08. Percentage of phagocytic cells in THP1- and Monomac-6-derived macrophages treated with galectin-4. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-4 alone, galectin-4+DTT, galectin-4+DTT+lactose, or left untreated, for a further 24 hours. Cells were incubated with phagocytosis assay reagent for two hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. A: Monomac-6-derived M0 cells. B: THP1-derived M0 cells. C: Monomac-6-derived M1 cells. D: THP1-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.2.5. Effects of galectin-7

As with galectin-4, galectin-7 (gal-7) was rare in GBM TM samples (3.2.2.2.5.) but common in GBM sections (4.2.2.3.4.). As this protein is also not widely studied in the context of GBM, it was considered worth further study in this series of experiments.

Figure 5.09 displays the results. Gal-7 had no discernible effect on M0 Monomac-6-derived macrophages, with mean average phagocytosis at 11.4%, 10.1% and 13.0% for cells treated with inactive, active and blocked protein respectively, compared to 10.6% in control cells ($p=0.9006$, Kruskal-Wallis test). Inactive and active gal-7 slightly stimulated M0 THP1-derived macrophage phagocytosis over control (13.6%, 12.4% and 10.2% respectively), which was reduced with blocked protein to 8.7% (B, $p=0.6559$, ANOVA). Mean phagocytosis in M1 Monomac-6-derived cells was reduced from 24.4% to 9.8%, 12.0% and 9.3% following exposure to inactive, active or blocked protein respectively (C, $p=0.0405$, ANOVA). Tukey's multiple comparisons test did not identify significantly differing pairs of means, but the differences between control cells and cells treated with inactive or blocked protein approached significance at $p=0.0581$ and 0.0543 respectively. In M1 THP-derived macrophages, active protein slightly increased mean phagocytosis from 13.9% in control cells to 16.9%, which mean is reduced by an outlier, without which the difference may have approached significance (D, $p=0.9130$, ANOVA).

In conclusion, galectin-7 may have had significantly suppressive effects on M1 Monomac-6-derived macrophages, while slightly promoting phagocytic behaviour in THP1-derived macrophages.

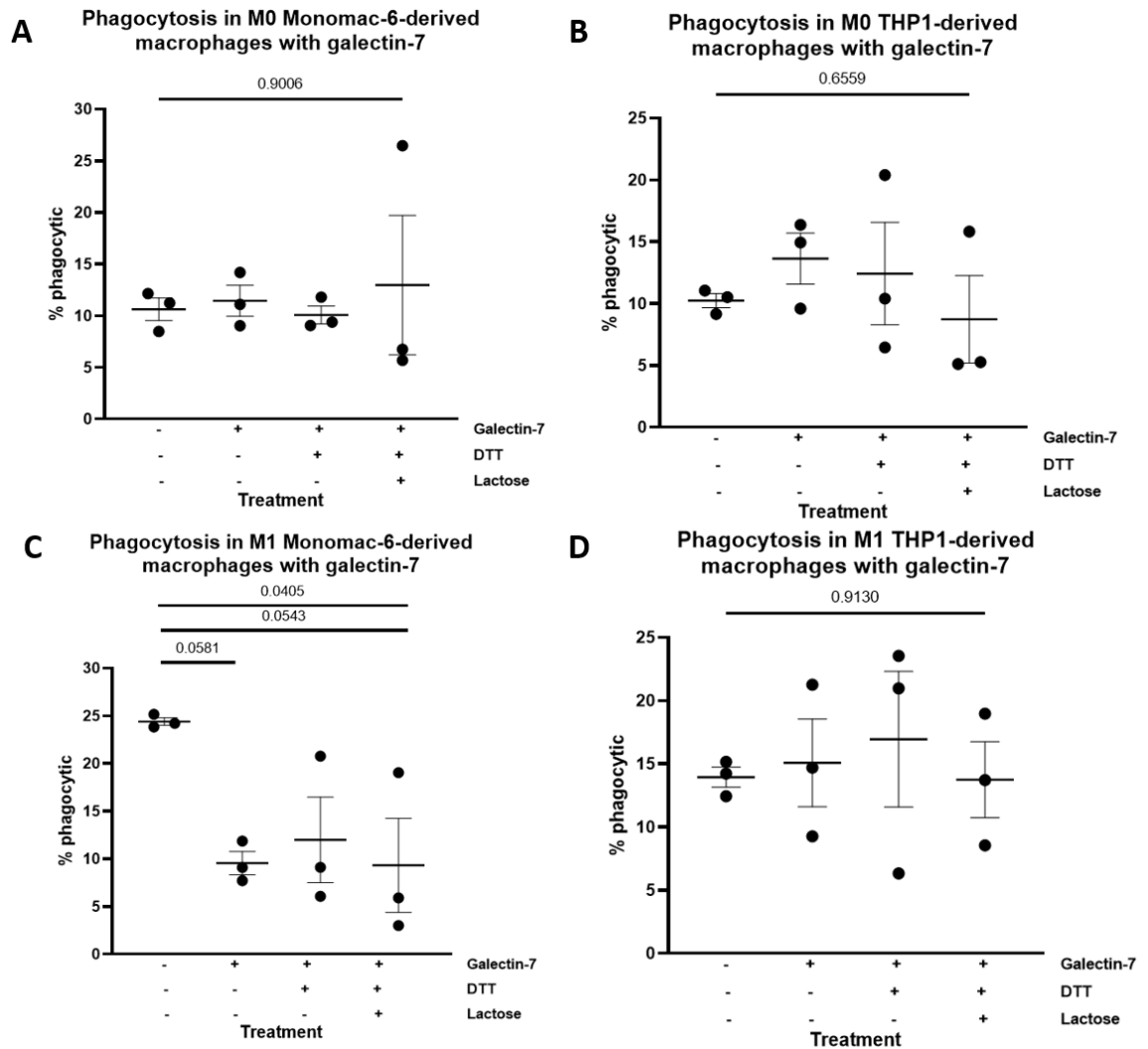


Figure 5.09. Percentage of phagocytic cells in THP1- and Monomac-6-derived macrophages treated with galectin-7. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-7 alone, galectin-7+DTT, galectin-7+DTT+lactose, or left untreated, for a further 24 hours. Cells were incubated with phagocytosis assay reagent for two hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. A: Monomac-6-derived M0 cells. B: THP1-derived M0 cells. C: Monomac-6-derived M1 cells. D: THP1-derived M1 cells. Data were analysed by one-way ANOVA (top result in panel C) followed by Tukey's multiple comparisons test (lower results in panel C). N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.2.6. Effects of CTGF

To conclude the individual protein experiments, cells were treated with CTGF, which was detectable in GBM TM and sections (3.2.2.2.6. and 4.2.2.3.5.). It must be noted here that the correction of CTGF used in these experiments mistakenly did not match the mean concentration of CTGF in GBM TM as determined by ELISA in 3.2.2.2.6; details are given in 5.3.3.

Results are shown in Figure 5.10. No significant effects were observed in either Monomac-6- or THP1-derived M0 cells (A and B, $p=0.6724$ and 0.6828 respectively, ANOVA). Mean phagocytosis was increased from 10.2 to 15.9% and 15.5% in M0 THP1-derived cells following exposure to CTGF or CTGF+DTT, which appeared to be purely outlier-driven. In THP1-derived M1 counterparts, mean phagocytosis changed from 13.9% to 10.0%, 15.1% and 12.4% in cells treated with CTGF, CTGF+DTT and CTGF+DTT+lactose respectively (D, $p=0.7297$, ANOVA). However, in M1 Monomac-6-derived cells (C), a significant suppression of phagocytosis was detected by ANOVA ($p=0.0033$). Tukey's multiple comparisons test identified significant differences between phagocytosis in control and CTGF only (24.4% and 11.1%, $p=0.0169$), CTGF+DTT (24.4% and 11.1%, $p=0.0169$) and CTGF+DTT+lactose groups (24.4% and 6.22%, $p=0.0027$).

Thus, it appeared that CTGF had a marked ability to suppress phagocytosis in M1 Monomac-6-derived macrophages but did not affect phagocytosis in M0 or THP1-derived cells. Furthermore, in Monomac-6-derived cells, the effect was seen regardless of DTT or lactose. As CTGF does not need to be maintained in an oxidised state and lacks a CRD, unlike galectins, these results are to be expected.

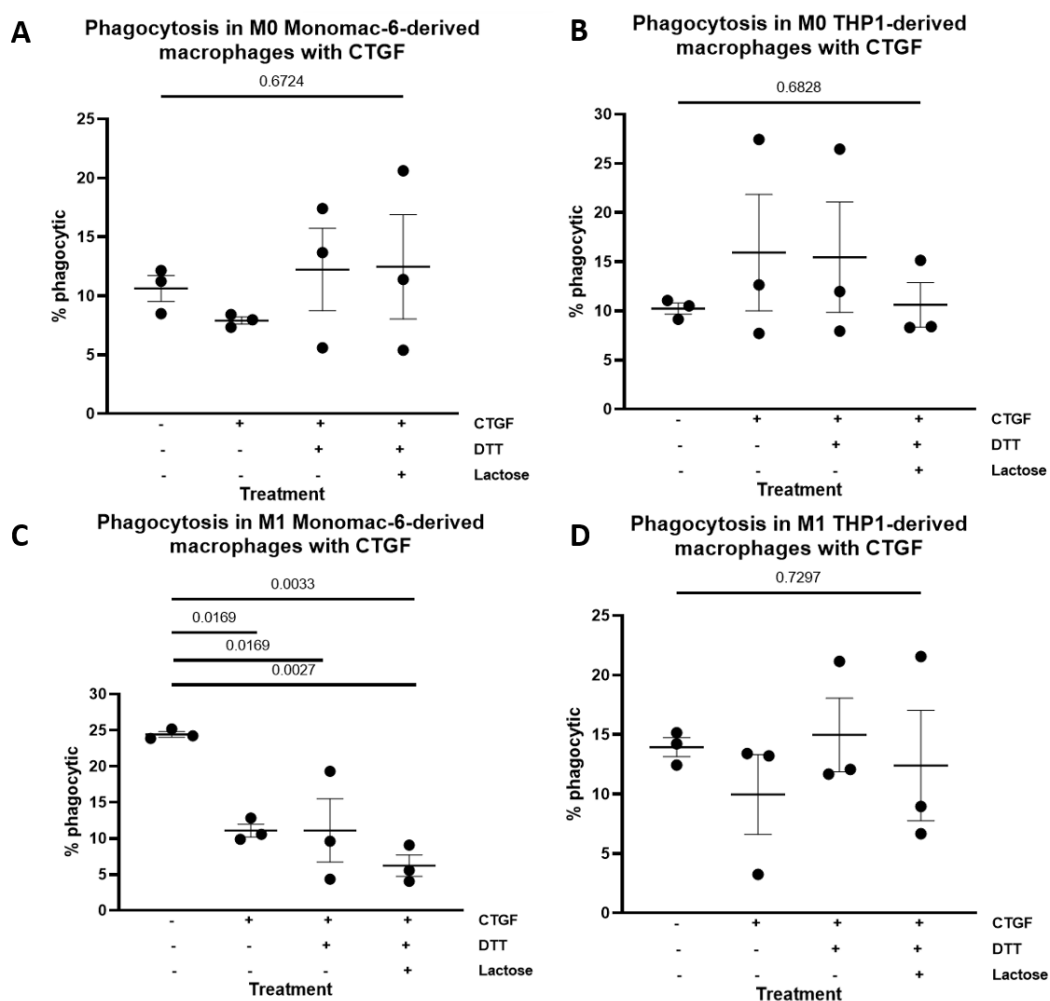


Figure 5.10. Percentage of phagocytic cells in THP1- and Monomac-6-derived macrophages treated with CTGF. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with CTGF alone, CTGF+DTT, CTGF+DTT+lactose, or left untreated, for a further 24 hours. Cells were incubated with phagocytosis assay reagent for two hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. A: Monomac-6-derived M0 cells. B: THP1-derived M0 cells. C: Monomac-6-derived M1 cells. D: THP1-derived M1 cells. Data were analysed by one-way ANOVA (top result in panel C) followed by Tukey's multiple comparisons test (lower results in panel C, significant results shown). N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.2.7. Effects of pooled protein

There is evidence that some galectins can act synergistically (Weinmann et al., 2018), and thus drawing conclusions from individual protein experiments about their relevance in the tumour microenvironment (TME) would be flawed. Thus, cells were next treated with pooled proteins at GBM-relevant concentrations. As in 5.2.2.6., the wrong concentration of CTGF was also used in these experiments.

Figure 5.11 displays the results. In Monomac-6-derived M0 cells (A), mean phagocytosis increased from 10.6% in control cells to 13.9% following pooled protein exposure, which appeared outlier-driven. DTT- or lactose-treated conditions were similar to control (9.3% and 11.7% respectively) and no significant change was observed ($p=0.7782$, ANOVA). M0 THP1-derived cells also showed increased mean phagocytosis in protein-only or blocked protein conditions, from 10.2% to 16.3% and 18.5% respectively; these were also outlier-driven and no significant result was returned by ANOVA (B, $p=0.5905$). In M1 Monomac-6-derived macrophages (C), pooled protein uniformly suppressed phagocytosis, from 24.4% (control) to 14.1%, 15.2% and 13.8% following exposure to pooled protein, active galectin and blocked galectin conditions respectively. This approached but did not reach significance ($p=0.0609$, ANOVA). Mean phagocytosis in THP1-derived M1 cells was increased in pooled protein and lactose-treated groups (20.9 and 20.8% compared to 13.9% in control and 14.4% with DTT); while not being obviously outlier-driven, the spread of these data is large and it does not reach significance (D, $p=0.4239$, ANOVA). From these data, it appeared that M1 Monomac-6-derived macrophages were uniquely vulnerable to suppression by pooled protein at GBM-relevant concentrations.

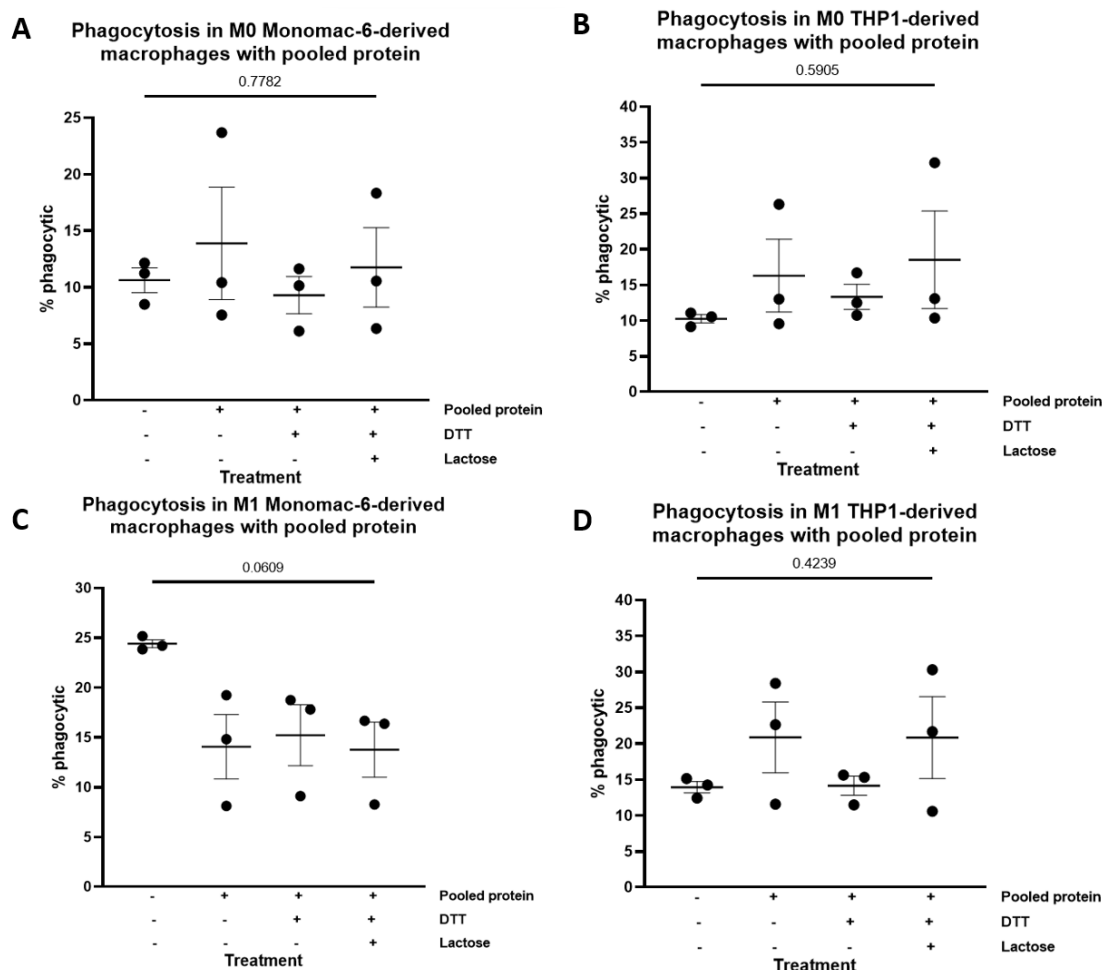


Figure 5.11. Percentage of phagocytic cells in THP1- and Monomac-6-derived macrophages treated with pooled protein. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with pooled protein alone, protein+DTT, protein+DTT+lactose, or left untreated, for a further 24 hours. Cells were incubated with phagocytosis assay reagent for two hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. A: Monomac-6-derived M0 cells. B: THP1-derived M0 cells. C: Monomac-6-derived M1 cells. D: THP1-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.2.8. Effects of glioma cell line supernatant

Following on from recombinant protein experiments, macrophages were exposed to a titration of supernatants taken from U251 and U373 glioma cultures after 96 hours' growth. While these supernatants would not perfectly recapitulate the GBM TME, or indeed the GBM secretome, they served as a more complex liquid model. Immunomodulatory proteins of interest were generally also detectable in supernatants and so this model may allow determination of whether specific effects on phagocytic behaviour were caused by individual proteins.

Results for the U251 supernatant titration are displayed in Figure 5.12. For Monomac-6-derived M0 cells, a significant ($p=0.0003$, ANOVA) increase in mean phagocytic activity following exposure to U251 supernatant was observed (A). Tukey's multiple comparisons test identified significant differences between control and 50% or 100% CS-treated cells (10.6 and 37.2% phagocytic, $p=0.0006$; 10.6 and 38.0% phagocytic, $p=0.0005$ respectively). Mean phagocytosis was also significantly higher in these than the 25% CS group (21.8% phagocytic, $p=0.0173$ and 0.0129 respectively). A significant change in mean phagocytosis was also identified by Kruskal-Wallis test in THP1-derived M0 macrophages ($p=0.0064$); the significantly different groups were found to be control and 100% CS-treated cells (10.2% and 32.1% phagocytic respectively, $p=0.0194$) by Dunn's multiple comparisons test (B).

For M1 Monomac-6-derived macrophages, no significant effect on phagocytosis was observed (C, $p=0.8921$, ANOVA). A significant upregulation was identified in THP1-derived M1 cells (D, $p=0.0114$, ANOVA); Tukey's multiple comparisons test

found the differing means to be control and 25% or 50% CS groups (13.9 and 32.5% phagocytic, $p=0.0163$; 13.9 and 32.6% phagocytic, $p=0.0155$).

From these data, it was concluded that U251 supernatant generally promoted phagocytosis. This effect was most pronounced in M0 cells, where either 50% or 100% supernatant significantly increased phagocytosis. In THP1-derived M1 macrophages, 25% or 50% supernatant was sufficient to significantly increase phagocytosis, which effect was not seen in 100% supernatant-treated cells. This may have been due to a high levels of a suppressive protein, or due to increased metabolic waste or depleted nutrients in the media, which THP1-derived M1 cells may have been more sensitive to than their M0 counterparts. Conversely, M1 Monomac-6-derived macrophages were unresponsive to U251 supernatant.

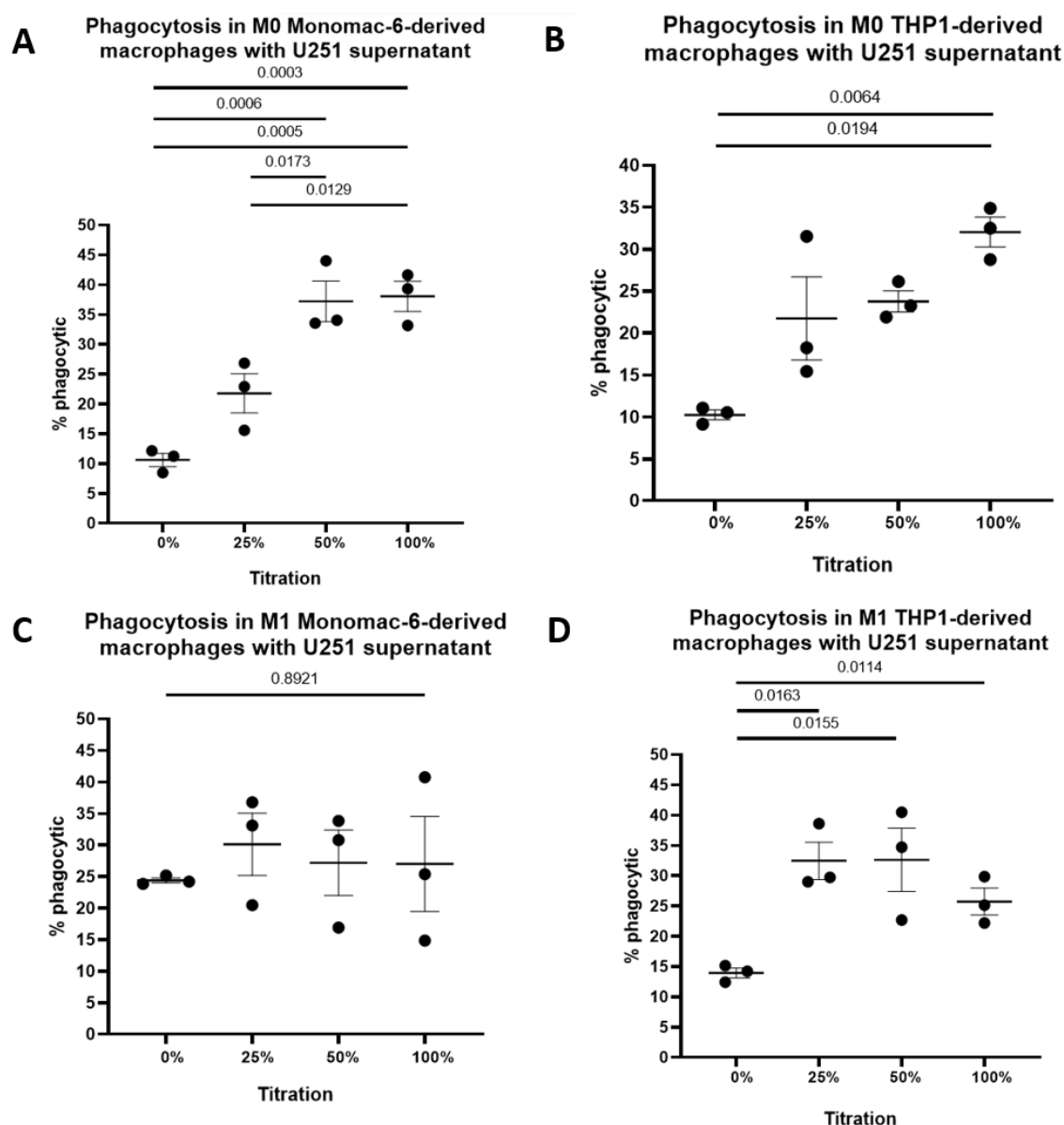


Figure 5.12. Percentage of phagocytic cells in THP1- and Monomac-6-derived macrophages treated with U251 conditioned media. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with 0%, 25%, 50% or 100% conditioned media for a further 24 hours. Cells were incubated with phagocytosis assay reagent for two hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. A: Monomac-6-derived M0 cells. B: THP1-derived M0 cells. C: Monomac-6-derived M1 cells. D: THP1-derived M1 cells. Data were analysed by one-way ANOVA (top of figure) and Tukey's multiple comparisons test (lower results in figure, significant results shown) except for panel B which was analysed by Kruskal-Wallis test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

The above experiment was then repeated using U373 cell line supernatant. Results are shown in Figure 5.13.

U373 supernatant promoted phagocytic behaviour in M0 macrophages of both cell lines, although this did not reach significance in THP1-derived macrophages (B, $p=0.1193$). However, in Monomac-6-derived macrophages (A), mean phagocytosis in control cells (10.6% phagocytic) was found to be significantly different from cells treated with 25% (32.9%, $p=0.0323$), 50% (37.1%, $p=0.0132$) or 100% (34.9%, $p=0.0212$) conditioned media using Tukey's multiple comparisons test. Differences between the three latter groups were not significant.

M1 macrophages derived from both cell lines also showed increased phagocytosis following exposure to U373 supernatant. For Monomac-6-derived macrophages (C), mean phagocytosis increased from 24.4% in control cells to 34.8%, 38.6% and 40.4% in cells treated with 25%, 50% and 100% U373 supernatant respectively; the difference in means approached but did not reach significance by ANOVA ($p=0.0660$). In THP1-derived macrophages (D), phagocytosis was also increased from 13.9% in control cells to 24.0%, 22.2% and 24.2% following exposure to 25%, 50% and 100% supernatant respectively, which did not approach significance ($p=0.1510$).

It was concluded that U373 supernatant also promoted phagocytosis, which was most pronounced in Monomac-6-derived M0 macrophages, and insignificant but consistent in THP1-derived and M1 cells.

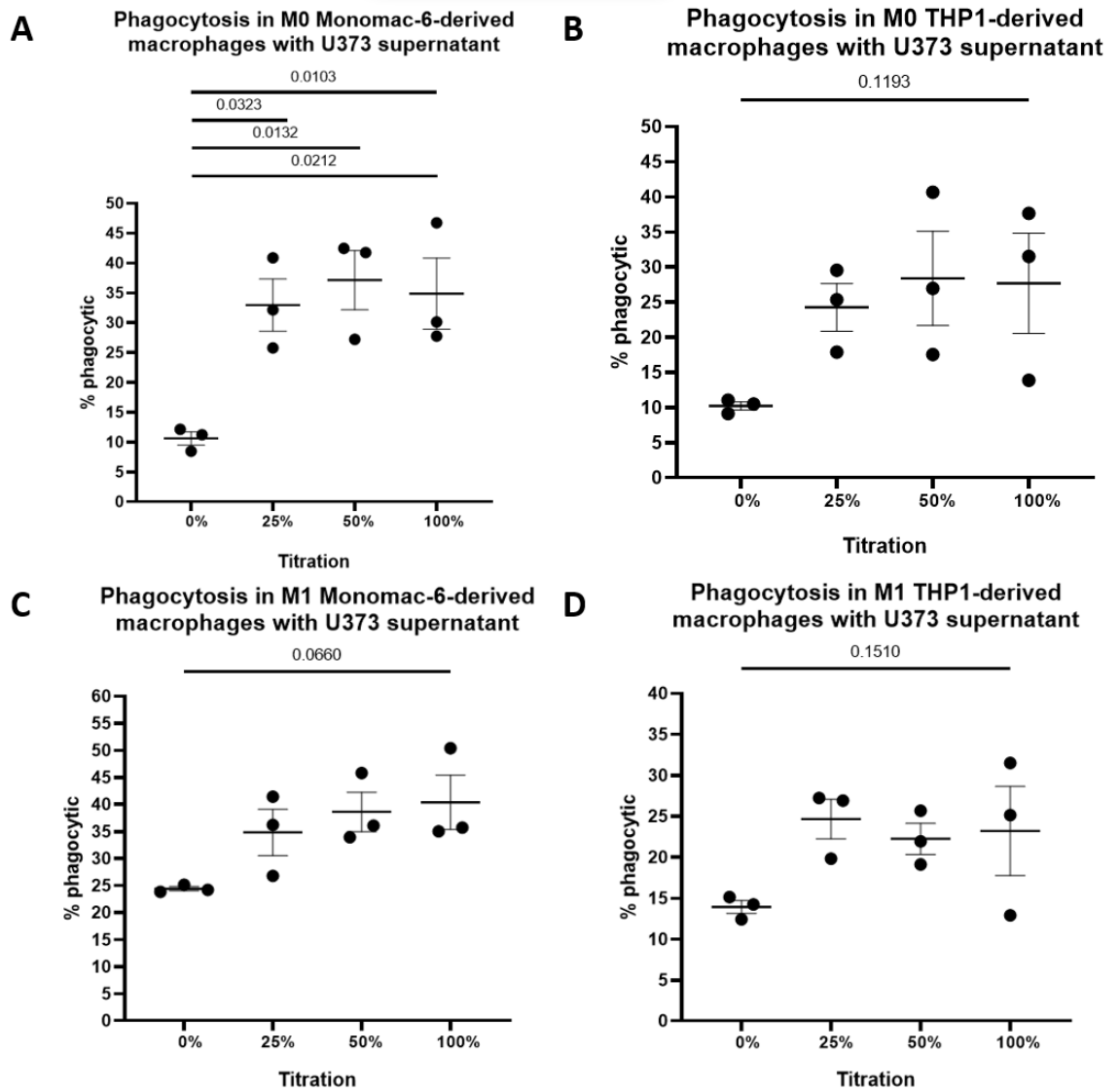


Figure 5.13. Percentage of phagocytic cells in THP1- and Monomac-6-derived macrophages treated with U373 conditioned media. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with 0%, 25%, 50% or 100% conditioned media for a further 24 hours. Cells were incubated with phagocytosis assay reagent for two hours and imaged on an Evos FL Auto 2 using transmitted light and an RFP filter. Counting was performed with ImageJ cell counter plugin to determine the percentage of phagocytic cells. A: Monomac-6-derived M0 cells. B: THP1-derived M0 cells. C: Monomac-6-derived M1 cells. D: THP1-derived M1 cells. Data were analysed by one-way ANOVA (top of figure) and Tukey's multiple comparisons test (lower results in figure, significant results shown). N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.3. Effects on antigen presentation-associated markers

Next, the effects of proteins of interest on antigen presentation-associated molecules were examined. For these experiments, both the percentage of cells positive for each marker and the mean fluorescence intensity (MFI) on positive cells were recorded. Intratumoural macrophages that cannot present antigen despite retaining tumour-directed phagocytic activity would – in the absence of other antigen-presenting cell (APC) populations – limit the development of T-cell-mediated immunity, and thus assist GBM immune escape.

The molecules studied were MHCII (HLA-DR/DP/DQ), which presents externally-encountered antigens following phagocytosis and antigen processing. MHCI, or HLA-A/B/C, molecules present antigens obtained from inside the cell, e.g. during viral infection. In anti-tumoural immunity, MHCI is only relevant in the context of transformed cells and dendritic cells, which can cross-present external antigens on MHCI. As tumour antigens presented by macrophages are obtained externally, they will be presented on MHCII.

Alongside the antigen-presenting molecules, the costimulatory ligands CD80 and CD86, which are required to trigger a full immune response by ligating CD28 on T-cells during antigen presentation, were also studied.

5.2.3.1. Untreated cells

Cells were treated with and without 100ng/mL LPS to ascertain whether M1 polarisation significantly influenced marker expression when compared to M0 cells. Cells were triple-stained for these experiments, but each marker was examined in isolation.

In THP1-derived macrophages (Figure 5.14), an average of 85.7% of M0 and 89.2% of M1 cells were MHCII+. Costimulatory ligand expression was much lower: 11.1% and 10.1% of M0 and M1 cells expressed CD80, respectively, and 16.7% and 15.8% of M0 and M1 cells were CD86+. No significant difference was found in percentage positivity for any of the three markers between M0 and M1 cells ($p=0.6940$, 0.6957 and 0.8752 , respectively).

As with THP-1 cells, Monomac-6-derived macrophages (Figure 5.15) expressed high levels of MHCII, with a mean average of 93.5% of M0 and 89.6% of M1 cells MHCII+. Again, costimulatory ligand expression was lower. 18.1% of M0 cells were CD80+, compared to 25.7% of M1 cells; and 21.3% and 20.0% of M0 and M1 cells were CD86+ respectively. None of these differences approached significance ($p=0.3817$, 0.4209 and 0.7484 , respectively).

From these data, it was concluded that both THP1- and Monomac-6-derived macrophages constitutively expressed MHCII, whereas a smaller percentage expressed costimulatory ligands, and that treatment with 100ng/mL LPS for 24 hours did not significantly affect expression of any of the studied proteins.

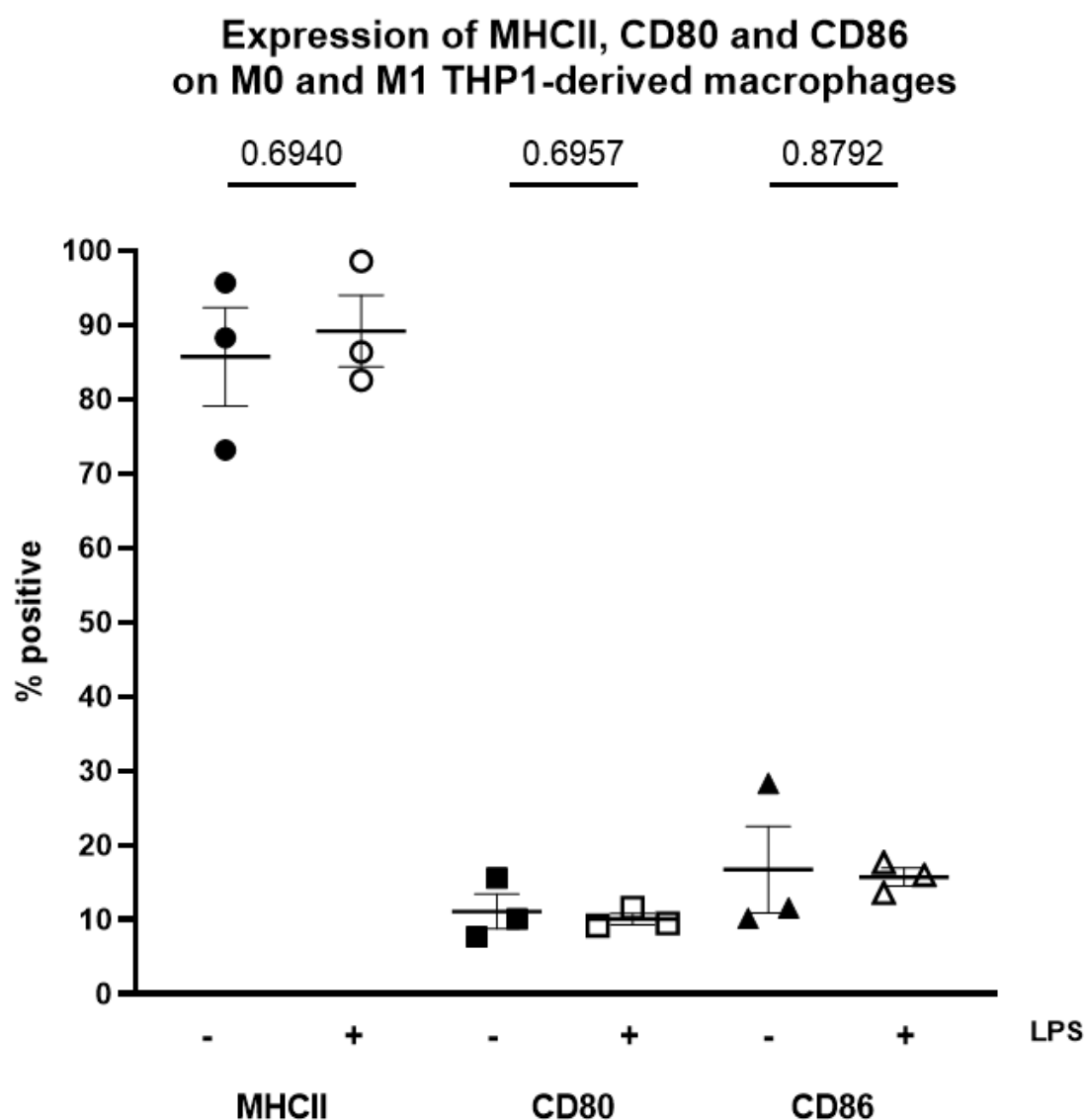


Figure 5.14. Expression of MHCII, CD80 and CD86 on M0 and M1 THP1-derived macrophages. THP1 cells were differentiated using 20ng/mL PMA, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were collected and stained with CD86-FITC, MHCII-PE and CD80-APC. Samples were analysed by flow cytometry. Data were analysed by unpaired 2-tailed t-test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

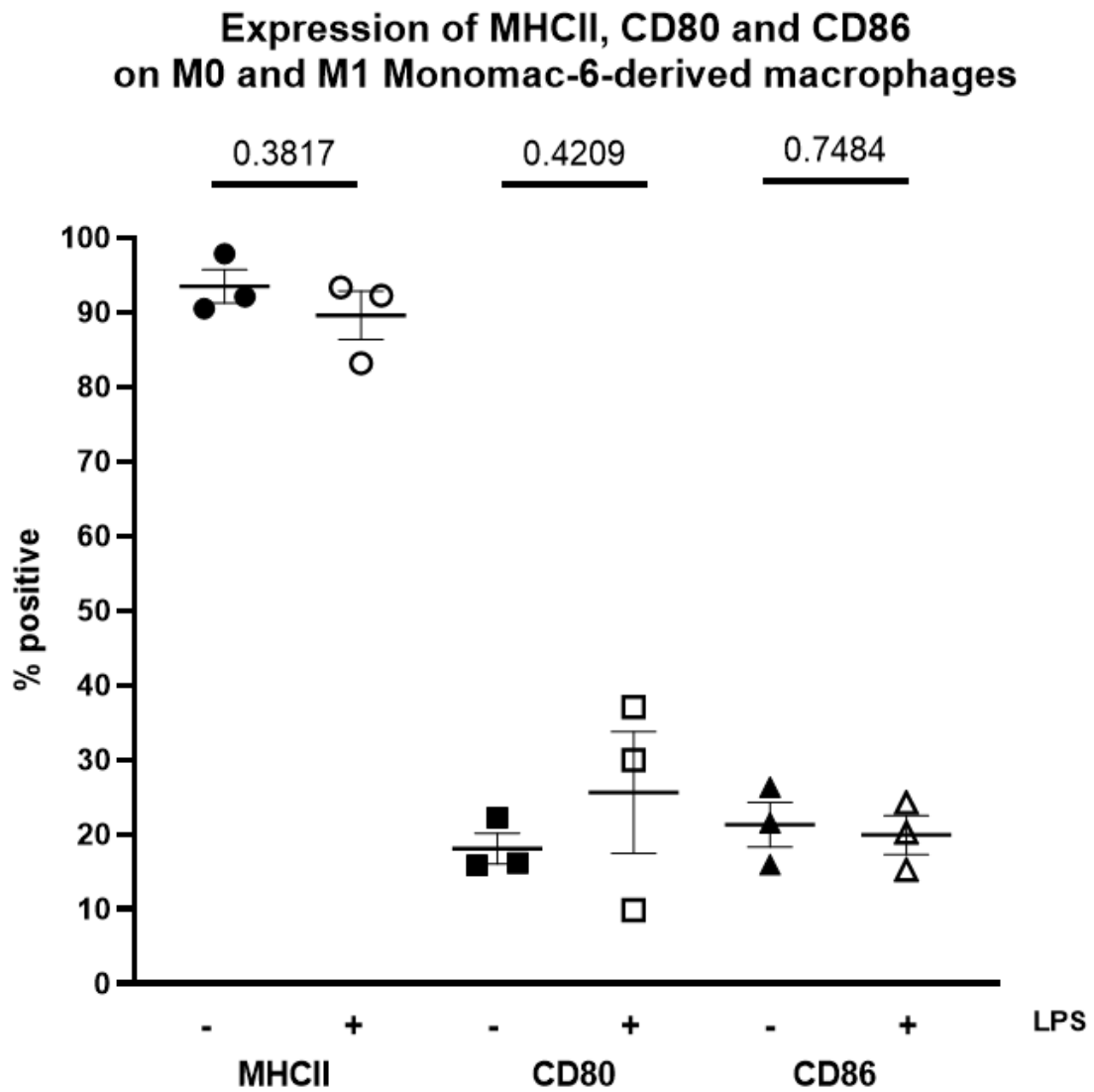


Figure 5.15. Expression of MHCII, CD80 and CD86 on M0 and M1 Monomac-6-derived macrophages. Monomac-6 cells were differentiated using 20ng/mL PMA, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were collected and stained with CD86-FITC, MHCII-PE and CD80-APC. Samples were analysed by flow cytometry. Data were analysed by unpaired 2-tailed t-test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.3.2. Effects of galectin-1

As described above, galectin-1 was commonly present in tumours (4.2.2.3.1.) and had the highest mean concentration of assayed proteins in GBM TM (3.2.2.2.2.). As the effects of this protein on phagocytosis had been studied, its effects on expression of antigen presentation-associated molecules were also studied to build a more complete picture of how galectin-1 in GBM may affect macrophage ability to interact with T-cells. Cells were stained simultaneously for all three markers, but markers were considered separately in these experiments.

5.2.3.2.1. MHCII expression

The expression of MHCII molecules was studied following exposure to physiological concentrations of active, inactive and blocked human galectin-1 using flow cytometry. Results for THP1-derived cells are shown in Figure 5.16.

Active gal-1 slightly, but insignificantly, suppressed MHCII percentage positivity from 85.7 to 83.0% on THP1-derived M0 macrophages; no significant changes were apparent (A, $p=0.6994$, ANOVA). MFI was more uniformly decreased at 77.6%, 82.6% and 72.2% of control for cells treated with inactive, active and blocked gal-1 respectively (B, $p=0.5498$, ANOVA).

In M1 counterparts, MHCII percentage positivity declined from 89.2% in control cells to 75.5%, 80.6 and 86.8% in inactive, active and blocked galectin groups respectively, which was found to be significant (C, $p=0.0441$, ANOVA). Tukey's multiple comparisons test revealed control and inactive gal-1 groups to be the significantly different pair of means ($p=0.0462$). These data suggest that oxidised, but not active, gal-1 significantly downregulates MHCII expression on M1 macrophages. However, MFI on the same cells was increased following exposure to gal-1, with little influence from DTT and lactose (mean averages 145.2%, 213.5% and 216.9% of control respectively. This upregulation approached but did not reach significance (D, $p=0.0704$, ANOVA).

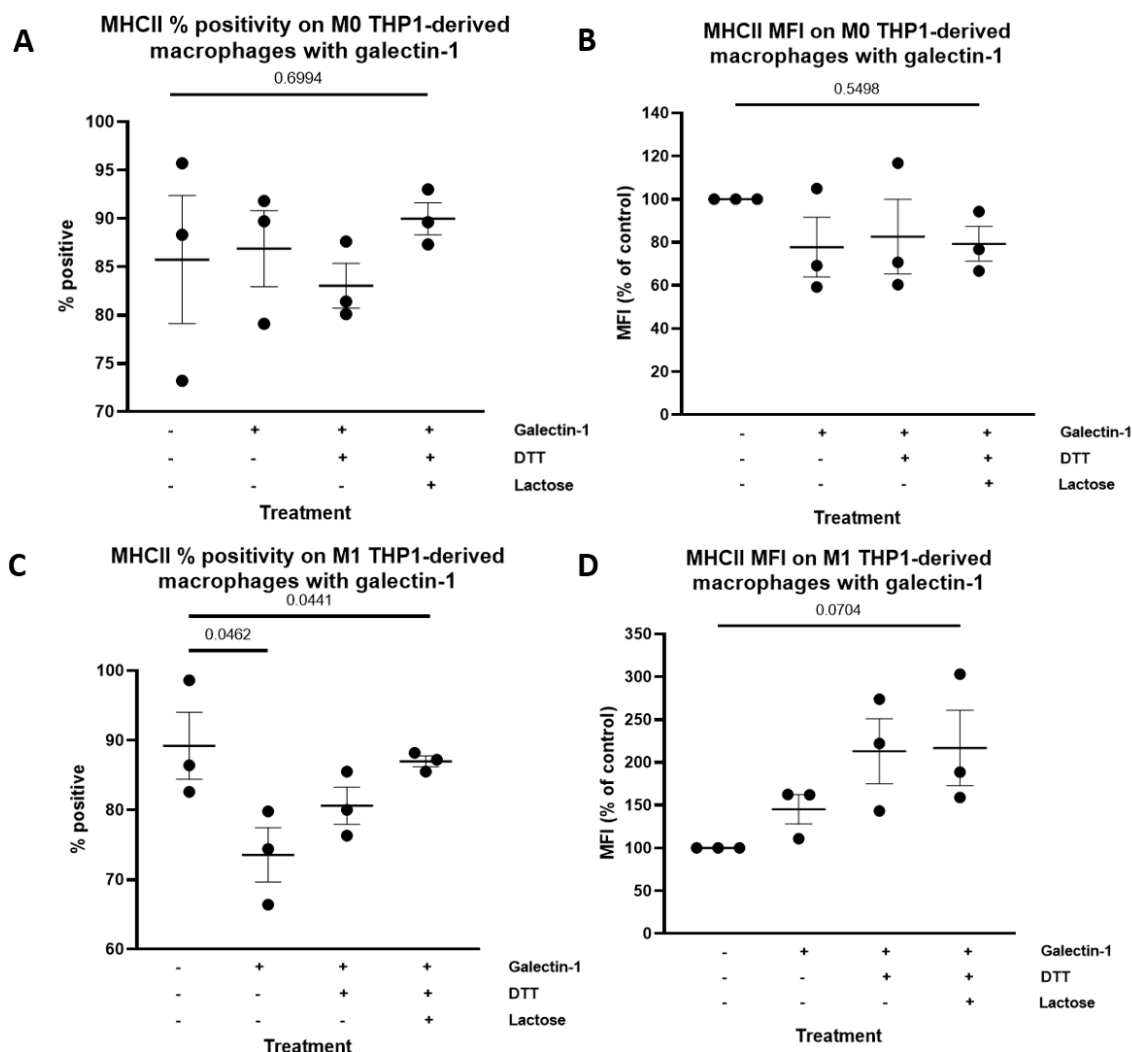


Figure 5.16. Expression of MHCII on M0 and M1 THP1-derived macrophages treated with galectin-1. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-1 alone, galectin-1+DTT, galectin-1+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with MHCII-PE and analysed by flow cytometry. A: Percentage positivity on THP1-derived M0 cells. B: MFI on THP1-derived M0 cells. C: Percentage positivity on THP1-derived M1 cells. D: MFI on THP1-derived M1 cells. Data were analysed by one-way ANOVA (top line in each panel) and Tukey's multiple comparisons test (lower line in panel C). N=3 for each experiment. Error bars=mean $\pm$ SEM.

Next, the expression of MHCII on Monomac-6-derived macrophages treated with galectin-1 was examined. Results are shown in Figure 5.17.

M0 cells trended toward reduced MHCII positivity, which appeared regardless of DTT or lactose (mean average 84.2%, 81.3% and 66.6% respectively, compared to 93.5% for control), which was not significant, possibly due to the large spread of these data (A, $p=0.1904$, ANOVA). MFI suppression was reduced to 68.9%, 61.4% and 81.2% of control for inactive, active and blocked protein groups respectively, which approached but did not reach significance (B, $p=0.0717$, ANOVA).

For M1 Monomac-6-derived macrophages, active gal-1 reduced MHCII percentage positivity from 89.6% (control) to 83.6%, though no changes in these cells approached significance (C, $p=0.5044$, ANOVA). The same trend was seen when examining MFI on MHCII-retaining cells: mean MFI was 109.2%, 77.7% and 105.9% of control for active, inactive and blocked protein groups respectively (D, $p=0.3914$, ANOVA).

It was concluded that oxidised gal-1 lowered the percentage of MHCII+ THP1-derived macrophages, which effect was significant for M1 macrophages ($p=0.0462$). MFI was increased on MHCII-retaining cells ($p=0.0704$), suggesting two subpopulations with differential responses to this protein. Monomac-6-derived M1 macrophages non-significantly reduced MHCII expression following exposure to active gal-1, whereas M0 macrophages were also vulnerable to MHCII suppression by oxidised and blocked galectin, resulting in uniformly decreased MFI ($p=0.0717$). However, these trends were minor and did not reach significance.

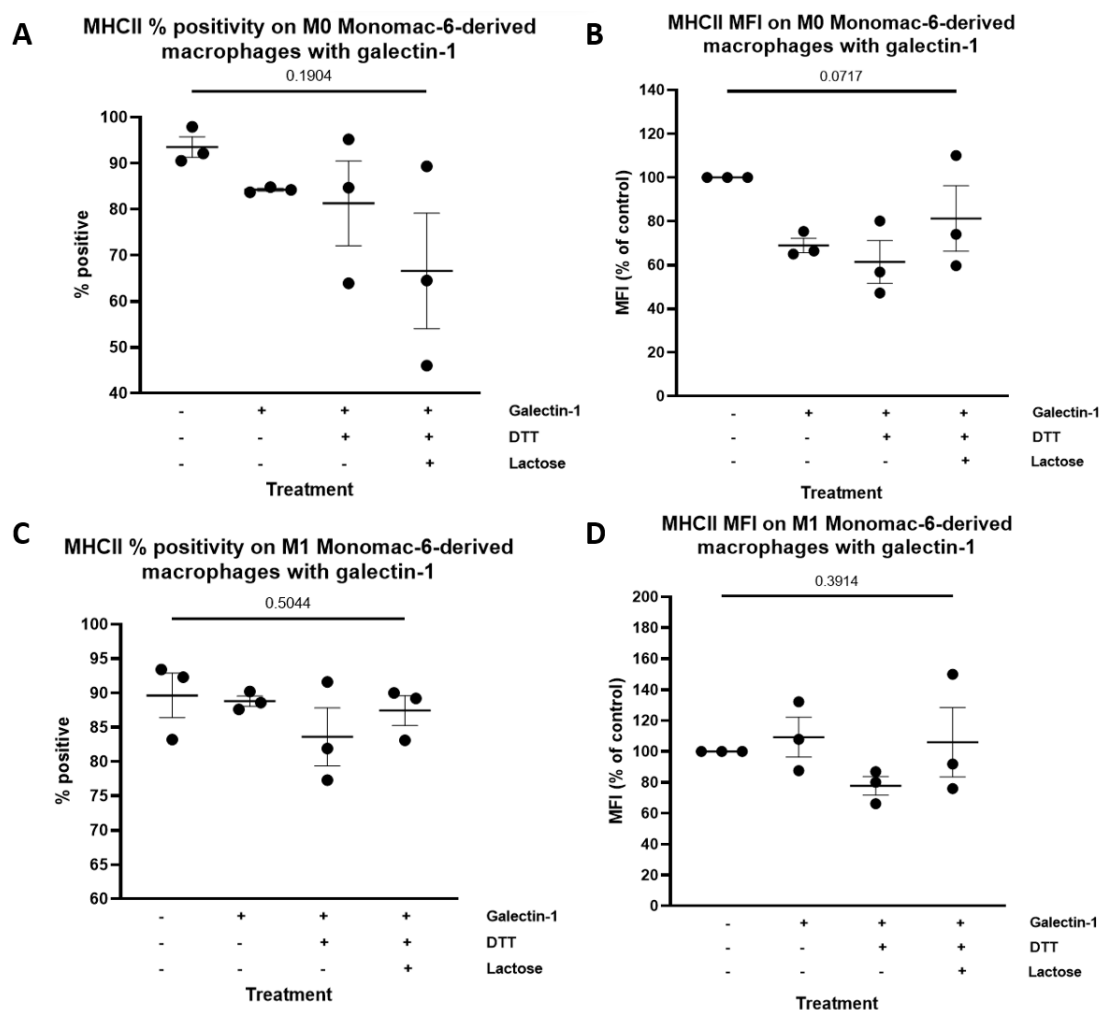


Figure 5.17. Expression of MHCII on Monomac-6-derived macrophages treated with galectin-1. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-1 alone, galectin-1+DTT, galectin-1+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with MHCII-PE and analysed by flow cytometry. A: Percentage positivity on Monomac-6-derived M0 cells. B: MFI on Monomac-6-derived M0 cells. C: Percentage positivity on Monomac-6-derived M1 cells. D: MFI on Monomac-6-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.3.2.2. CD80 expression

Next, the expression of CD80 molecules was studied on both THP1- and Monomac-6-derived macrophages following exposure to physiological concentrations of active, inactive and blocked human galectin-1. Results are shown in Figure 5.18.

THP1-derived M0 macrophages treated with inactive or blocked gal-1 showed increased mean CD80 percentage positivity (16.2% and 17.2% respectively, compared to 11.1% for control); these changes did not approach significance (A, $p=0.4931$, ANOVA). MFI on CD80+ cells was not affected by inactive or active gal-1 but was increased to 210.5% of control in the blocked group. The spread of data in this condition was large and the differences in means did not approach significance (B, $p=0.7388$, Kruskal-Wallis test).

In M1 macrophages, CD80 percentage positivity was upregulated by inactive, active and blocked gal-1, from 10.1% on control cells to 28.9%, 33.5% and 38.2% respectively (C, $p=0.0005$, ANOVA). Tukey's multiple comparisons test showed that all three conditions differed significantly from the mean ($p=0.0068$, 0.0017 and 0.0005 respectively). A concurrent but insignificant ($p=0.1550$, ANOVA) increase in MFI was observed on CD80+ cells (D).

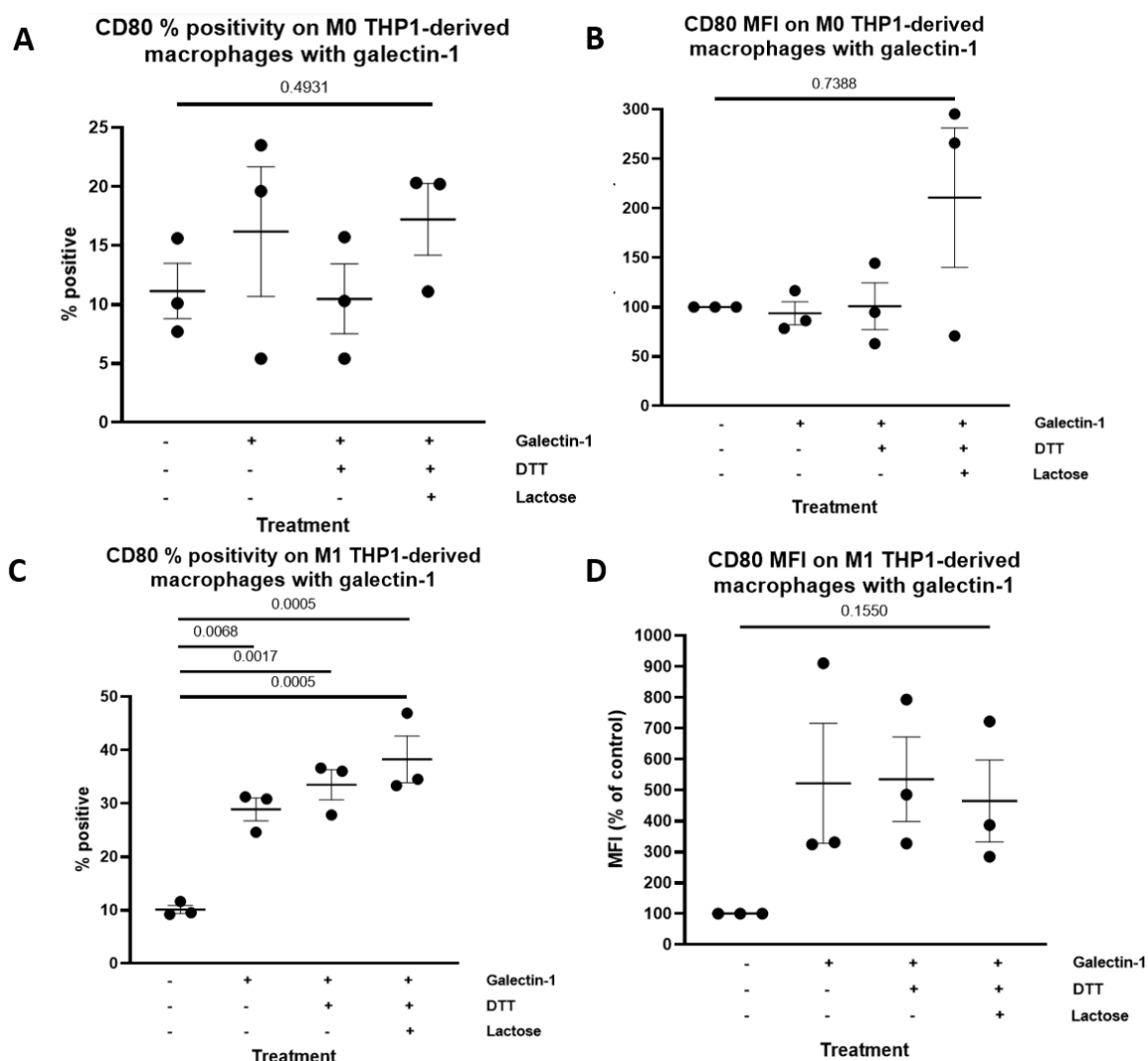


Figure 5.18. Expression of CD80 on THP1-derived macrophages treated with galectin-1. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-1 alone, galectin-1+DTT, galectin-1+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with CD80-APC and analysed by flow cytometry. A: Percentage positivity on THP1-derived M0 cells. B: MFI on THP1-derived M0 cells. C: Percentage positivity on THP1-derived M1 cells. D: MFI on THP1-derived M1 cells. Data were analysed by one-way ANOVA (top line in each panel) and Tukey's multiple comparisons test (lower lines in panel C) except panel B, which was analysed by Kruskal-Wallis test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

Next, CD80 expression in Monomac-6-derived macrophages following treatment with GBM-relevant concentrations of galectin-1 was studied. Results are shown in Figure 5.19.

In M0 macrophages, gal-1 uniformly decreased mean CD80 percentage positivity from 18.1% on control cells to 12.6%, 11.6% and 10.5% following exposure to inactive, active and blocked gal-1 respectively (A, $p=0.5201$, ANOVA). Mean MFI on CD80-retaining cells treated with active and blocked gal-1 decreased to 79.8% and 84.6% of control, and was increased to 128.1% in the inactive treatment group due to one outlier (B, $p=0.4281$, Kruskal-Wallis test). These data suggested a uniform but insignificant reduction in CD80 expression on M0 Monomac-6-derived macrophages.

In M1 macrophages, mean CD80 percentage positivity was 36.8%, 29.3% and 48.9% following exposure to inactive, active and blocked gal-1 respectively, compared to 25.7% on control cells (C, $p=0.1918$, ANOVA). Control and active gal-1 groups each had an outlier lowering the mean average, without which percentage positivity would likely have been uniform overall. Mean MFI on CD80+ cells was increased in the inactive and blocked gal-1 groups to 238.9% and 298.3% of control respectively, compared to 102.8% on cells treated with active gal-1; however, these increased values were driven by one outlier in each group (D, $p=0.5857$, ANOVA).

It was concluded that GBM-relevant concentrations of gal-1 were sufficient to upregulate the percentage of CD80+ THP1-derived M1 macrophages, and non-CRD-dependent interactions may have played a role in this. Unlike percentage

positivity, increased MFI on CD80+ THP1-derived M1 cells did not approach significance, suggesting gal-1 more effectively promoted CD80 expression in negative macrophages than boosted CD80 on cells that were already positive. Monomac-6-derived M1 cells appeared largely unresponsive to gal-1, and M0 cells showed an insignificant decline in percentage positivity, suggesting differential effects of exogenous gal-1 between cell lines.

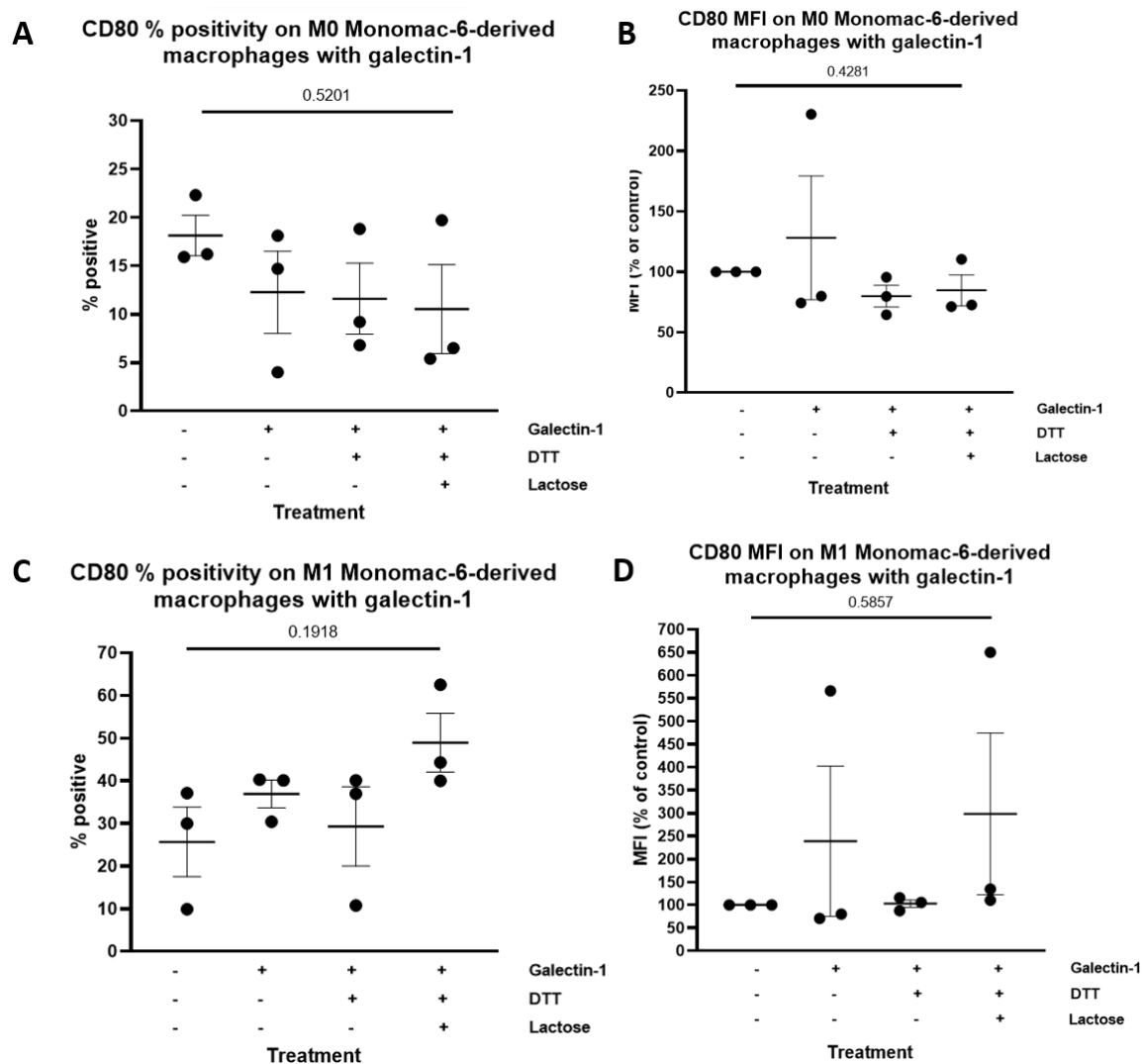


Figure 5.19. Expression of CD80 on Monomac-6-derived macrophages treated with galectin-1. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-1 alone, galectin-1+DTT, galectin-1+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with CD80-APC and analysed by flow cytometry. A: Percentage positivity on Monomac-6-derived M0 cells. B: MFI on Monomac-6-derived M0 cells. C: Percentage positivity on Monomac-6-derived M1 cells. D: MFI on Monomac-6-derived M1 cells. Data were analysed by one-way ANOVA, except for panel B which was analysed by Kruskal-Wallis test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.3.2.3. CD86 expression

Finally, an examination of CD86 expression following exposure to physiological concentrations of active, inactive and blocked human galectin-1 was performed. Results for THP1-derived macrophages are shown in Figure 5.20.

Gal-1 insignificantly decreased mean CD86 percentage positivity from 16.7% on control cells to 7.2%, 9.0% and 14.3% on cells treated with inactive, active and blocked protein respectively (A, $p=0.2233$, ANOVA). MFI on CD86+ cells was relatively unchanged with inactive or active gal-1 (98.1% and 93.98% of control respectively), but decreased to 55.7% of control following exposure to blocked galectin (B, $p=0.5041$, Kruskal-Wallis test).

Gal-1 did not significantly alter CD86 percentage positivity on M1 macrophages (C; $p=0.2241$, ANOVA). However, CD86 MFI was upregulated on all M1 cells exposed to gal-1, at 373.0%, 375.3% and 369.8% of control for cells exposed to inactive, active and blocked protein respectively (D, $p=0.0040$, ANOVA). Tukey's multiple comparisons test showed that all three experimental conditions were significantly different from the control ($p=0.0082$, 0.0078 and 0.0088 for cells exposed to inactive, active and blocked protein respectively).

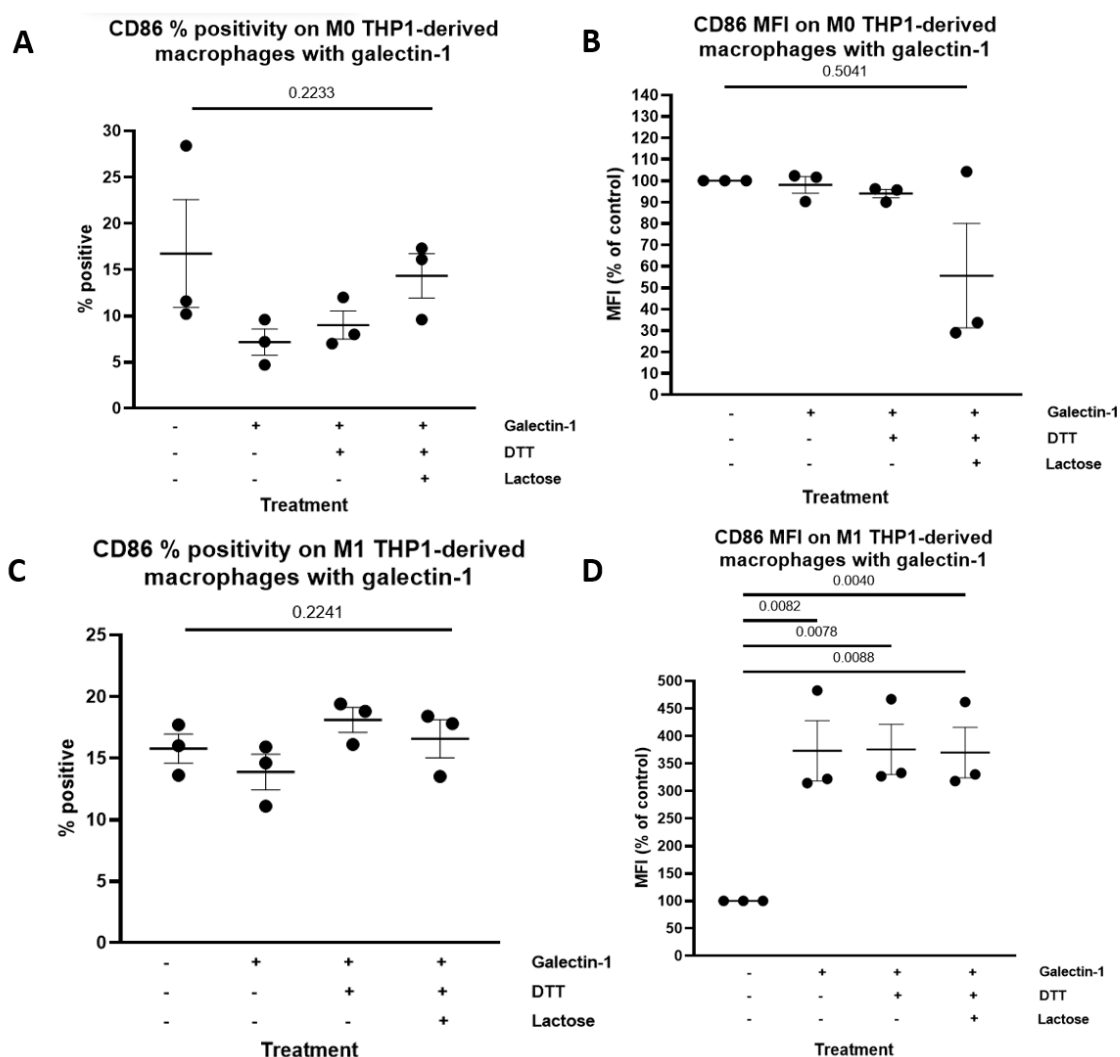


Figure 5.20. Expression of CD86 on THP1-derived macrophages treated with galectin-1. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-1 alone, galectin-1+DTT, galectin-1+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with CD86-FITC and analysed by flow cytometry. A: Percentage positivity on THP1-derived M0 cells. B: MFI on THP1-derived M0 cells. C: Percentage positivity on THP1-derived M1 cells. D: MFI on THP1-derived M1 cells. Data were analysed by one-way ANOVA (top line in each panel) and Tukey's multiple comparisons test (lower lines in panel D) except for panel B, which was analysed by Kruskal-Wallis test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

Galectin-1-based experiments were concluded by studying CD86 expression in M0 and M1 Monomac-6-derived macrophages, following exposure to GBM-relevant concentrations of galectin-1. Data are shown in Figure 5.21.

M0 cells showed decreased CD86 percentage positivity following exposure to inactive, active or blocked gal-1, from 21.3% on control cells to 13.6%, 13.9% and 9.4% respectively (A, $p=0.0988$, ANOVA). MFI on CD86+ cells increased to 187.9%, 196.6% and 241.5% of control following exposure to inactive, active or blocked protein; however, the spread of these data was comparatively large, and the differences did not approach significance (B, 0.5106, ANOVA).

On M1 cells, mean CD86 positivity was increased from 20.0% to 34.4%, 39.8% and 36.9% by inactive, active and blocked gal-1 to respectively (C, 0.2306, ANOVA). Each of the experimental groups had a wide data spread with one value close to control, which reduced the mean value. Mean MFI on CD86+ cells was 150.8%, 99.7% and 142.2% of control for cells exposed to inactive, active and blocked protein. Increased averages in inactive and blocked groups were driven by one outlier each, making the observed increases insignificant (D, $p=0.4691$, ANOVA).

These data suggested that gal-1, at GBM-relevant concentrations, slightly decreased the percentage of CD86-expressing M0 macrophages from both THP1 and Monomac-6 cell lines. On M1 THP1-derived cells, gal-1 significantly increased CD86 expression on CD86+ cells, which appeared to be CRD-independent. Conversely, the trend in Monomac-6-derived M1 cells was towards a greater percentage of CD86+ cells with no increase in MFI.

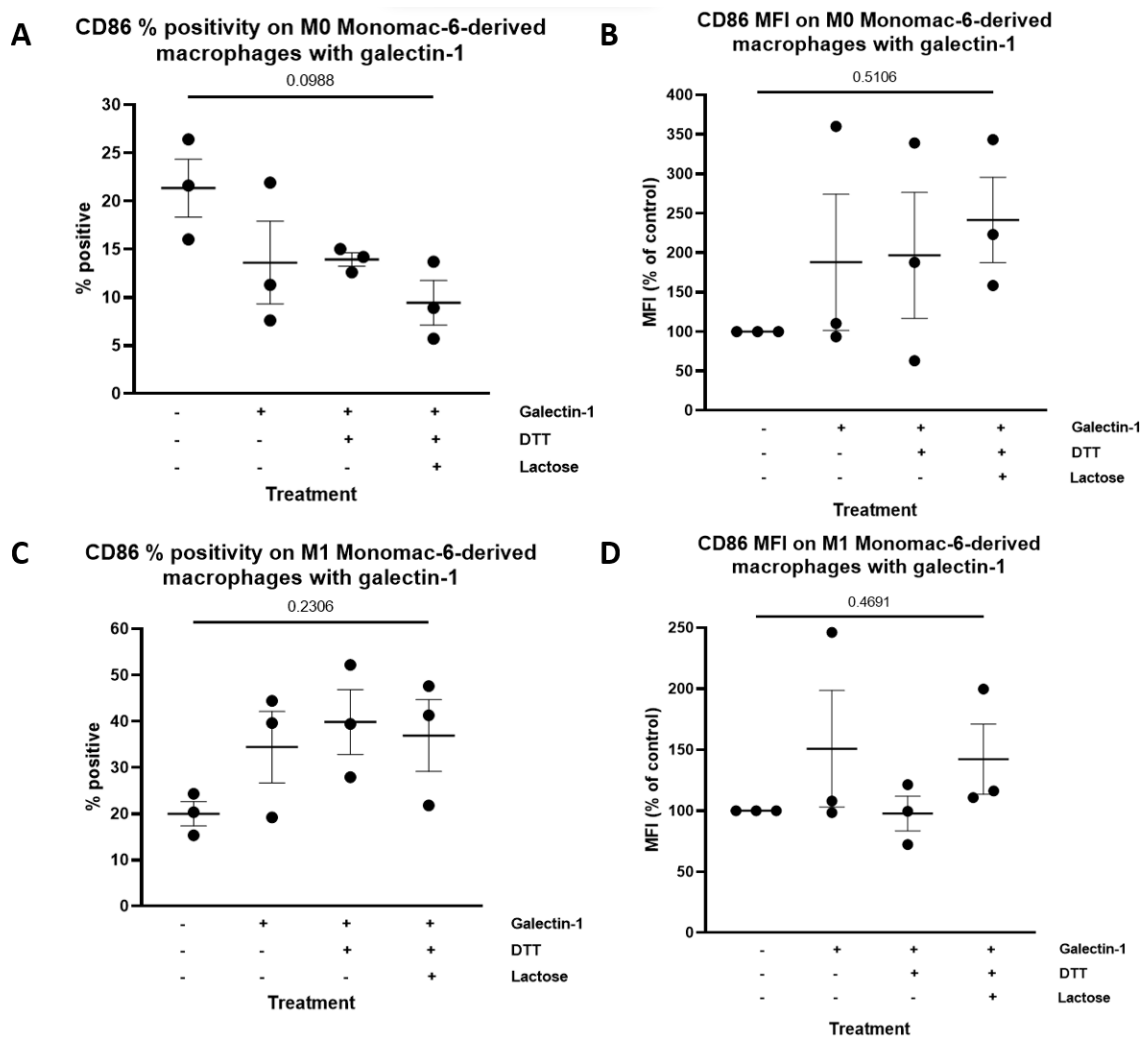


Figure 5.21. Expression of CD86 on Monomac-6-derived macrophages treated with galectin-1. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with galectin-1 alone, galectin-1+DTT, galectin-1+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with CD86-FITC and analysed by flow cytometry. A: Percentage positivity on Monomac-6-derived M0 cells. B: MFI on Monomac-6-derived M0 cells. C: Percentage positivity on Monomac-6-derived M1 cells. D: MFI on Monomac-6-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.3.3. Effects of pooled protein

In the previous section, THP1- and Monomac-6-derived macrophages were treated with GBM-relevant concentrations of human galectin-1, and resultant changes in MHCII, CD80 and CD86 expression were studied. To examine the effects of immunomodulatory proteins together, these experiments were repeated using pooled gal-1,-3, -4 and 7 at GBM-relevant concentrations, and CTGF at an incorrect concentration as also occurred in 5.2.2.6. and 5.2.2.7. Cells in these experiments were stained for all three markers, but markers were examined separately.

5.2.3.3.1. MHCII expression

Initially, MHCII expression was studied on both M0 and M1 THP1-derived macrophages following exposure to physiological concentrations of pooled protein. Results are shown in Figure 5.22.

M0 cells showed no significant response to pooled proteins. Inactive and active galectin groups reduced mean MHCII percentage positivity from 85.7 to 80.7% and 76.3% respectively, which was outlier-driven, rendering any change insignificant (A, $p=0.9249$, ANOVA). Mean MFI was slightly reduced in this experiment to 86.8%, 93.1% and 72.2% of control for active, inactive and blocked protein groups respectively (B, $p=0.6015$, ANOVA); these data indicated a slight but insignificant downregulation of MHCII.

In M1 THP1-derived macrophages, mean MHCII percentage positivity was reduced from 89.2% on control cells to 80.2%, 83.7% and 72.3% following exposure to inactive, active and blocked protein respectively, which did not reach significance (C, $p=0.1031$, ANOVA). Conversely, mean MFI on MHCII-retaining cells increased to 188.2%, 164.5% and 172.2% of control for cells treated with inactive, active and blocked protein respectively, which approached significance (D, $p=0.0664$, ANOVA). Thus, it appears that pooled protein increased MHCII expression intensity on MHCII+ cells.

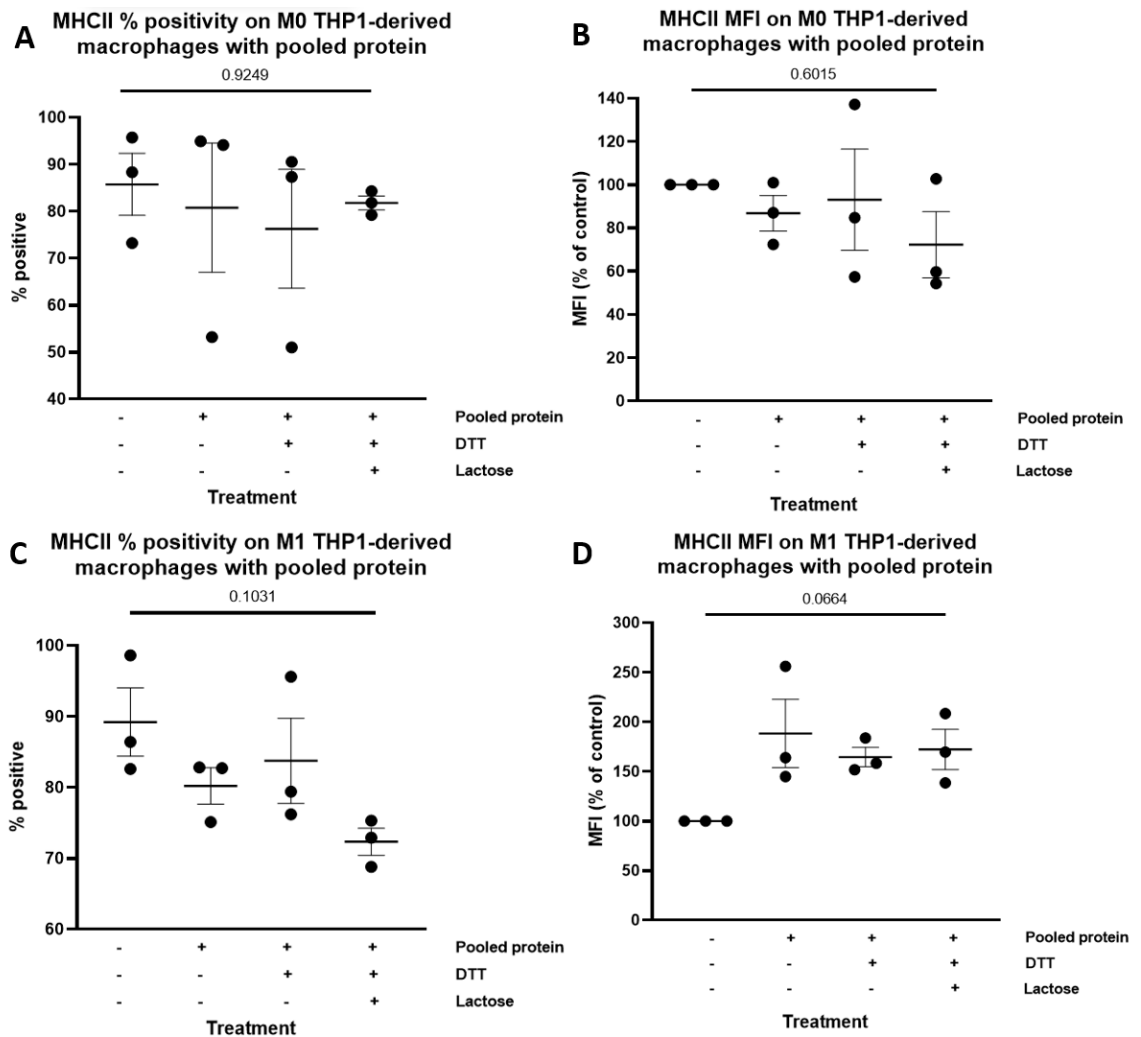


Figure 5.22. Expression of MHCII on THP1-derived macrophages treated with pooled protein. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with pooled protein alone, protein+DTT, protein+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with MHCII-PE and analysed by flow cytometry A: Percentage positivity on THP1-derived M0 cells. B: MFI on MHCII+ THP1-derived M0 cells. C: Percentage positivity on THP1-derived M1 cells. D: MFI on MHCII+ THP1-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

Following on from THP1 experiments, an analysis of MHCII expression in Monomac-6-derived macrophages was performed. Results are shown in Figure 5.23.

In the M0 Monomac-6-derived group, inactive, active and blocked galectin exposure reduced mean MHCII positivity to 90.9%, 85.2% and 90.6% respectively, compared to 93.5% for control cells. Reduced means in inactive and active groups were driven by outliers and the differences did not approach significance (A, $p=0.7786$, ANOVA). Mean MFI on MHCII-retaining cells also showed an insignificant decrease to 69.4%, 84.4% and 88.9% of control following exposure to inactive, active and blocked protein (B, $p=0.1144$, ANOVA), indicating a non-significant downregulation of MHCII expression intensity on M0 Monomac-6-derived macrophages.

On M1 macrophages, pooled protein resulted in an insignificant but non-outlier-driven reduction of mean MHCII percentage positivity, from 89.6% on control cells to 85.6%, 82.2% and 82.4% of cells exposed to inactive, active and blocked galectin conditions respectively (C, $p=0.4758$, ANOVA). MFI on MHCII+ macrophages was consistent at 109.4%, 101% and 96.1% of control on inactive, active and blocked protein groups (D, $p=0.1490$, ANOVA). These trends also did not approach significance, but the downregulation of MHCII percentage positivity was consistent, suggesting pooled protein may have promoted a slight loss of MHCII.

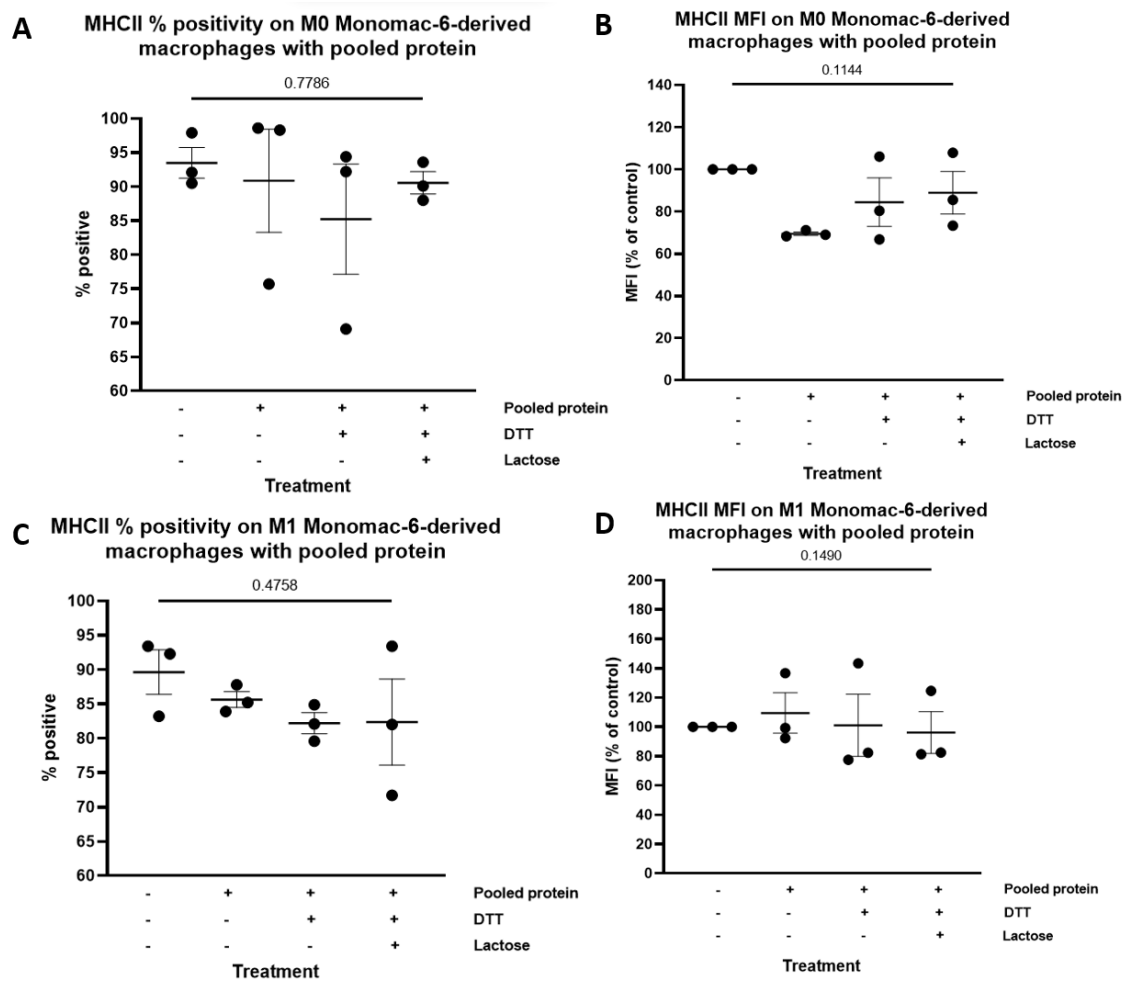


Figure 5.23. Expression of MHCII on Monomac-6-derived macrophages treated with pooled protein. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with pooled protein alone, protein+DTT, protein+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with MHCII-PE and analysed by flow cytometry. A: Percentage positivity on Monomac-6-derived M0 cells. B: MFI on MHCII+ Monomac-6-derived M0 cells. C: Percentage positivity on Monomac-6-derived M1 cells. D: MFI on MHCII+ Monomac-6-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.3.3.2. *CD80 expression*

Next, CD80 expression was studied following exposure to pooled protein. Results for THP1-derived cells are shown in Figure 5.24.

A slight increase in mean CD80 percentage positivity was noted following exposure to active galectin. Mean CD80 positivity was 11.1% on control cells and 11.7%, 18.0% and 15.2% following exposure to inactive, active and galectin respectively (A, $p=0.7619$, ANOVA). Mean MFI on CD80+ cells was relatively unchanged, at 136.7%, 96.9% and 90.8% of control on inactive, active and pooled protein groups (B, $p=0.8577$, Kruskal-Wallis test); the high value in the inactive group was driven by one outlier. These differences did not approach significance, but suggested CD80 percentage positivity upregulation by active galectins.

CD80 percentage positivity on M1 cells increased from 10.1% to 31.9%, 58.2% and 26.6% when exposed to inactive, active and blocked pooled protein respectively (C, $p=0.0104$, ANOVA). Tukey's multiple comparisons test showed control and active galectin groups to be significantly different ($p=0.0070$). Mean MFI on CD80+ cells increased to 499.6%, 356.4% and 608.3% of control in inactive, active and blocked galectin groups respectively (D, $p=0.0044$, ANOVA). Significantly different pairs of means were found to be control and inactive ($p=0.0044$) and control and blocked ($p=0.0038$) galectin groups by Tukey's multiple comparisons test.

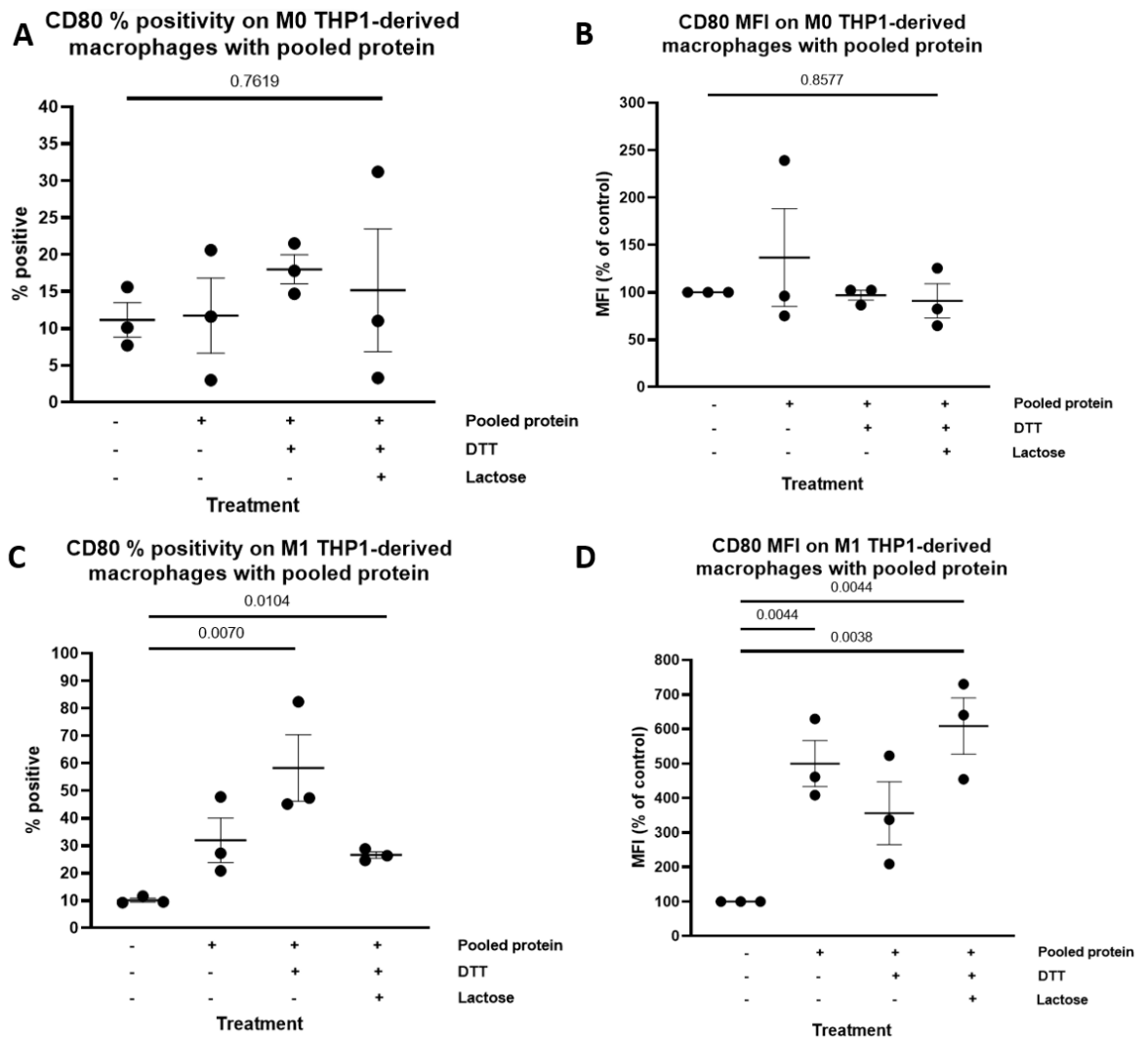


Figure 5.24. Expression of CD80 on THP1-derived macrophages treated with pooled protein. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with pooled protein alone, protein+DTT, protein+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with CD80-APC and analysed by flow cytometry. A: Percentage positivity on THP1-derived M0 cells. B: MFI on CD80+ THP1-derived M0 cells. C: Percentage positivity on THP1-derived M1 cells. D: MFI on CD80+ THP1-derived M1 cells. Data were analysed by one-way ANOVA (top line in each panel) and Tukey's multiple comparisons test (lower lines in panels C and D) except for panel B, which was analysed by Kruskal-Wallis test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

Next, CD80 expression was examined in Monomac-6-derived macrophages following treatment with GBM-relevant concentrations of pooled proteins. Results are shown in Figure 5.25.

Mean CD80 percentage positivity on M0 cells was not significantly affected by pooled protein treatments (A, $p=0.9290$, ANOVA). Mean MFI as a percentage of control was 83.6%, 95.0% and 98.8% for cells treated with inactive, active and blocked protein (B, $p=0.7925$, ANOVA).

M1 Monomac-6-derived macrophages approximately doubled mean CD80 percentage positivity, from 25.7% to 45.3%, 41.8% and 56.0% on cells treated with inactive, active or blocked protein respectively, though the latter was outlier-driven (C, $p=0.2184$, ANOVA). MFI on CD80+ cells was 186.9%, 239.6% and 258.5% of control for inactive, active or blocked groups respectively, which did not approach significance because in every case the mean was elevated by one outlier (D, $p=0.7769$, ANOVA).

These data showed that M1 THP1- and Monomac-6-derived cells may both have upregulated CD80 in response to pooled protein. CTGF appeared to increase CD80 MFI on THP1-derived cells in opposition to active galectins, and pooled protein slightly suppressed CD80 expression on M0 Monomac-6-derived macrophages.

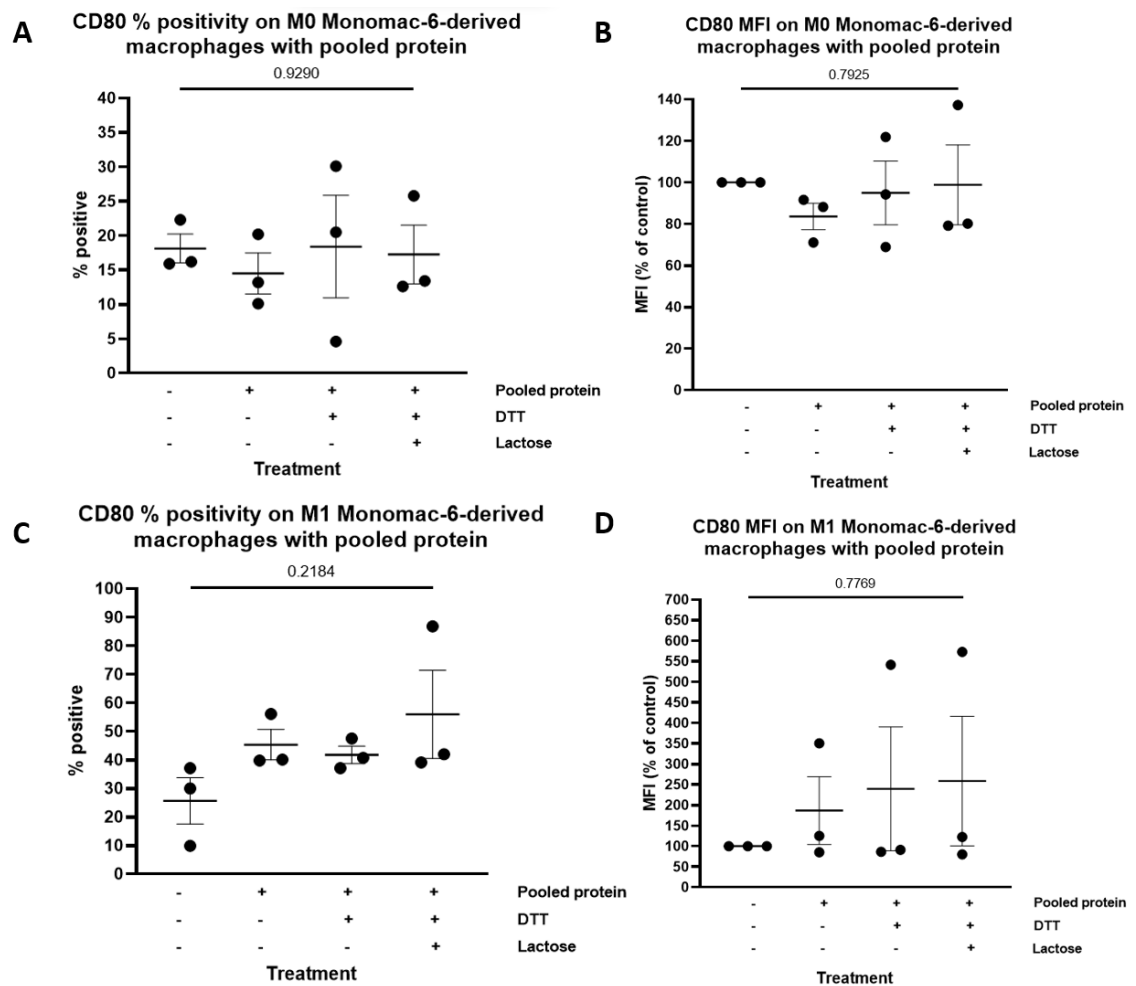


Figure 5.25. Expression of CD80 on Monomac-6-derived macrophages treated with pooled protein. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with pooled protein alone, protein+DTT, protein+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with CD80-APC and analysed by flow cytometry. A: Percentage positivity on Monomac-6-derived M0 cells. B: MFI on CD80+ Monomac-6-derived M0 cells. C: Percentage positivity on Monomac-6-derived M1 cells. D: MFI on CD80+ Monomac-6-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.3.3.3. *CD86 expression*

To conclude experiments on the effects of pooled protein on antigen-presentation molecules, the expression of CD86 molecules on both THP1- and Monomac-6-derived macrophages was studied. Results for THP1-derived cells are shown in Figure 5.26.

Mean CD86 percentage positivity on THP1-derived M0 macrophages was 16.7% in control cells and 16.9%, 12.3% and 14.7% in inactive, active and blocked galectin groups (A, $p=0.9166$, ANOVA). MFI on CD86+ cells was also relatively unaltered at 117.3%, 103.5% and 110.7% of control for inactive, active and blocked protein treatments respectively; increased mean MFI in the inactive galectin group was outlier-driven (B, $p=0.5640$, Kruskal-Wallis test).

Mean CD86 percentage positivity on M1 THP1-derived macrophages was also relatively static, from 15.8% on control cells to 21.4% (outlier-driven), 14.0% and 13.2% following exposure to inactive, active and blocked treatments respectively (C, $p=0.5220$). Mean MFI on CD86+ cells was 402.3%, 327.5% and 333.2% of control following exposure to inactive, active and blocked galectin conditions respectively (D, $p=0.0228$, ANOVA). Tukey's multiple comparisons test revealed that control and inactive groups were significantly different ($p=0.0205$).

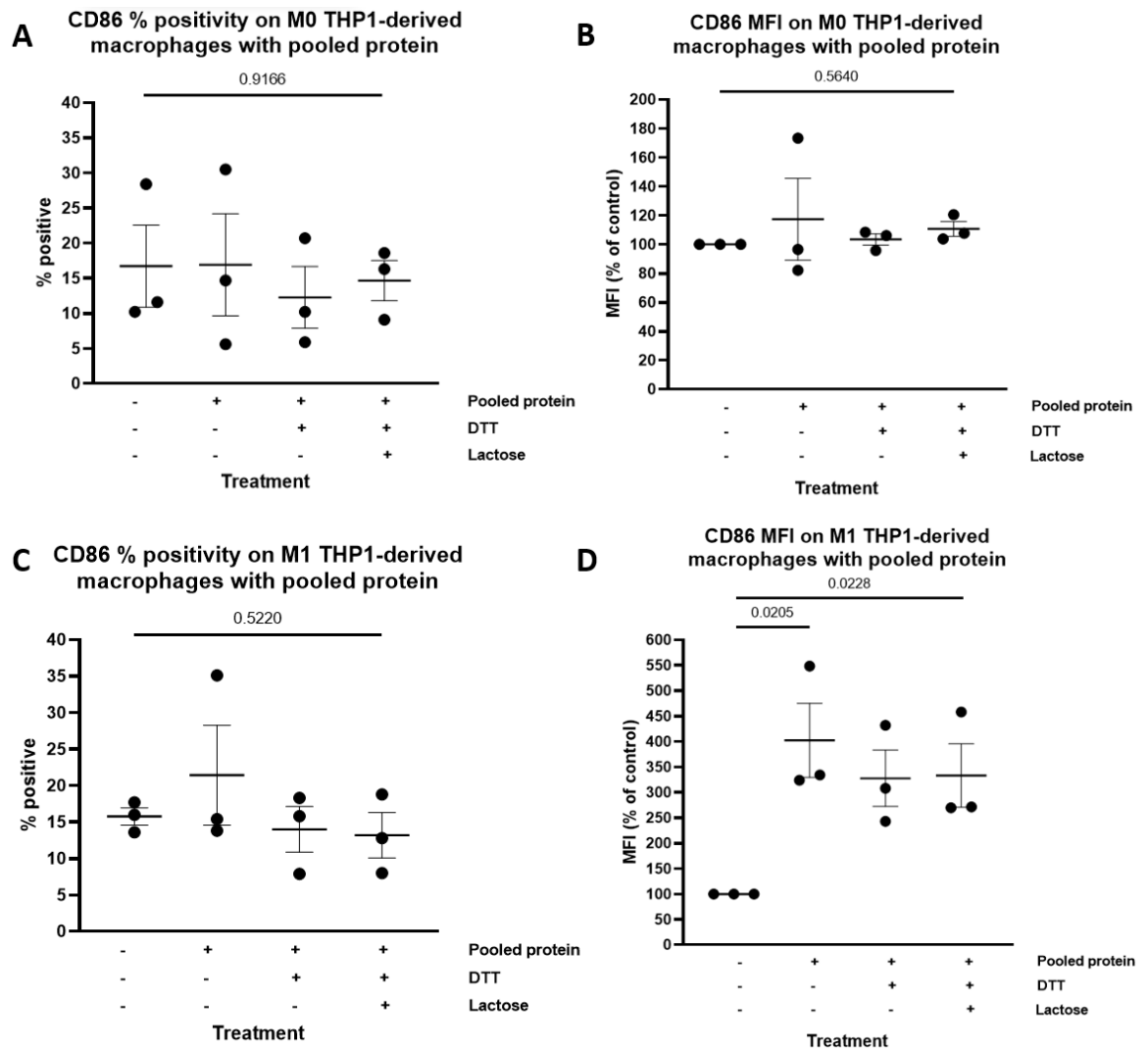


Figure 5.26. Expression of CD86 on THP1-derived macrophages treated with pooled protein. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with pooled protein alone, protein+DTT, protein+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with CD86-FITC and analysed by flow cytometry. A: Percentage positivity on THP1-derived M0 cells. B: MFI on CD86+ THP1-derived M0 cells. C: Percentage positivity on THP1-derived M1 cells. D: MFI on CD86+THP1-derived M1 cells. Data were analysed by one-way ANOVA (top line in each panel) and Tukey's multiple comparisons test (lower line in panel D) except panel B, which was analysed by Kruskal-Wallis test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

Finally, CD86 expression on Monomac-6-derived macrophages in response to pooled proteins was examined. These results are shown in Figure 5.27.

Mean CD86 percentage positivity decreased from 21.3% on control M0 cells to 14.6%, 16.8% and 13.1% for cells treated with inactive, active or blocked protein respectively (A, $p=0.4425$, ANOVA). Mean MFI on CD86-retaining cells was 91.7%, 175.2% and 119.4% of control in inactive, active and blocked protein groups respectively (B, $p=0.9569$, Kruskal-Wallis test). Increased MFI in the active galectin group was outlier-driven.

In M1 counterparts, mean CD86 percentage positivity was 20.0% on control cells and 27.9%, 28.1% and 30.4% following exposure to inactive, active and blocked protein respectively (C, $p=0.5099$, ANOVA). Mean CD86 MFI on positive cells was 139.6%, 146.5% and 160.8% of control for the same groups (D, $p=0.6440$, ANOVA). Increased MFI in the inactive and active protein groups is outlier-driven; although there may have been a genuine trend towards increased MFI in the blocked protein group, no firm conclusion could be drawn from these data.

Both THP1- and Monomac-6-derived M0 macrophages were unresponsive to pooled protein. However, while it was insufficient to change the percentage of CD86+ M1 cells, pooled protein treatment consistently increased MFI on THP1 M1 CD86+ cells. Additionally, data indicated a slight increase in CD86 percentage positivity on MM6 M1, though the trend did not approach significance.

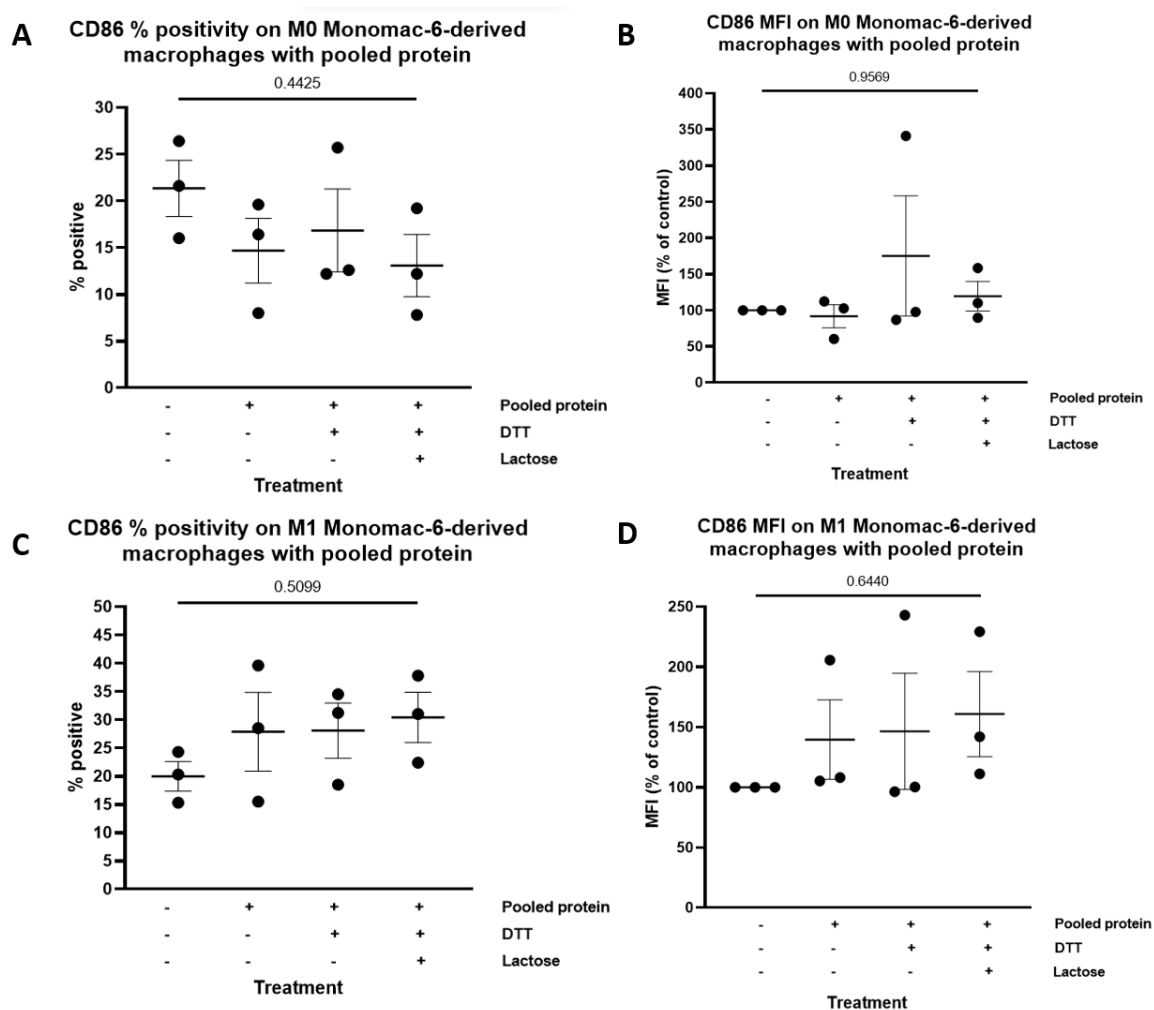


Figure 5.27. Expression of CD86 on Monomac-6-derived macrophages treated with pooled protein. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with pooled protein alone, protein+DTT, protein+DTT+lactose, or left untreated, for a further 24 hours. Cells were collected, stained with CD86-FITC and analysed by flow cytometry. A: Percentage positivity on Monomac-6-derived M0 cells. B: MFI on CD86+ Monomac-6-derived M0 cells. C: Percentage positivity on Monomac-6-derived M1 cells. D: MFI on CD86+ Monomac-6-derived M1 cells. Data were analysed by one-way ANOVA except for panel B, which was analysed by Kruskal-Wallis test. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.2.3.4. Effects of glioma cell line supernatant on MHCII expression

In the above sections, the expression of antigen presentation-associated molecules on the surface of THP1- and Monomac-6-derived macrophages was studied after exposure to GBM-relevant concentrations of galectin-1 and pooled protein. To conclude this series of experiments, MHCII expression was examined after macrophages were exposed to a titration of U373 96-hour supernatant.

Initially, MHCII expression on THP1-derived macrophages following exposure to a U373 supernatant titration was examined. Results are shown in Figure 5.28.

M0 macrophages showed no significant response to U373 supernatant in terms of mean MHCII percentage positivity (A, $p=0.9167$, ANOVA). MFI on MHCII⁺ cells was 119.0%, 87.4% and 78.7% of control following exposure to 25%, 50% and 100% U373 CS, which did approach significance (B, $p=0.0581$, ANOVA). These results were indicative of a global reduction in MHCII intensity without affecting the percentage of MHCII⁺ cells; however, the spread of data was large (panel A) and no firm conclusion could be based on it.

M1 THP1-derived macrophages showed a slight increase in MHCII percentage positivity from a mean average of 49.8% on control cells to 57.9%, 78.2% and 70.0% following exposure to 25%, 50% and 100% U373 supernatant respectively (C, $p=0.8167$, ANOVA). The spread of data in this panel was extremely broad and no firm conclusions were drawn from it. MHCII MFI under the same conditions was 106.4%, 159.4% and 124.8% of control; the last two values were outlier-driven and it appeared that without those, MFI would have been very consistent (D, $p=0.6735$, ANOVA).

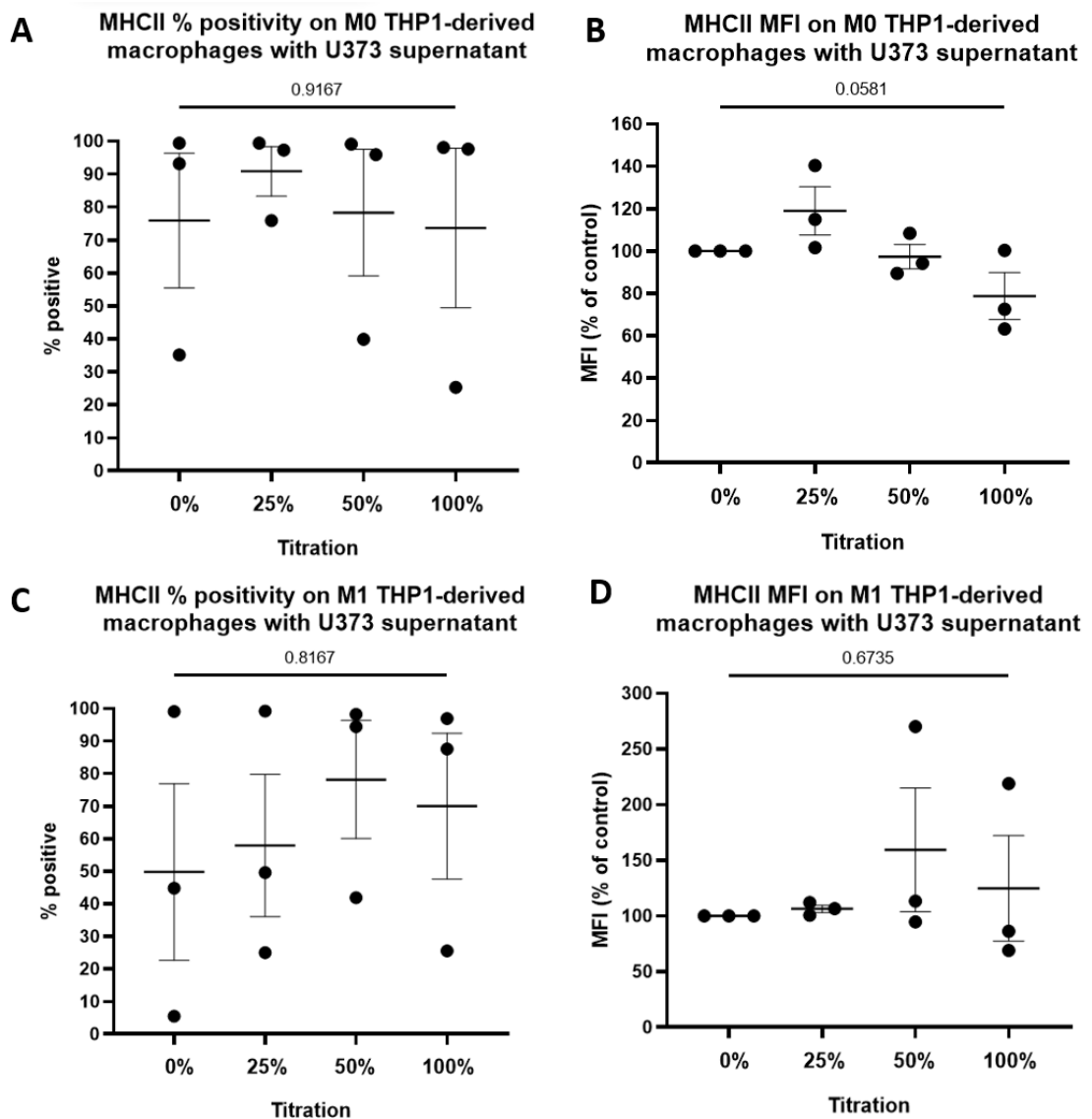


Figure 5.28. Expression of MHCII on THP1-derived macrophages treated with U373 conditioned media. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with 0%, 25%, 50% or 100% conditioned media for a further 24 hours. Cells were collected, stained with MHCII-PE and analysed by flow cytometry A: Percentage positivity on THP1-derived M0 cells. B: MFI on MHCII+ THP1-derived M0 cells. C: Percentage positivity on THP1-derived M1 cells. D: MFI on MHCII+ THP1-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

Next, MHCII expression on Monomac-6-derived macrophages following exposure to U373 supernatant was examined. Results are shown in Figure 5.29.

M0 macrophages showed consistent mean percentage positivity at all concentrations assayed, from 96.8% on control cells to 98.3%, 97.2% and 96.3% on cells treated with 25%, 50% and 100% U373 supernatant (A, $p=0.8645$, ANOVA). Mean MFI on MHCII+ cells was also consistent, at 103.4%, 104.5% and 100.7% of control MFI under the same conditions (B, $p=0.9991$, ANOVA). M1 cells also showed consistent MHCII percentage positivity, at 98.6%, 98.0% and 93.4% of cells treated with 25%, 50% and 100% U373 CS. No significant differences were identified between these and control cells, which were 90.9% MHCII+ (C, $p=0.3209$, ANOVA). The mean MFI on M1 MHCII+ cells was 131.6%, 147.4% and 85.7% of control for cells treated with 25%, 50% and 100% U373 CS (D, $p=0.6599$, ANOVA); values in the 25% and 50% groups were outlier-driven and it appeared that otherwise, MFI values would have been more consistent.

In conclusion, MHCII on Monomac-6-derived macrophages was unresponsive to U373 supernatant, as was its expression on M1 THP-1-derived macrophages. It was possible that U373 supernatant promoted a global reduction in MHCII expression on THP1-derived M0 macrophages, which conclusion would need verification with further experimental work.

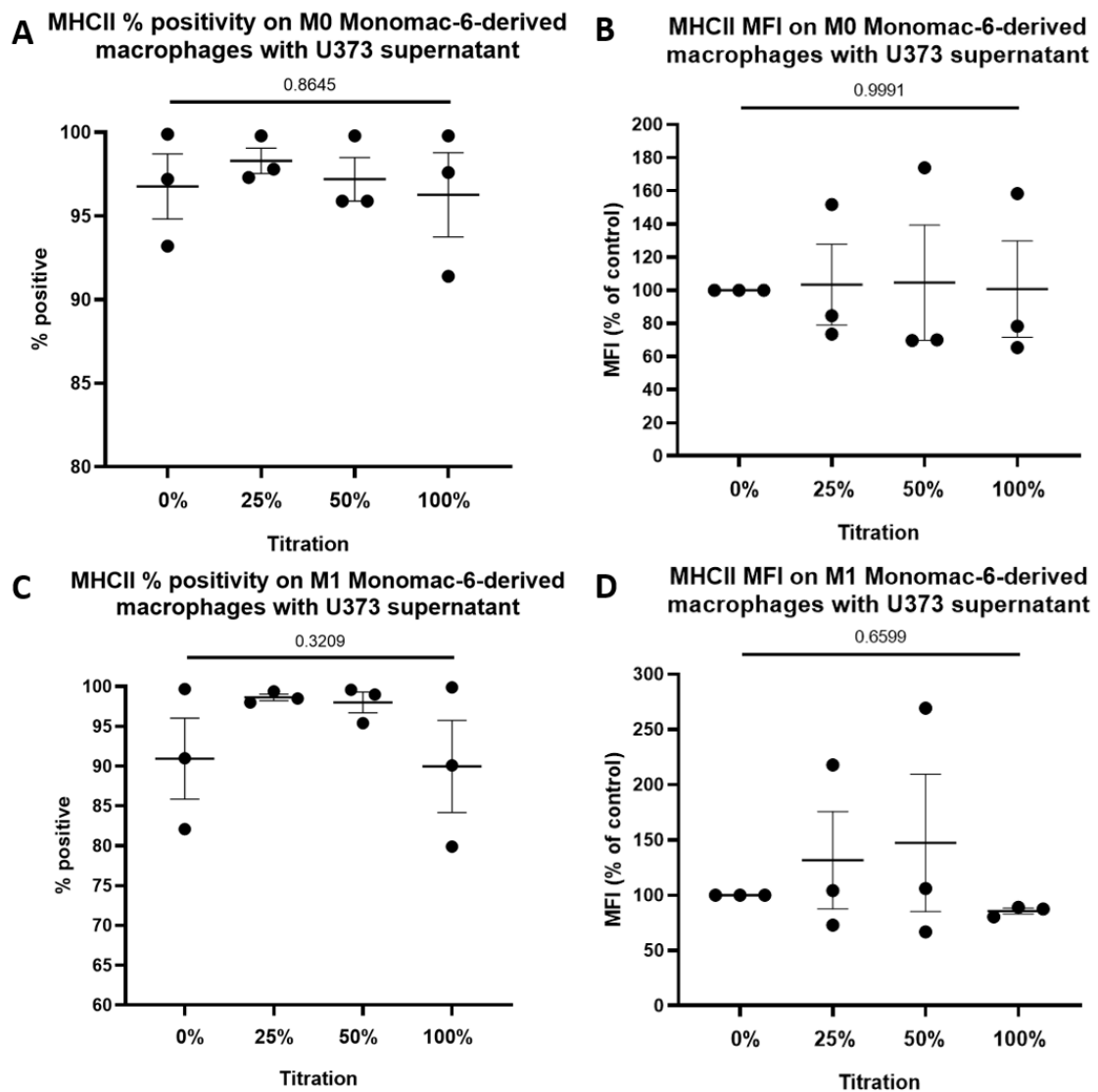


Figure 5.29. Expression of MHCII on Monomac-6-derived macrophages treated with U373 conditioned media. Cells were seeded in media containing 20ng/mL PMA for 24 hours, rested for 24 hours and either stimulated with 100ng/mL LPS for a further 24 hours or rested for a further 24 hours. Cells were then treated with 0%, 25%, 50% or 100% conditioned media for a further 24 hours. Cells were collected, stained MHCII-PE and analysed by flow cytometry A: Percentage positivity on Monomac-6-derived M0 cells. B: MFI on MHCII+ Monomac-6-derived M0 cells. C: Percentage positivity on Monomac-6-derived M1 cells. D: MFI on MHCII+ Monomac-6-derived M1 cells. Data were analysed by one-way ANOVA. N=3 for each experiment. Error bars=mean $\pm$ SEM.

5.3. Discussion and conclusions

Many experiments have been performed to assay the effects of recombinant human galectins – particularly galectin-1 – on leukocytes, often T-cells. However, many papers use high concentrations of the protein of interest, often with little justification as to why these concentrations are chosen or any indication of physiological relevance. Galectin-1 in particular behaves differently at different concentrations: below approximately 10 μ M, it dissociates to monomers (mgal-1), and otherwise exists as a dimer (dgal-1) (Perillo et al., 1995). Galectin-3 can form pentamers, mediated through interactions of its disordered N-terminal tail (Ahmad et al., 2004), which is dependent on ligands or high concentration. Many experiments either do not acknowledge this, or use high concentrations of galectin – particularly galectin-1 – with no justification. In many cases, the concentration of gal-1 is high enough that it would exist in solution as a dimer, and these results should not be extrapolated to apply to mgal-1 without justification.

Experiments in 3.2.2.2.2. determined a mean average of gal-1 secreted into TM from 23 GBM tumours of 11.2ng/mL; at this concentration, galectin-1 likely exists as a monomer. Thus, inferences cannot be drawn from experiments using higher concentrations of galectin-1 about what may occur in the GBM TME. To this end, in the work described herein, macrophages were exposed to GBM-relevant concentrations of target immunomodulatory proteins, individually and in combination, and resultant changes in phagocytosis and MHCII, CD80 and CD86 expression were analysed.

The human monocyte cell lines THP1 and Monomac-6 were used in these experiments, both of which are derived from acute myeloid leukaemia patients (AML). However, THP1 was isolated from an infant patient (one year old) whereas Monomac-6 was isolated from an adult (64 years old). AML is a heterogeneous cancer, comprising multiple clinical entities as of the 2016 World Health Organisation (WHO) classification guidelines (Arber et al., 2016). Paediatric AML is more likely to arise *de novo* compared to adult AML, which is often secondary to exposure to environmental risk factors, notably chemo- or radiotherapy, or a prior myelodysplasia diagnosis. Paediatric AML also shows distinct chromosomal and epigenetic abnormalities and reduced somatic mutations compared to adult cancers (Ma et al., 2018; Newcombe, Gibson and Keeshan, 2018). While the THP1 line is widely used in macrophage assays, the Monomac-6 line is one of the few that accurately recapitulates many features of adult monocytes and thus, in the context of GBM which primarily affects older patients, is likely to be a more representative model. However, the THP1 cell line was also useful in this series of experiments to compare potentially different behaviours between adult- and infant-derived macrophages in an identical context, which may be of benefit to future researchers.

5.3.1. Effects on phagocytosis

Results in 5.2.2. showed that M1 macrophages of both lines were significantly more phagocytic than M0 counterparts ($p=0.0196$, THP1; 0.0003 , Monomac-6, unpaired two-tailed t-test). However, no individual protein significantly promoted phagocytosis at GBM-relevant concentrations, whereas glioma cell line supernatants strongly promoted phagocytosis. Additionally, it is impossible to conclude from this series of experiments that pro-phagocytic effects of CS are unique to GBM: similar effects have also been documented by Wu et al., 2014, following treatment of THP1-derived cells with CS from colorectal cancer cell lines. It is possible that cell line supernatants generally promote phagocytosis. Galectins were not examined by Wu et al., and so increased phagocytosis post-CS exposure cannot be definitively attributed to these proteins..

THP1-derived macrophages were the most likely to show increased phagocytosis; slight but insignificant trends were noted when cells were treated with gal-1 (M0 and M1), gal-3 (M0 only), gal-4 (M1 only) and gal-7 (M0 and M1). Following exposure to CTGF, M1 THP1-derived cells exhibited insignificantly reduced phagocytosis, while M0 cells were unresponsive. When treated with pooled protein, slightly increased phagocytosis was noted in both M1 and M0 groups following exposure to inactive or blocked galectin conditions; however, the spread of these data was quite large and it could not be conclusively stated that these conditions promoted phagocytosis. However, if this were found to be the case, the comparatively reduced phagocytosis in the active galectin group would imply a synergistic phagocytosis-suppressing effect of active galectins, in contrast to individual pro-phagocytic effects. While these data would appear at odds with the slightly reduced phagocytosis seen in cells

treated with CTGF alone, non-CRD-mediated signalling may also have played a role here, thus allowing for increased phagocytic activity under conditions with active CTGF and oxidised galectin.

Oxidised gal-1, while lacking carbohydrate-binding activity, still has other biological functions and is able to promote axonal regeneration (Inagaki et al., 2000). In rat macrophages, 10ng/mL oxidised gal-1 is able to counter LPS-mediated M1 polarisation, resulting in reduced pro-inflammatory cytokine secretion (Kogawa et al., 2011). This is not dissimilar from the concentration used in this series of experiments (11ng/mL). It is possible that oxidised gal-1 affects LPS-induced M1 macrophage behaviour beyond pro-inflammatory cytokine secretion and can also affect phagocytosis and surface marker expression. However, human macrophages were not used in this study and these areas of macrophage biology were not studied; this hypothesis would need validation through further experimental work.

In the case of Monomac-6-derived macrophages, however, M0 cells were broadly unresponsive to both individual and pooled protein. No obvious trends could be discerned from M0 data except insignificantly increased phagocytosis when exposed to gal-1 and reduced phagocytosis when exposed to gal-4. M1 cells, conversely, showed reduced phagocytosis under every condition. This reached significance for gal-7 ($p=0.0405$, ANOVA, no significantly different pairs of means identified by Tukey's multiple comparisons test) and CTGF ($p=0.0169$, 0.0169 and 0.0027 for inactive, active and blocked galectin groups compared to control respectively). Suppressed phagocytosis following exposure to pooled protein also approached significance ($p=0.0609$, ANOVA). It appeared that adult M1

macrophages may be uniquely vulnerable to suppression of phagocytosis through a variety of soluble effectors.

However, when cell lines were exposed to glioma cell line supernatants, an increased percentage of phagocytic cells was observed. U251 supernatant promoted phagocytosis in Monomac-6-derived M0 cells ($p=0.0006$ and 0.0005 for 50% and 100% supernatant compared to control, respectively), THP1-derived M0 cells ($p=0.0324$ and 0.0021 for 50% and 100% supernatant compared to control, respectively) and THP1-derived M1 cells ($p=0.0163$ and 0.0155 for 25% and 50% U251 CS, Tukey's multiple comparisons test). These data indicated a potent pro-phagocytic effect of U251 supernatant, which regardless was not sufficient to promote phagocytosis in Monomac-6-derived M1 macrophages. A similar, though lesser, pro-phagocytic effect was seen in U373 treatments. In these experiments, only M0 Monomac-6-derived macrophages were significantly more phagocytic ($p=0.0323$, 0.0132 and 0.0212 for 25%, 50% and 100% CS, Tukey's multiple comparisons test). In all other conditions, U373 promoted phagocytosis to an insignificant degree, although it did approach significance in Monomac-6-derived M1 macrophages by ANOVA ($p=0.0660$).

Several conclusions can be drawn from these results. Firstly, Monomac-6-derived M1 macrophages were vulnerable to inhibition of their phagocytic activity, and may have been resistant to promotion of phagocytosis even under conditions that promoted such behaviour in other macrophages. Secondly, that even where significant suppressive effects existed for the soluble proteins in isolation, these were countered by the presence of unknown pro-phagocytic proteins in U251 and

U373 supernatant, which may also apply to the TME. Finally, at GBM-relevant concentrations, many of the individual proteins of interest induced trends in phagocytic behaviour that failed to reach significance.

Very little to no data about galectin-mediated effects on phagocytosis in human cells is available. Exogenous gal-3 has been found to promote phagocytosis at concentrations below 1 μ M by cross-linking macrophage cell surface receptors with targets (Caberoy et al., 2012), whereas these experiments found reduced phagocytosis. The above study used Jurkat cells as a phagocytic target, whereas this work used *E. coli*, which may be less effectively cross-linked, resulting in no positive phagocytic trend. Another study found gal-1, -3 and -4 to not affect phagocytosis; however, this work used 50 μ g/mL and human monocytes (Paclik et al., 2011), and thus is not directly comparable the above series of experiments.

5.3.2. Effects on antigen presentation molecules

It was found that gal-1 reduced MHCII expression on both THP1- and Monomac-6-derived macrophages. M0 cells from both lines downregulated MHCII regardless of oxidation or blocking, which approached significance in Monomac-6-derived cells; M1 cells only responded to active gal-1, which reached significance in THP1-derived cells. Of interest here was the discovery that MFI increased on MHCII-retaining cells, implying that subpopulations of THP1-derived M1 cells existed. Regardless, it was clear from these data that gal-1 suppressed overall MHCII percentage positivity. CD80 was unaffected by gal-1 exposure except in the case of THP1-derived M1 cells, which significantly acquired CD80 regardless of oxidation or blocking. Conversely, CD86 was insignificantly suppressed on M0 cells and promoted on M1 cells – except for THP1-derived M1 macrophages, upon which MFI was significantly increased regardless of oxidation or blocking.

Next, the effects of pooled proteins were examined. MHCII responded similarly, with global downregulation except in THP1-derived M1 cells, where reduced percentage positivity and increased MFI on the MHCII-retaining subpopulation was again observed. M1 THP1-derived macrophages responded in the same way to pooled protein as gal-1 in terms of CD80: this costimulatory molecule was upregulated, though only by active gal-1. The opposite was seen in terms of MFI, suggesting that CTGF promoted CD80 expression on CD80+ cells, which was opposed by other proteins. In Monomac-6-derived experiments, M0 cells showed an insignificant decline, and M1 cells an insignificant increase, in CD80 percentage positivity. Thus, the ability of pooled proteins to promote CD80 acquisition in M1 cells applied to some extent to both cell lines. CD86 MFI was upregulated on THP1-derived M1 cells

regardless of galectin oxidation or blocking; the same effect was seen with gal-1 alone. For Monomac-6-derived cells, similar trends were also seen between gal-1 and pooled protein treatment, wherein a clear but non-significant upregulation of CD86 on M1 cells was seen.

It was concluded from these experiments that GBM-relevant concentrations of gal-1 were sufficient to cause immunomodulatory effects on monocyte-derived macrophages *in vitro*. Gal-1 appeared to be the primary protein responsible for MHCII and CD86 modulation, but other factors appeared to affect CD80 expression. Gal-1's suppressive effects *in vivo* may be mediated primarily through reduction of MHCII, which would reduce T-cell stimulation in the event of cell-cell interaction. The same trends were observed with pooled protein treatment, and so it was concluded that the other proteins of interest had no effect on MHCII and CD86 expression. It must be noted that while the trend of downregulated MHCII was clear, these data did not reach significance.

Other work has found gal-1 to upregulate CD80 and MHCII on human monocytes, although it prevented MHCII expression in the presence of a pro-inflammatory stimulus. However, human macrophages did not alter CD80 expression in response to gal-1. This work used a far higher concentration of galectin: 50µg/mL (Paclik et al., 2011). However, exogenous gal-1 as low as 0.4µg/mL can reduce MHCII expression on mouse macrophages in response to pro-inflammatory stimuli; (Barrionuevo et al., 2007). In the GBM TME, murine TAMs have been found to increase MHCII and CD80 expression (Bowman et al., 2016). However, work in human GBM found low CD80 and 86 positivity (Kmieciak et al., 2013). TAM behaviour

may not be consistent across different organisms, and different exogenous protein concentrations will affect results further. However, the results from the above experiments were in line with a general potential downregulation in MHCII. Further, it is clear that THP1-derived M1 macrophages showed unique sensitivity to immunomodulatory proteins that was not reflected in the adult monocyte line. These cells responded to exogenous gal-1 and pooled protein in a pro-inflammatory manner through acquisition of CD80 and upregulated CD86, and a subpopulation upregulated MHCII, which changes were not observed in Monomac-6-derived macrophages.

Comparison with U373 96-hour CS revealed that further immunomodulatory factors were clearly at play. MHCII was steady on all cells treated with CS except for THP1-derived M0 cells, which showed global downregulation.

Thus, the immunomodulatory effects of gal-1 were broadly opposed by unknown factors found in U373 CS. It is worth noting that the spread of these data tended to be larger than in recombinant protein experiments, which may have masked more subtle trends. If U373 supernatant is indeed more representative of the TME, it is possible that antigen-presenting molecules on intratumoural macrophages are not strongly affected. To confirm this hypothesis, further experiments utilising GBM TM would be required.

It was also demonstrated herein that adult- and infant-derived macrophages showed different responses to identical conditions. As Monomac-6 cells more closely recapitulate the adult monocyte phenotype and thus may be a better model for

extrapolation to GBM TAMs, it was interesting that these cells were broadly unresponsive to recombinant proteins or CS. No obviously immunosuppressive changes in antigen-presentation molecules were apparent. Thus, it is possible that intratumoural TAM-mediated T-cell suppression is not mediated primarily through disrupted antigen presentation, but other mechanisms.

5.3.3. Conclusions and limitations

Many of the proteins of interest – CTGF, gal-1, gal-3, gal-4 and gal-7 – resulted in downwards-trending phagocytosis in Monomac-6-derived M1 cells, which was significant for CTGF and gal-7. It thus appeared that pro-inflammatory adult macrophages may be uniquely vulnerable to suppression of their phagocytic abilities, which would necessarily inhibit the development of anti-tumoural immunity. Notably, gal-1 alone significantly promoted phagocytosis in every other macrophage phenotype studied. This protein also had broadly suppressive effects on MHCII expression. These data suggested that gal-1 may be highly relevant to inhibition of macrophage-mediated anti-tumoural immunity. In addition, reduced intratumoural antigen presentation would limit the retention of GBM-specific T-cells activated in LNs. However, CS – which may be a closer model of the GBM TME than protein-based experiments – was pro-phagocytic and did not downregulate MHCII. While at first these results appeared at odds, this may not be the case.

That is, given gal-1's nearly-significant prevalence in the PVN ($p=0.0844$), TAMs that remained in this niche may have impaired phagocytosis and antigen presentation, resulting in reduced T-cell retention. TAMs that successfully infiltrated the tumour, which appeared to be the majority as TAMs were preferentially ($p=0.0087$) located away from the PVN, would have been exposed to different immunomodulatory proteins during migration, likely resulting in different behaviours in different intratumoural niches. Thus, phagocytosis may be promoted in the tumour bulk (TB), resulting in potential phagocytic destruction of B-cells. This also suggested the possibility of temporal or sequential exposure to immunomodulatory proteins affecting TAM behaviour: exposure to gal-1 in the PVN followed by gal-3 in

the TB may have different effects than concurrently encountering both. Such questions could not be addressed by this series of experiments, but may merit further study. Additionally, this analysis was limited by the use of mean average protein concentrations in liquid media. Regional protein concentrations in the tumour may be considerably different.

Overall, downregulation of antigen presentation did not appear to be a significant means through which GBM could inhibit intratumoural immunity. Instead, it seemed likely that TAMs furthered immunosuppression through modulation of other behaviours, of which phagocytosis may be a likely target. However, inhibited antigen presentation may be regionally important in the PVN. Isolation of TAMs from different intratumoural regions, followed by more complex characterisation that reaches beyond M1/M2 phenotypes to examine multiple aspects of macrophage behaviour, may illuminate new aspects of the GBM TME.

It must be noted here that an error was made in both individual and pooled protein experiments with regards to the concentration of CTGF used. In 3.2.2.2.6, eight of 23 GBM TM samples were found to contain CTGF detectable by ELISA, with a mean average of 246.0pg/mL across all 23 samples. In 5.2.1.1., Table 5.01 shows the mean concentration of CTGF used in the following assays to be 925.7pg/mL – almost four times higher. It was determined that data from a meningioma TM sample, which had been omitted from the results in Chapter 3, had been mistakenly included in place of one of the eight positive GBM samples. This meningioma contained far higher concentrations of CTGF than any assayed GBM and thus considerably increased the mean concentration when erroneously included in the

calculation. Although no changes in macrophage behaviour resulting from CTGF exposure were documented, it would have been erroneous to extrapolate any results to the GBM TME.

These data also showed that, in terms of both phagocytosis and antigen presentation, adult- and infant-derived human macrophages behaved differently in response to identical stimuli *in vitro*. These are two of the primary behaviours of human macrophages, and are important in both the cells' own ability to control localised infectious or malignant disease, and their ability to stimulate T-cell responses to said disease. Macrophages are highly plastic cells which carry out a diverse range of functions, and this discrepancy between cells isolated from patients of different ages may extend beyond the limited factors examined here to affect a range of behaviours. Thus, there is potentially a pitfall in extrapolating results from one patient age group to another. It is known that the human immune system undergoes multiple pronounced changes through life, which has direct clinical relevance in some contexts and also adds to the difficulty of developing robust and relevant models of macrophage behaviour. The THP1 cell line is one of the most widely used monocyte cell lines and is exclusively used in many experiments examining monocyte and macrophage biology; however, in some contexts, selection of other cell lines may be more appropriate.

These data also highlighted the dangers of extrapolating from *in vitro* results. While Monomac-6-derived M1 macrophages showed suppressed phagocytic behaviour when exposed to individual proteins, exposure to glioma cell line supernatants promoted phagocytosis. It would clearly have been erroneous to describe media

with one recombinant protein added as representative of the TME; however, it would clearly also have been wrong to suggest that if a given protein suppressed phagocytosis in isolation, it would do the same in the GBM TME. In these experiments, CS was used as a simplistic model of the TME secretome, with the understanding that it would not accurately recapitulate all the *in vivo* features, and a marked difference was seen between individual protein and supernatant experiments. Thus, caution is necessary when extrapolating the results of simplified *in vitro* models to *in vivo* environments without the use of other, more complex models as a comparison.

In addition to being limited by the initial small number of GBM TM samples and the comparatively few parameters assessed here, this dataset was also limited in that it relies on extrapolation of macrophage effects on T-cell function, which results could not be confirmed *in vitro*. In addition, the mechanisms through which gal-1 and other proteins may have affected macrophage behaviour are as-yet uncharacterised.

6: DISCUSSION

6.1. Distribution of intratumoural immune populations

6.1.1. T-cells, B-cells and potential intratumoural lymphoid tissue

In the 50 GBM dataset described herein, 2235 T-cells were observed across 42 GBM (4.2.1.3.2.). While a high percentage (84.0%) of tumours were T-cell+, T-cells did not appear to account for the majority of cells in any tumour examined. However, more T-cells were observed than B-cells (171; 4.2.1.3.3.) and tumour-associated macrophages (TAMs) (1318; 4.2.1.3.4.).

Other reports have found T-cells to comprise a minority of immune effectors in GBM by mass cytometry (Fu et al., 2020). A flow cytometry (FC)-based analysis found 8% and 0.5-1% of cells within GBM to be T- and B-cells respectively, which was similar to this work's observed 1:13 ratio (González-Tablas Pimenta et al., 2021). T-cells are overall considered to account for only small amounts of cells within GBM; perhaps 5%. T-cells may have been over-represented in the above results compared to tumour-associated macrophages (TAMs) and B-cells because of the number of sections assayed. Multiple sections from some tumours were stained for CD3 in combination with, for example, immune checkpoint (IC) molecules and CD56; the former of which was not performed for TAMs, and neither of which were performed for B-cells. Indeed, when only triple-stained tumours were included in the analysis, an increased prevalence of TAMs was found compared to B-cells ($p=0.0114$) and T-cells (0.0646) (4.2.1.3.5.).

In terms of intratumoural localisation, each cell type was considered independently. More T-cells were observed in the perivascular niche (PVN) compared to the tumour bulk (TB) to a degree which approached significance ($p=0.0932$). Other authors have found T-cells to be primarily located in the perinecrotic zone and cellular tumour using gene expression data (Qian et al., 2018). Kmiecik et al. in 2013 noted high heterogeneity across both CD4+ and CD8+ phenotypes, with some tumours showing high infiltration and others showing infiltration limited to the perivascular (PV) region. It is worth noting that these results analysed cell presence and niche (PVN or TB) as defined by distance from the nearest vessel. These results were not always comparable to those of other authors, who have often reported cells per unit area without absolute counts, or stratify intratumoural regions as necrotic, perinecrotic, perivascular, infiltrating margin *et cetera*. This analysis was less heavily stratified – partially due to the absence of haematoxylin and eosin stains, which are the standard for defining intratumoural niches for further study – partially as the sections were not full tumour sections and thus did not include, or did not reliably include, infiltrating margin and adjacent normal-appearing brain tissue.

B-cells have often been described as rare both in terms of number – less than 1% of infiltrating cells (Kmiecik et al., 2013) – and tumour positivity; Martinez-Lage et al. in 2019 identified B-cells in just four of 98 GBM using immunohistochemistry (IHC). In this dataset, B-cell positivity was 45.8% of 48 GBM assayed, and this might be explained by use of the marker CD19 as compared to Martinez-Lage et al.'s use of CD20. CD19 is a pan-B-cell marker, whereas plasma cells are CD20-; however, CD20 has been widely used in GBM studies to identify B-cells, and as these authors reported lower positivity, it is possible that GBM is infiltrated largely by plasma cells

which were undetected in other analyses. This would also account for the multiple Ig fragments in Gkanatsiou's mass spectrometry data (2022) (3.2.1.1.2.).

Of particular interest was the 2021 paper by Mauldin et al., who found 8% of their 77 GBM specimens to contain perivascular B- and T-cell aggregates that they suggested to be tertiary lymphoid structures (TLS); this was not dissimilar to the 22% of samples in this dataset that contained T-cell aggregates, and the images in their paper appeared remarkably similar. No B-cells were observed in the aggregates; in combination with the PV presence of galectin-1 (gal-1), this suggested that some of the observed T-cell aggregations may have been caused by gal-1-mediated inhibited motility. If such structures were TLS, the differential results could potentially be explained by the use of different markers: the above work used CD3 and CD19, whereas Mauldin et al. used CD8 and CD20. They also observed improved overall survival in patients with higher proliferating B- and CD8+ T-cells, showing a role for both direct cytotoxicity and antibody-mediated immunity. Regrettably, the presence of T-cell subsets in this GBM dataset could not be evaluated; however, the discovery of immune effector aggregates in multiple GBM was certainly of interest. It is possible that patients with TLS, already having a basal level of anti-tumoural immunity, would particularly benefit from therapies that alleviate the immunosuppressive tumour microenvironment (TME).

6.1.2. Macrophages

In terms of macrophage prevalence, these results were somewhat divergent from other authors. In total, 1318 TAMs were observed across 36 GBM for a mean average of 36.6 cells per positive tumour (4.2.1.3.4.) as determined by CD68 staining.

Other authors have found higher occurrences in their datasets. Fu et al., 2020 found mononuclear phagocytes to comprise approximately 60% of immune effectors using mass cytometry on cells isolated from 13 GBM. The mononuclear phagocyte category includes monocytes and dendritic cells and thus is a broader term than macrophage. However, Gonzáles-Tablas Pimenta et al. also identified approximately 60% of immune effectors to be TAMs by FC in their 2021 paper. Another group found the proportion of TAMs to be subject to intertumoural heterogeneity, ranging from under 10 to over 50% of cells (Zeiner et al., 2019). It is clear from this limited selection of recent papers that the dataset described above contained a notably reduced TAM incidence.

There were several potential explanations for this discrepancy. Firstly, unlike microscopy-based analyses, FC studies can use several macrophage-associated markers to identify TAMs, whereas IHC and standard immunofluorescence (IF) are generally limited to one or two. Further weight was lent to a marker-related underestimate by the finding from two separate groups that macrophage markers were not redundant. In 2019, both Zeiner et al. and Kvisten et al. noted that Iba1 stained more intratumoural macrophages than CD68; thus, use of the CD68 marker in these experiments may have led to an underestimate of TAM prevalence.

Furthermore, it appeared that TAM presence varies with the subtype, recurrence status and tumoural region examined. Greater TAM presence in mesenchymal GBM has been indicated by both IHC and data from The Cancer Genome Atlas (Martinez-Lage et al., 2019); this association with the mesenchymal subtype was also described specifically for M2-like TAMs (Zhou et al., 2022). A smaller study of paired samples from 15 GBM patients identified a greater prevalence of macrophages in recurrent than primary tumours: in this study, approximately 10% of cells in primary and 20% in recurrent GBM expressed CD68 (Loussouarn et al., 2023), whereas other authors have observed the opposite (Fu et al., 2020). Other authors have found TAMs to be significantly more present in central areas versus the infiltrating margin, and to be rare in highly necrotic areas (Kvisten et al., 2019).

Patient information pertaining to GBM subtype or primary/recurrent status was not available for this dataset. Furthermore, the tumour sections were not whole-tumour sections and did not include tumour-adjacent brain tissue; as such, it was not possible to ascertain whether the regions studied were derived from the central or marginal tumour. It is entirely plausible that consideration of this data, in combination with the understanding that some TAMs may have been undetected by CD68 staining, would elucidate exactly why this dataset showed reduced macrophages in comparison to other work. However, these results may well be reflective of heterogeneity, as is also observed in other datasets.

6.2. Potential roles of perivascular galectin-1

6.2.1. Other soluble proteins

A discrepancy was observed in target protein presence in GBM transport media (TM) and tissue sections. With the exception of galectin-3 (gal-3), all five proteins of interest were present in under half of TM samples assayed. However, all five were present in the majority of GBM tissue sections, except CTGF, which was found in 50%. These results at first appeared discordant, but in 3.2.1.1.2., secretory regulation of various proteins and protein families was identified. While mass spectrometry data was only sufficient to identify differential regulation between directly secreted and extracellular vesicles (EV)-packaged proteins, it was likely that this regulation extends further, such that proteins could also be synthesised without secretion. Certain proteins – for example, galectins 4 and 7 and CTGF – may have been widely synthesised, but not widely secreted, and thus were detectable by IF but not enzyme-linked immunosorbent assay (ELISA). Unless these proteins were bound to the surface of intratumoural cells, they would likely have no effect on infiltrating immune effectors. It was therefore possible that galectins 4 and 7 were less valuable targets for further study than gal-1 and -3 and the IC molecules galectin-9 (gal-9) and PD-L1.

6.2.2. Galectin-1, galectin-9 and T-cell extravasation

In 4.2.1.3.2., a marked PV presence of T-cells which approached significance ($p=0.0932$) was documented; this was in contrast to B-cells and TAMs, which were significantly more likely to be found in the TB (4.2.1.3.3, $p=0.0053$; 4.2.1.3.4., $p=0.0087$). In 4.2.3.3.1., a nearly-significant ($p=0.0844$) presence of gal-1+ cells in the PVN was also noted. This suggested that T-cells, unlike other immune effector phenotypes, were uniquely subject to galectin-1-mediated inhibition of migration. Alongside gal-1, PV galectin-9 (gal-9) – a ligand for the immune checkpoint molecule TIM-3 – was also noted (4.2.3.2.), although this protein was also present in TB and the distribution did not approach significance. Thus, the relevance these proteins may play in promoting T-cell aggregation was of interest.

Human umbilical vein endothelial cells (HUVECs), which constitutively express both proteins, upregulated gal-1 and translocated gal-9 to the cell membrane after exposure to tumour cell line conditioned media (CM) (Thijssen, Hulsmans and Griffioen, 2008). Head and neck cancer (HNC)-relevant concentrations of gal-1 increased expression of gal-9 and PD-L1 on tumour endothelial cells (ECs), resulting in reduced T-cell transmigration *in vitro* and providing an explanation for the inverse correlation between gal-1 expression and CD4+ T-cell infiltration in this cancer. Gal-1-null tumours were more permissive of T-cell infiltration. Combined PD-1 and TIM-3 blockade improved T-cell infiltration *in vivo*; these authors also found that gal-1 blockade sensitised tumours to anti-PD-1 therapy. This permitted CD8+ T-cell infiltration, resulting in inhibited tumour growth and metastasis in a pre-clinical mouse model (Nambiar et al., 2019).

Conversely, pre-treatment with soluble gal-9 promoted T-cell adhesion to ECs *in vitro* (Mansour et al., 2022). Soluble gal-1 was not identified in the mass spectrometry dataset and its secretion by primary GBM does not appear to have been documented in the literature. Some tissue sections appeared to contain gal-9+ ECs, which may facilitate T-cell adhesion; however, it could not be stated definitively that gal-9 positivity on ECs would promote T-cell adhesion in the same manner as pre-exposure to soluble gal-9, or that it would be sufficient to outweigh the counter-migratory effect of gal-1 on those same cells.

Additionally, stimulating HUVEC gal-1 expression using tumour cell line CM allowed them to deposit gal-1 into extracellular matrix (ECM). In these experiments, T-cells failed to transmigrate across gal-1+ HUVECs in the absence of inhibited adhesion or cell death (He and Baum, 2006). It can be concluded from this that, even if gal-9 expression on vascular ECs – as was observed in some GBM tissue sections – facilitated T-cell adhesion, gal-1 expression on the same cells would likely inhibit extravasation.

6.2.3. Inhibited T-cell migration

As described in the above section, both gal-1 and gal-9 expression on ECs may have inhibited T-cell extravasation; however, T-cells were observed in the majority of GBM examined, so other immunosuppressive mechanisms must be at play.

Gal-9 has been found to inhibit leukocyte migration by binding to the chemotactic protein CXCL12 (Mansour et al., 2022) and its strong perivascular presence in these sections may have inhibited CXCL12-mediated T-cell migration, resulting in aggregation. CXCL12, also known as SDF1, has been known to be expressed in GBM for over two decades (Rempel et al., 2000); thus it can be assumed that intratumoural CXCL12, which is a T-cell chemoattractant, would promote T-cell infiltration if it were not likely bound to gal-9.

Other work has shown gal-9 to induce T-cell apoptosis independently of TIM-3; it was recognised by multiple receptors, including CD40 (Gooden et al., 2013 and references therein). These authors found that after several days of exposure to low gal-9 concentrations, surviving T-cells began to proliferate without displaying an exhausted phenotype. This study, contrary to other published results, found that gal-9 was able to induce expansion of the Th1 and memory subsets. Other work suggested that gal-1 facilitates Th2 infiltration into tissue by binding the PDI enzyme on the cell surface (Bi et al., 2011). This mechanism may also be relevant to the results described herein.

Additionally, the finding that tumour cell line CM was sufficient to promote gal-1 ECM deposition by HUVECs (He and Baum, 2006) may partially explain the PV presence

of gal-1. In these experiments by He and Baum, gal-1 deposition in the ECM inhibited T-cell migration even independently of cell death. This occurred through the gal-1/CD43 interaction preventing the redistribution of CD43 to the uropod: a structure at the rear of migrating leukocytes that is anchored to the ECM by CD43 and other molecules to provide stability (He and Baum, 2006). Prevention of T-cell migration via inhibited CD43 redistribution would also explain why T-cells appeared to be retained in tissue sections despite a paucity of antigen-presenting cells (APCs), which would usually result in T-cell exit from the tissue if their cognate antigen is not recognised. Thus it appeared likely that in GBM, T-cell migration throughout the tumour may have been inhibited by gal-1 deposition.

6.2.4. Galectin-9 expression may be promoted by pro-inflammatory T-cells

Gal-9 has been found to be expressed in varied cell types in response to pro-inflammatory stimuli, including IFN γ . IFN γ produced by TILs promoted gal-9 expression on APCs, implying that T-cell and APC contacts could result in suppression instead of activation (Li et al., 2012). Other authors have found pro-inflammatory molecules promoted gal-9 expression in mesenchymal stromal cells, resulting in similar defects in T-cell activation (Gieseke et al., 2013). Nambiar et al. in their 2019 paper also documented upregulation of intratumoural gal-9 and PD-L1 by IFN γ secretion when gal-1 was knocked out in a murine HNC model to permit T-cell infiltration.

Gal-9 – like gal-1 – is immunosuppressive, which conferred a worse prognosis in recurrent nasopharyngeal carcinoma (Chen et al., 2017) and hepatocellular carcinoma (HCC) via its interaction with TIM-3 (Li et al., 2012). Gal-9 has been found to induce T-cell death *in vitro* more potently than gal-1, and recognises different glycosylation; its efficient induction of apoptosis may depend on the flexible linker domain between its two carbohydrate recognition domains (CRDs), which facilitates crosslinking (Bi et al., 2008).

Overall, it appeared that tumour tissue may respond to an influx of pro-inflammatory T-cells by producing gal-9 to specifically dampen their function, which mechanism has been suggested in a non-tumoural context by Anderson and Anderson, 2006. This theory was further supported by the gal-9/TIM-3 axis's specific regulation of Th1, Tc1 and Th17, but not Th2, cells *in vitro* and *in vivo* (Monney et al., 2002; Zhu et al., 2005). Thus it is possible that GBM, and potentially other solid tumours, may

compensate for T-cell infiltration across gal-1+ ECs by co-opting pro-inflammatory signals to induce IC expression.

6.2.5. Inhibited antigen presentation

Gal-1 in the PVN may affect other cell types. While evidence exists of its ability to suppress T-cell extravasation, the same does not seem to exist for B-cells; regardless, this possibility could not be discounted.

BMDMs have likely encountered gal-1 as they extravasate. As described in 5.2.2.2., Monomac-6-derived M1 macrophages were vulnerable to phagocytic suppression by GBM-relevant concentrations of gal-1. Gal-1 also caused consistent, though insignificant, MHCII downregulation (5.2.3.2.1.), which was in line with data seen by other researchers, where gal-1 suppressed MHCII upregulation on human and murine macrophages in response to pro-inflammatory stimuli (Barrionuevo et al., 2007; Paclik et al., 2011). It is possible that even cells retaining some pro-inflammatory function, when localised in the PVN, show inhibited antigen presentation. As this niche is key for T-cell activation and retention in tissue, such inhibition could result in suppressed T-cell-mediated anti-tumoural immunity.

This hypothesis is dependent on extrapolation from *in vitro* experiments using immortalised cells to the far more complex *in vivo* environment, and thus requires considerable further work. Exposure to PV gal-1 would likely occur around the time of monocyte-to-macrophage transition. Gal-1 has been found to have differential effects on both human cell types (Paclik et al., 2011), and its effect during differentiation appears to be completely undocumented. Furthermore, given the altered phenotype of peripheral monocytes in human GBM patients (Harshyne et al., 2016, Gustafson et al., 2010), even extrapolation from healthy subjects may be unreliable in this context.

6.3. The roles of intratumoural macrophages

6.3.1. Phagocytosis and B-cell immunosuppression

The results suggested that, in addition to their known roles in supporting GBM growth and gliomagenesis, TAMs may also support immune escape by consuming infiltrating B-cells. There was no evidence that the same does not occur with T-cells, and indeed, one instance of what appeared to be T-cell-directed phagocytosis was observed (Figure 4.24, 4.2.1.4.2.), as well as several incidences of CD19-CD68 colocalisation, and none of CD3-CD68 colocalisation. It appeared that phagocytosis of T-cells may have occurred less frequently than that of B-cells, which might be partially explained by the significant presence of B-cells and TAMs in the tumour bulk.

Conversely, no instances of B-cell-directed phagocytosis were observed, and the possibility that B-cells died in the tumour bulk and were then scavenged, rather than being directly engulfed, could not be ruled out. It was also possible that T- and B-cells were both subject to phagocytosis, but that CD3 was degraded more quickly than CD19 in the phagosomes and lysosomes, thus rendering T-cell engulfment harder to detect.

This theory was also supported by the finding that U373 cell line supernatant, but not individual target proteins, promoted phagocytic behaviour in Monomac-6-derived macrophages (5.2.2. and subsections therein), which is an adult monocyte cell line that retains many of the features and behaviours of non-immortalised monocytes.

In this series of experiments, gal-1 insignificantly promoted phagocytosis in M0 cells while suppressing it in M1 cells. While the M1/M2 model has been found to apply poorly to GBM TAMs (Szulzewsky et al., 2015; Zeiner et al., 2019), it is possible that macrophages which retained pro-inflammatory function would lose phagocytic activity following gal-1 exposure. M0 macrophages have also been noted in GBM by Zhou et al., 2022; in the above experiments, M0 Monomac-6-derived macrophages showed a trend towards increased and decreased phagocytosis following exposure to GBM-relevant concentrations of galectin-1 and galectin-7, respectively, although neither trend approached significance. It possible that phagocytosis was promoted by PV galectin-1 in M0 macrophages following extravasation.

6.3.2. Potential role of galectins in mediating phagocytosis

The ability of galectins to induce phosphatidylserine (PS) exposure independently of apoptosis on the outer membrane of T-cells has been described for dimeric, but not monomeric, galectin-1 (mgal-1 and dgal-1) (Dias-Baruffi et al., 2003), gal-4 (Stowell et al., 2007) and gal-3 (Stowell et al., 2008); furthermore, alkylated gal-1-induced PS exposure has been found to be reversible (Stowell et al., 2009).

Recognition of PS has been found to be critical for macrophage engulfment of apoptotic cells (Fadok et al., 2000), and indeed, in their 2003 paper, Dias-Baruffi et al. found dgal-1-induced PS exposure promoted phagocytic clearance of human T-cell lines by murine macrophages. It is worth noting here, however, that their mgal-1 was a mutant protein, and that they observed gal-1 to behave as a dimer at concentrations as low as 2 μ M. Regardless, these data implied that crosslinking induced by dgal-1 may be highly important for PS exposure.

At first glance, it appeared that intratumoural exposure to gal-1, -3 and -4 may result in high levels of PS exposure, thus promoting phagocytic clearance of non-apoptotic T-cells. However, gal-1 and gal-4 have been found to cause PS exposure only on activated primary T-cells with prior dithiothreitol (DTT) treatment (Stowell et al., 2007). Gal-3, conversely, bound separate receptors to gal-1 and induced both PS exposure and apoptosis in activated T-cells (Stowell et al., 2008).

Therefore, intratumoural gal-3 may be a primary means through which T-cells are marked for phagocytic clearance by TAMs. It appears that no similar research into galectin-mediated PS exposure at the B-cell surface has been conducted.

6.3.3. CTGF+ macrophages may be micro-RNA-induced

The high prevalence of CTGF in macrophages was of particular interest. As described in Table 4.24, 4.2.2.4.5., almost 80% of CTGF-expressing cells also costained with CD68 in sections where both antibodies were used.

Work by Zhao et al., 2022 showed a role for the micro-RNA miR-27a-3p in supporting GBM growth. This micro-RNA was secreted in U87-derived EVs, and promoted an M2 phenotype and IL-10 secretion in THP1-derived macrophages. Their paper described miR-27a-3p-mediated modulation of EZH1 and KDM3A, resulting in greater CTGF expression and M2 polarisation. This was prevented by CTGF knockdown (KD). These CTGF+ macrophages promoted GBM cell line growth and invasion both *in vitro* and *in vivo*.

A direct comparison between their work and the results herein was impossible for several reasons: Gkanatsiou's mass spectrometry analysis did not include micro-RNA; direct extrapolation between cell lines and their non-immortalised counterparts is subject to error; and Zhao et al.'s analysis of M1/M2 polarisation used only two phenotypic markers. However, if the same mechanism occurred in primary human tumours, it would explain the high percentage of CTGF+ macrophages observed in tissue sections. Additionally, the promotion of IL-10 secretion would have suppressive effects on T-cell activity, thus representing another potential way in which TAMs could suppress T-cell-mediated immunity in the absence of phagocytosis. IL-10 has been used in laboratory settings to induce the anti-inflammatory M2 phenotype, and thus a positive feedback loop of anti-inflammatory TAM polarisation may exist within GBM.

6.4. Future work

While this thesis has addressed several questions pertinent to the current understanding of GBM, it has also raised new questions which require further work to address.

Among the most obvious of these is the role of B-cells within GBM: this understudied population was widely present in the examined tissue sections, and mass spectrometry data – rich in Ig fragments – indicated they may be functional. However, the effect of secreted immunomodulatory proteins on B-cells in GBM was not addressed. Thus, suggested future work includes flow cytometry-based analysis of B-cell function and viability following treatment with recombinant galectins and CTGF at GBM-relevant concentrations, in much the same way as cell line-derived macrophages were studied in Chapter 5. This series of experiments could also include analysis of different immunoglobulin isotype secretion by ELISA.

Furthermore, data in Chapter 4 suggested inhibition of T-cell extravasation by gal-1 and possibly gal-9 in the PVN – a hypothesis that also merits closer examination. Proposed experiments include T-cell migration assays through Matrigel, which simulates the ECM, and across cultured endothelial cells, with prior and concurrent exposure to GBM-relevant gal-1 and gal-9. In combination with a B-cell-focused series of assays, these experiments may also explain the differences in T- and B-cell infiltration observed in human GBM.

Data on gal-9 in GBM is extremely limited, and the preliminary dataset examined herein was sufficient to indicate gal-9's widespread presence. Use of a larger

dataset to examine gal-9 presence, intratumoural distribution and cellular sources is a valuable avenue for further study, as the sample size in this thesis was not large enough to draw any firm conclusions from.

Finally, the GBM TME is often held to be unique in terms of its immunomodulatory effects. When GBM and LGG were compared in terms of target protein secretion using ELISA, several differences were seen (Chapter 3). Including other intracranial tumours such as meningioma in the dataset would allow further characterisation of GBM as a clinical entity, and may also reveal similarities between different immunosuppressive TMEs, potentially informing the development and use of future immunotherapies.

APPENDIX: PROOF OF ETHICAL APPROVAL



21 September 2020

Dear Rye Greywood

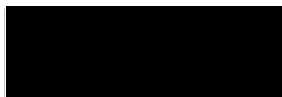
RE: Immunosuppression in Glioblastoma

Thank you for submitting your ethics application form to the Life Sciences Ethics Committee for review.

I am pleased to inform you that your project 'Immunosuppression in Glioblastoma' was discussed by the Committee on 9th July 2020 and approval was granted by Chair's Action 21st September 2020, following receipt of requested amendments: REC number **LSEC/201920/KA/157**.

The Committee wishes you every success in your research project.

Kind regards,



Professor Tracy Warr
Professor of Neuro-oncology and Director of Research Institute of Healthcare Sciences
Chair of Life Sciences Ethics Committee
Faculty of Science and Engineering
University of Wolverhampton

REFERENCES

- Aarvak T, Chabaud M, Miossec P, Natvig JB, 1999. IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. *J Immunol*;162(3):1246-51.
- Adams H, Chaichana KL, Avendaño J, Liu B, Raza SM, Quiñones-Hinojosa A, 2013. Adult cerebellar glioblastoma: understanding survival and prognostic factors using a population-based database from 1973 to 2009. *World Neurosurg*;80(6):e237-e243. DOI: 10.1016/j.wneu.2013.02.010. Epub 2013 Feb 6.
- Ahmad N, Gabius HJ, André S, Kaltner H, Sabesan S, Roy R, Liu B, Macaluso F, Brewer CF, 2004. Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. *J Biol Chem*;279(12):10841-7.
- Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM, 2007. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci*;10(12):1538-43.
- Akashi K, Traver D, Miyamoto T, Weissman IL, 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*;404(6774):193-7.
- Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, Ma J, Tezuka K, Yagita H, Okumura K, 2005. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. *J Immunol*;175(4):2340-8.
- Alban TJ, Alvarado AG, Sorensen MD, Bayik D, Volovetz J, Serbinowski E, Mulkearns-Hubert EE, Sinyuk M, Hale JS, Onzi GR, McGraw M, Huang P, Grabowski MM, Wathen CA, Ahluwalia MS, Radivoyevitch T, Kornblum HL, Kristensen BW, Vogelbaum MA, Lathia JD, 2018. Global immune fingerprinting in glioblastoma patient peripheral blood reveals immune-suppression signatures associated with prognosis. *JCI Insight*;3(21):e122264. Epub 2018 Nov 2. DOI: 10.1172/jci.insight.122264.
- Alban TJ, Bayik D, Otvos B, Rabljenovic A, Leng L, Jia-Shiun L, Roversi G, Lauko A, Momin AA, Mohammadi AM, Peereboom DM, Ahluwalia MS, Matsuda K, Yun K, Bucala R, Vogelbaum MA, Lathia JD, 2020. Glioblastoma myeloid-derived suppressor cell subsets express differential macrophage migration inhibitory factor receptor profiles that can be targeted to reduce immune suppression. *Front Immunol*;11:1191. Epub 2020 Jun 18. DOI: 10.3389/fimmu.2020.01191.
- Alkabie S, Castrodad-Molina R, Heck KA, Mandel J, Hutton GJ, 2021. The concurrence of multiple sclerosis and glioblastoma. *Mult Scler Relat Disord*;50:102877. DOI: 10.1016/j.msard.2021.102877. Epub 2021 Mar 2.
- Allen RC, Armitage RJ, Conley ME, Rosenblatt H, Jenkins NA, Copeland NG, Bedell MA, Edelhoff S, Disteche CM, Simoneaux DK, Fanslow WC, Belmont J, Spriggs MK, 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science*;259(5097):990-3.

Allende ML, Tuymetova G, Lee BG, Bonifacino E, Wu YP, Proia RL, 2010. S1P1 receptor directs the release of immature B cells from bone marrow into blood. *J Exp Med*;207(5):1113-24.

Alliot F, Godin I, Pessac B, 1999. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Brain Res Dev Brain Res*;117(2):145-52.

Almqvist N, Mårtensson IL, 2012. The pre-B cell receptor; selecting for or against autoreactivity. *Scand J Immunol*;76(3):256-62.

Amirian ES, Zhou R, Wrensch MR, Olson SH, Scheurer ME, Il'yasova D, Lachance D, Armstrong GN, McCoy LS, Lau CC, Claus EB, Barnholtz-Sloan JS, Schildkraut J, Ali-Osman F, Sadetzki S, Johansen C, Houlston RS, Jenkins RB, Bernstein JL, Merrell RT, Davis FG, Lai R, Shete S, Amos CI, Melin BS, Bondy ML, 2016. Approaching a scientific consensus on the association between allergies and glioma Risk: A report from the glioma international case-control study. *Cancer Epidemiol Biomarkers Prev*;25(2):282-90.

Amoozgar Z, Kloepper J, Ren J, Tay RE, Kazer SW, Kiner E, Krishnan S, Posada JM, Ghosh M, Mamessier E, Wong C, Ferraro GB, Batista A, Wang N, Badeaux M, Roberge S, Xu L, Huang P, Shalek AK, Fukumura D, Kim HJ, Jain RK, 2021. Targeting Treg cells with G1TR activation alleviates resistance to immunotherapy in murine glioblastomas. *Nat Commun*;12(1):2582. DOI: 10.1038/s41467-021-22885-8. Epub 2021 May 11.

Anderson AC, Anderson DE, 2006. TIM-3 in autoimmunity. *Curr Opin Immunol*;18(6):665-9.

Andoh M, Koyama R, 2021. Microglia regulate synaptic development and plasticity. *Dev Neurobiol*;81(5):568-90.

Andrew A, Strebel K, 2010. HIV-1 Vpu targets cell surface markers CD4 and BST-2 through distinct mechanisms. *Mol Aspects Med*;31(5):407-17.

Anfossi N, André P, Guia S, Falk CS, Roetynck S, Stewart CA, Bresó V, Frassati C, Reviron D, Middleton D, Romagné F, Ugolini S, Vivier E, 2006. Human NK cell education by inhibitory receptors for MHC class I. *Immunity*;25(2):331-42.

Anghileri E, Castiglione M, Nunziata R, Boffano C, Nazzi V, Acerbi F, Finocchiaro G, Eoli M, 2016. Extraneural metastases in glioblastoma patients: two cases with YKL-40-positive glioblastomas and a meta-analysis of the literature. *Neurosurg Rev*;39(1):37-45.

Annunziato F, Cosmi L, Santarlaschi V, Maggi L, Liotta F, Mazzinghi B, Parente E, Fili L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S, 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med*;204(8):1849-61.

Annunziato F, Manetti R, Tomasévic I, Guidizi MG, Biagiotti R, Giannò V, Germano P, Mavilia C, Maggi E, Romagnani S, 1996. Expression and release of LAG-3-encoded protein by human CD4⁺ T cells are associated with IFN-gamma production. *FASEB J*;10(7):769-76.

Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M, Vardiman JW, 2016. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*;127(20):2391-405.

Armstrong GT, Liu Q, Yasui Y, Huang S, Ness KK, Leisenring W, Hudson MM, Donaldson SS, King AA, Stovall M, Krull KR, Robison LL, Packer RJ, 2009. Long-term outcomes among adult survivors of childhood central nervous system malignancies in the Childhood Cancer Survivor Study. *J Natl Cancer Inst*;101(13):946-58.

Arrieta VA, Dmello C, McGrail DJ, Brat DJ, Lee-Chang C, Heimberger AB, Chand D, Stupp R, Sonabend AM, 2023. Immune checkpoint blockade in glioblastoma: from tumor heterogeneity to personalized treatment. *J Clin Invest*;133(2):e163447. DOI: 10.1172/JCI163447. Epub 2023 Jan 17.

Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S, Detmar M, Wiig H, Alitalo K, 2015. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J Exp Med*;212(7):991-9.

Astorgues-Xerri L, Riveiro ME, Tijeras-Raballand A, Serova M, Neuzillet C, Albert S, Raymond E, Faivre S, 2014. Unraveling galectin-1 as a novel therapeutic target for cancer. *Cancer Treat Rev*;40(2):307-19.

Bacher M, Schrader J, Thompson N, Kuschela K, Gerns D, Waeber G, Schlegel J, 2003. Up-regulation of macrophage migration inhibitory factor gene and protein expression in glial tumor cells during hypoxic and hypoglycemic stress indicates a critical role for angiogenesis in glioblastoma multiforme. *Am J Pathol*;162(1):11-7.

Bahmanyar S, Montgomery SM, Hillert J, Ekbom A, Olsson T, 2009. Cancer risk among patients with multiple sclerosis and their parents. *Neurology*;72(13):1170-7.

Bahua M, Vidaud D, Jenkins RB, Bièche I, Kimmell DW, Assouline B, Smith JS, Alderete B, Cayuela JM, Harpey JP, Caille B, Vidaud M, 1998. Germ-line deletion involving the INK4 locus in familial proneness to melanoma and nervous system tumors. *Cancer Res*;58(11):2298-303.

Bailey SL, Schreiner B, McMahon EJ, Miller SD, 2007. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4⁺ T(H)-17 cells in relapsing EAE. *Nat Immunol*;8(2):172-80.

Baixeras E, Huard B, Miossec C, Jitsukawa S, Martin M, Hercend T, Auffray C, Triebel F, Piatier-Tonneau D, 1992. Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. *J Exp Med*;176(2):327-37.

Balss J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A, 2008. Analysis of the IDH1 codon 132 mutation in brain tumors. *Acta Neuropathol*;116(6):597-602.

Barami K, Lyon L, Conell C, 2017. Type 2 diabetes mellitus and glioblastoma multiforme-assessing risk and survival: results of a large retrospective study and systematic review of the literature. *World Neurosurg*;106:300-7.

Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R, 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*;439(7077):682-7.

Barrionuevo P, Beigier-Bompadre M, Ilarregui JM, Toscano MA, Bianco GA, Isturiz MA, Rabinovich GA, 2007. A novel function for galectin-1 at the crossroad of innate and adaptive immunity: galectin-1 regulates monocyte/macrophage physiology through a nonapoptotic ERK-dependent pathway. *J Immunol*;178(1):436-45.

Barrow H, Guo X, Wandall HH, Pedersen JW, Fu B, Zhao Q, Chen C, Rhodes JM, Yu LG, 2011. Serum galectin-2, -4, and -8 are greatly increased in colon and breast cancer patients and promote cancer cell adhesion to blood vascular endothelium. *Clin Cancer Res*;17(22):7035-46.

Bartholomäus I, Kawakami N, Odoardi F, Schläger C, Miljkovic D, Ellwart JW, Klinkert WE, Flügel-Koch C, Issekutz TB, Wekerle H, Flügel A, 2009. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature*;462(7269):94-8.

Bassing CH, Swat W, Alt FW, 2002. The mechanism and regulation of chromosomal V(D)J recombination. *Cell*;109 Suppl:S45-55.

Bayik D, Bartels CF, Lovrenert K, Watson DC, Zhang D, Kay K, Lee J, Lauko A, Johnson S, Lo A, Silver DJ, McGraw M, Grabowski M, Mohammadi AM, Veglia F, Fan Y, Vogelbaum MA, Scacheri P, Lathia JD, 2022. Distinct cell adhesion signature defines glioblastoma myeloid-derived suppressor cell subsets. *Cancer Res*;82(22):4274-87. Erratum in: *Cancer Res*;83(10):1757.

Becknell B, Caligiuri MA, 2005. Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Adv Immunol*;86:209-39.

Beier CP, Kumar P, Meyer K, Leukel P, Bruttel V, Aschenbrenner I, Riemenschneider MJ, Fragoulis A, Rümmele P, Lamszus K, Schulz JB, Weis J, Bogdahn U, Wischhusen J, Hau P, Spang R, Beier D, 2012. The cancer stem cell subtype determines immune infiltration of glioblastoma. *Stem Cells Dev*;21(15):2753-61.

Belcaid Z, Phallen JA, Zeng J, See AP, Mathios D, Gottschalk C, Nicholas S, Kellett M, Ruzevick J, Jackson C, Albesiano E, Durham NM, Ye X, Tran PT, Tyler B, Wong JW, Brem H, Pardoll DM, Drake CG, Lim M, 2014. Focal radiation therapy combined with 4-1BB activation and CTLA-4 blockade yields long-term survival and a protective antigen-specific memory response in a murine glioma model. *PLoS One*;9(7):e101764. DOI: 10.1371/journal.pone.0101764. Epub 2014 Jul 11.

Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD, 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*;27(1):20-1.

Benz C, Conley MR, Kent DG, Wohrer S, Cortes A, Aghaeepour N, Ma E, Mader H, Rowe K, Day C, Treloar D, Brinkman RM, Eaves CJ, 2012. Haematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell*;10(3):273-83.

Berghoff AS, Kiesel B, Widhalm G, Rajky O, Ricken G, Wöhrer A, Dieckmann K, Filipits M, Brandstetter A, Weller M, Kurscheid S, Hegi ME, Zielinski CC, Marosi C, Hainfellner JA, Preusser M, Wick W, 2015. Programmed death ligand 1 expression and tumor-infiltrating lymphocytes in glioblastoma. *Neuro Oncol*;17(8):1064-75.

Bernard A, Lamy L, Alberti I, 2002. The two-signal model of T-cell activation after 30 years. *Transplantation*;73(1 Suppl):S31-S35.

Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK, 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*;441(7090):235-8.

Bettini M, Szymczak-Workman AL, Forbes K, Castellaw AH, Selby M, Pan X, Drake CG, Korman AJ, Vignali DA, 2011. Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. *J Immunol*;187(7):3493-8.

Beutler B, 2004. Innate immunity: an overview. *Mol Immunol*;40(12):845-859.

Bi S, Earl LA, Jacobs L, Baum LG, 2008. Structural features of galectin-9 and galectin-1 that determine distinct T cell death pathways. *J Biol Chem*;283(18):12248-58.

Bi S, Hong PW, Lee B, Baum LG, 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proc Natl Acad Sci U S A*;108(26):10650-5.

Binh NH, Satoh K, Kobayashi K, Takamatsu M, Hatano Y, Hirata A, Tomita H, Kuno T, Hara A, 2013. Galectin-3 in preneoplastic lesions of glioma. *J Neurooncol*;111(2):123-32.

Björkström NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, Björklund AT, Flodström-Tullberg M, Michaëlsson J, Rottenberg ME, Guzmán CA, Ljunggren HG, Malmberg KJ, 2010. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood*;116(19):3853-64.

Bogie JF, Timmermans S, Huynh-Thu VA, Irrthum A, Smeets HJ, Gustafsson JÅ, Steffensen KR, Mulder M, Stinissen P, Hellings N, Hendriks JJ, 2012. Myelin-derived lipids modulate macrophage activity by liver X receptor activation. *PLoS One*;7(9):e44998. DOI: 10.1371/journal.pone.0044998. Epub 2012 Sep 12.

Bogie JF, Jorissen W, Mailleux J, Nijland PG, Zelcer N, Vanmierlo T, Van Horssen J, Stinissen P, Hellings N, Hendriks JJ, 2013. Myelin alters the inflammatory phenotype of macrophages by activating PPARs. *Acta Neuropathol Commun*;1:43. DOI: 10.1186/2051-5960-1-43. Epub 2013 Aug 2.

Bondy ML, Scheurer ME, Malmer B, Barnholtz-Sloan JS, Davis FG, Il'yasova D, Kruchko C, McCarthy BJ, Rajaraman P, Schwartzbaum JA, Sadetzki S, Schlehofer B, Tihan T, Wiemels JL, Wrensch M, Buffler PA; Brain Tumor Epidemiology Consortium, 2008. Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium. *Cancer*;113(7 Suppl):1953-68.

Bowman RL, Klemm F, Akkari L, Pyonteck SM, Sevenich L, Quail DF, Dhara S, Simpson K, Gardner EE, Iacobuzio-Donahue CA, Brennan CW, Tabar V, Gutin PH, Joyce JA, 2016. Macrophage ontogeny underlies differences in tumor-specific education in brain malignancies. *Cell Rep*;17(9):2445-59.

Bräuner EV, Andersen ZJ, Andersen CE, Pedersen C, Gravesen P, Ulbak K, Hertel O, Loft S, Raaschou-Nielsen O, 2013. Residential radon and brain tumour incidence in a Danish cohort. *PLoS One*;8(9):e74435. DOI: 10.1371/journal.pone.0074435. Epub 2013 Sep 16.

Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Förster R, 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med*;192(11):1545-52.

Brennan CW, Verhaak RG, McKenna A, Campos B, Nounshmehr H, Salama SR, Zheng S, Chakravarty D, Sanborn JZ, Berman SH, Beroukhi R, Bernard B, Wu CJ, Genovese G, Shmulevich I, Barnholtz-Sloan J, Zou L, Vegesna R, Shukla SA, Ciriello G, Yung WK, Zhang W, Sougnez C, Mikkelsen T, Aldape K, Bigner DD, Van Meir EG, Prados M, Sloan A, Black KL, Eschbacher J, Finocchiaro G, Friedman W, Andrews DW, Guha A, Iacocca M, O'Neill BP, Foltz G, Myers J, Weisenberger DJ, Penny R, Kucherlapati R, Perou CM, Hayes DN, Gibbs R, Marra M, Mills GB, Lander E, Spellman P, Wilson R, Sander C, Weinstein J, Meyerson M, Gabriel S, Laird PW, Haussler D, Getz G, Chin L; TCGA Research Network, 2013. The somatic genomic landscape of glioblastoma. *Cell*;155(2):462-77. Erratum in: *Cell*;157(3):753.

Brenner AV, Linet MS, Fine HA, Shapiro WR, Selker RG, Black PM, Inskip PD, 2002. History of allergies and autoimmune diseases and risk of brain tumors in adults. *Int J Cancer*;99(2):252-9.

Brenner AV, Butler MA, Wang SS, Ruder AM, Rothman N, Schulte PA, Chanock SJ, Fine HA, Linet MS, Inskip PD, 2007. Single-nucleotide polymorphisms in selected cytokine genes and risk of adult glioma. *Carcinogenesis*;28(12):2543-7.

Bresalier RS, Yan PS, Byrd JC, Lotan R, Raz A, 1997. Expression of the endogenous galactose-binding protein galectin-3 correlates with the malignant potential of tumors in the central nervous system. *Cancer*;80(4):776-87.

Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, Ostberg JR, Blanchard MS, Kilpatrick J, Simpson J, Kurien A, Priceman SJ, Wang X, Harshbarger TL, D'Apuzzo M, Ressler JA, Jensen MC, Barish ME, Chen M, Portnow J, Forman SJ, Badie B, 2016. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. *N Engl J Med*;375(26):2561-9.

Burch JD, Craib KJ, Choi BC, Miller AB, Risch HA, Howe GR, 1987. An exploratory case-control study of brain tumors in adults. *J Natl Cancer Inst*;78(4):601-9.

Burmeister Y, Lischke T, Dahler AC, Mages HW, Lam KP, Coyle AJ, Kroczeck RA, Hutloff A, 2008. ICOS controls the pool size of effector-memory and regulatory T cells. *J Immunol*;180(2):774-82.

Caberoy NB, Alvarado G, Bigcas JL, Li W, 2012. Galectin-3 is a new MerTK-specific eat-me signal. *J Cell Physiol*;227(2):401-7.

Camby I, Belot N, Rorive S, Lefranc F, Maurage CA, Lahm H, Kaltner H, Hadari Y, Ruchoux MM, Brothi J, Zick Y, Salmon I, Gabius HJ, Kiss R, 2001. Galectins are differentially expressed in supratentorial pilocytic astrocytomas, astrocytomas, anaplastic astrocytomas and glioblastomas, and significantly modulate tumor astrocyte migration. *Brain Pathol*;11(1):12-26.

Camby I, Le Mercier M, Lefranc F, Kiss R, 2006. Galectin-1: a small protein with major functions. *Glycobiol*;16(11):137R-157R.

Carsetti R, Köhler G, Lamers MC, 1995. Transitional B cells are the target of negative selection in the B cell compartment. *J Exp Med*;181(6):2129-40.

Campion CG, Labrie M, Lavoie G, St-Pierre Y, 2013. Expression of galectin-7 is induced in breast cancer cells by mutant p53. *PLoS One*;8(8):e72468. DOI: 10.1371/journal.pone.0072468. Epub 2013 Aug 14.

Caza T, Landas S, 2015. Functional and phenotypic plasticity of CD4(+) T cell subsets. *Biomed Res Int*;2015:521957. DOI: 10.1155/2015/521957. Epub 2015 Oct 25.

Cedeno-Laurent F, Trujillo JR, 2011. Gliomas and brain lymphomas in HIV-1/AIDS patients: reflections from a 20-year follow up in Mexico and Brazil. *Microbiol Res*;2(1):e11. DOI: 10.4081/mr.2011.e11. Epub 2011 Jun.

Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N, 2012. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*;2(5):401-4. Erratum in: *Cancer Discov*;2(10):960. Database available at: <http://cbiportal.org>

Chai E, Zhang L, Li C, 2019. LOX-1+ PMN-MDSC enhances immune suppression which promotes glioblastoma multiforme progression. *Cancer Manag Res*;11:7307-15.

Chakraborty A, Staudinger C, King SL, Erickson FC, Lau LS, Bernasconi A, Luscinskas FW, Perlyn C, Dimitroff CJ, 2020. Galectin-9 bridges human B cells to vascular endothelium while programming regulatory pathways. *J Autoimmun*;117:102575. DOI: 10.1016/j.jaut.2020.102575. Epub 2020 Dec 4.

Chambless LB, Parker SL, Hassam-Malani L, McGirt MJ, Thompson RC, 2012. Type 2 diabetes mellitus and obesity are independent risk factors for poor outcome in patients with high-grade glioma. *J Neurooncol*;106(2):383-9.

Chang AL, Miska J, Wainwright DA, Dey M, Rivetta CV, Yu D, Kanojia D, Pituch KC, Qiao J, Pytel P, Han Y, Wu M, Zhang L, Horbinski CM, Ahmed AU, Lesniak MS, 2016. CCL2 produced by the glioma microenvironment is essential for the recruitment of regulatory T cells and myeloid-derived suppressor cells. *Cancer Res*;76(19):5671-82.

Chávez-Galán L, Olleros ML, Vesin D, Garcia I, 2015. Much more than M1 and M2 macrophages, there are also CD169(+) and TCR(+) macrophages. *Front Immunol*;6:263. DOI: 10.3389/fimmu.2015.00263. Epub 2015 May 26.

Chazaud B, 2014. Macrophages: supportive cells for tissue repair and regeneration. *Immunobiology*;219(3):172-8.

Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL, 2004. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol*;173(2):945-54.

Chen L, 2004. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol*;4(5):336-347.

Chen HY, Fermin A, Vardhana S, Weng IC, Lo KF, Chang EY, Maverakis E, Yang RY, Hsu DK, Dustin ML, Liu FT, 2009. Galectin-3 negatively regulates TCR-mediated CD4+ T-cell activation at the immunological synapse. *Proc Natl Acad Sci U S A*;106(34):14496-501.

Chen HJ, Zheng ZC, Yuan BQ, Liu Z, Jing J, Wang SS, 2012. The effect of galectin-3 genetic variants on the susceptibility and prognosis of gliomas in a Chinese population. *Neurosci Lett*;518(1):1-4.

Chen C, Duckworth CA, Fu B, Pritchard DM, Rhodes JM, Yu LG, 2014. Circulating galectins -2, -4 and -8 in cancer patients make important contributions to the increased circulation of several cytokines and chemokines that promote angiogenesis and metastasis. *Br J Cancer*;110(3):741-52.

Chen TC, Chen CH, Wang CP, Lin PH, Yang TL, Lou PJ, Ko JY, Wu CT, Chang YL, 2017. The immunologic advantage of recurrent nasopharyngeal carcinoma from the viewpoint of Galectin-9/Tim-3-related changes in the tumour microenvironment. *Sci Rep*;7(1):10349. DOI: 10.1038/s41598-017-10386-y. Epub 2017 Sep 4.

Chen Z, Hambardzumyan D, 2018. Immune microenvironment in glioblastoma subtypes. *Front Immunol*;9:1004. DOI: 10.3389/fimmu.2018.01004. Epub 2018 May 8.

Chen Q, Han B, Meng X, Duan C, Yang C, Wu Z, Magafurov D, Zhao S, Safin S, Jiang C, Cai J, 2019. Immunogenomic analysis reveals LGALS1 contributes to the immune heterogeneity and immunosuppression in glioma. *Int J Cancer*;145(2):517-30.

Cheung LC, Strickland DH, Howlett M, Ford J, Charles AK, Lyons KM, Brigstock DR, Goldschmeding R, Cole CH, Alexander WS, Kees UR, 2014. Connective tissue growth factor is expressed in bone marrow stromal cells and promotes interleukin-7-dependent B lymphopoiesis. *Haematologica*;99(7):1149-56.

Chi X, Li Y, Qiu X, 2020. V(D)J recombination, somatic hypermutation and class switch recombination of immunoglobulins: mechanism and regulation. *Immunology*;160(3):233-47.

Chiao EY, Coghill A, Kizub D, Fink V, Ndlovu N, Mazul A, Sigel K, 2021. The effect of non-AIDS-defining cancers on people living with HIV. *Lancet Oncol.*;22(6):e240-e253. DOI: 10.1016/S1470-2045(21)00137-6. Epub 2021 Jun.

Chiocca EA, Yu JS, Lukas RV, Solomon IH, Ligon KL, Nakashima H, Triggs DA, Reardon DA, Wen P, Stopa BM, Naik A, Rudnick J, Hu JL, Kumthekar P, Yamini B, Buck JY, Demars N, Barrett JA, Gelb AB, Zhou J, Lebel F, Cooper LJN, 2019. Regulatable interleukin-12 gene therapy in patients with recurrent high-grade glioma: Results of a phase 1 trial. *Sci Transl Med*;11(505):eaaw5680. DOI: 10.1126/scitranslmed.aaw5680. Epub 2019 Aug 14.

Chongsathidkiet P, Jackson C, Koyama S, Loebel F, Cui X, Farber SH, Woroniecka K, Elsamadicy AA, Dechant CA, Kemeny HR, Sanchez-Perez L, Cheema TA, Souders NC, Herndon JE, Coumans JV, Everitt JI, Nahed BV, Sampson JH, Gunn MD, Martuza RL, Dranoff G, Curry WT, Fecci PE, 2018. Sequestration of T cells in bone marrow in the setting of glioblastoma and other intracranial tumors. *Nat Med*;24(9):1459-68.

Chou SY, Yen SL, Huang CC, Huang EY, 2018. Galectin-1 is a poor prognostic factor in patients with glioblastoma multiforme after radiotherapy. *BMC Cancer*;18(1):105. DOI: 10.1186/s12885-018-4025-2. Epub 2018 Jan 30.

Choy W, Lagman C, Lee SJ, Bui TT, Safaee M, Yang I, 2016. Impact of human immunodeficiency virus in the pathogenesis and outcome of patients with glioblastoma multiforme. *Brain Tumor Res Treat*;4(2):77-86.

Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, Mackay CR, 2004. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol*;173(1):68-78.

Clayton KL, Haaland MS, Douglas-Vail MB, Mujib S, Chew GM, Ndhlovu LC, Ostrowski MA, 2014. T cell Ig and mucin domain-containing protein 3 is recruited to the immune synapse, disrupts stable synapse formation, and associates with receptor phosphatases. *J Immunol*;192(2):782-91.

Cloughesy TF, Mochizuki AY, Orpilla JR, Hugo W, Lee AH, Davidson TB, Wang AC, Ellingson BM, Rytlewski JA, Sanders CM, Kawaguchi ES, Du L, Li G, Yong WH, Gaffey SC, Cohen AL, Mellinghoff IK, Lee EQ, Reardon DA, O'Brien BJ, Butowski NA, Nghiemphu PL, Clarke JL, Arrillaga-Romany IC, Colman H, Kaley TJ, de Groot JF, Liao LM, Wen PY, Prins RM, 2019. Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med*;25(3):477-86.

Coghill AE, Engels EA, Schymura MJ, Mahale P, Shiels MS, 2018. Risk of breast, prostate, and colorectal cancer diagnoses among HIV-infected individuals in the United States. *J Natl Cancer Inst*;110(9):959-66.

Cohen MH, Shen YL, Keegan P, Pazdur R, 2009. FDA drug approval summary: bevacizumab (Avastin) as treatment of recurrent glioblastoma multiforme. *Oncologist*;14(11):1131-8.

Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, Carson WE, Caligiuri MA, 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood*;97(10):3146-51.

Costa BM, Caeiro C, Guimarães I, Martinho O, Jaraquemada T, Augusto I, Castro L, Osório L, Linhares P, Honavar M, Resende M, Braga F, Silva A, Pardal F, Amorim J, Nabiço R, Almeida R, Alegria C, Pires M, Pinheiro C, Carvalho E, Lopes JM, Costa P, Damasceno M, Reis RM, 2010. Prognostic value of MGMT promoter methylation in glioblastoma patients treated with temozolomide-based chemoradiation: a Portuguese multicentre study. *Oncol Rep*;23(6):1655-62.

Cote DJ, Samanic CM, Smith TR, Wang M, Smith-Warner SA, Stampfer MJ, Egan KM, 2021. Alcohol intake and risk of glioma: results from three prospective cohort studies. *Eur J Epidemiol*;36(9):965-74.

Croft M, Carter L, Swain SL, Dutton RW, 1994. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med*;180(5):1715-28.

Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, Jais JP, D'Cruz D, Casanova JL, Trouillet C, Geissmann F, 2010. Human CD14^{dim} monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity*;33(3):375-86.

Cserr HF, Harling-Berg CJ, Knopf PM, 1992. Drainage of brain extracellular fluid into blood and deep cervical lymph and its immunological significance. *Brain Pathol*;2(4):269-76.

Cui Y, Hill AW, 2016. Atopy and specific cancer sites: a review of epidemiological studies. *Clin Rev Allergy Immunol*;51(3):338-52.

Currie S, Urich H, 1974. Concurrence of multiple sclerosis and glioma. *J Neurol Neurosurg Psychiatry*;37(5):598-605.

Dalmaso AP, Martinez C, Sjodin K, Good RA, 1963. Studies on the role of the thymus in immunobiology: reconstitution of immunologic capacity in mice thymectomized at birth. *J Exp Med*;118(6):1089-109.

Danella Polli C, Alves Toledo K, Franco LH, Sammartino Mariano V, de Oliveira LL, Soares Bernardes E, Roque-Barreira MC, Pereira-da-Silva G, 2013. Monocyte migration driven by galectin-3 occurs through distinct mechanisms involving selective interactions with the extracellular matrix. *ISRN Inflamm*;2013:259256. DOI: 10.1155/2013/259256. Epub 2013 Feb 25.

Davis F, Il'yasova D, Rankin K, McCarthy B, Bigner DD, 2011. Medical diagnostic radiation exposures and risk of gliomas. *Radiat Res*;175(6):790-6.

Debray C, Vereecken P, Belot N, Teillard P, Brion JP, Pandolfo M, Pochet R, 2004. Multifaceted role of galectin-3 on human glioblastoma cell motility. *Biochem Biophys Res Commun*;325(4):1393-8.

DeKruyff RH, Bu X, Ballesteros A, Santiago C, Chim YL, Lee HH, Karisola P, Pichavant M, Kaplan GG, Umetsu DT, Freeman GJ, Casasnovas JM, 2010. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. *J Immunol*;184(4):1918-30.

de la Rosa M, Rutz S, Dorninger H, Scheffold A, 2004. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol*;34(9):2480-8.

Demotte N, Stroobant V, Courtoy PJ, Van Der Smissen P, Colau D, Luescher IF, Hivroz C, Nicaise J, Squifflet JL, Mourad M, Godelaine D, Boon T, van der Bruggen P, 2008. Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. *Immunity*;28(3):414-24.

Demotte N, Wieërs G, Van Der Smissen P, Moser M, Schmidt C, Thielemans K, Squifflet JL, Weynand B, Carrasco J, Lurquin C, Courtoy PJ, van der Bruggen P, 2010. A galectin-3 ligand corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. *Cancer Res*;70(19):7476-88.

de Vrij J, Maas SL, Kwappenberg KM, Schnoor R, Kleijn A, Dekker L, Luider TM, de Witte LD, Litjens M, van Strien ME, Hol EM, Kroonen J, Robe PA, Lamfers ML, Schilham MW, Broekman ML, 2015. Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells. *Int J Cancer*;137(7):1630-42.

Deltour I, Auvinen A, Feychting M, Johansen C, Klaeboe L, Sankila R, Schüz J, 2012. Mobile phone use and incidence of glioma in the Nordic countries 1979-2008: consistency check. *Epidemiology*;23(2):301-7.

den Braber I, Mugwagwa T, Vrisekoop N, Westera L, Mögling R, de Boer AB, Willems N, Schrijver EH, Spierenburg G, Gaiser K, Mul E, Otto SA, Ruiter AF, Ackermans MT, Miedema F, Borghans JA, de Boer RJ, Tesselaar K, 2012. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. *Immunity*;36(2):288-97.

Detry O, Honoré P, Hans MF, Delbouille MH, Jacquet N, Meurisse M, 2000. Organ donors with primary central nervous system tumor. *Transplantation*;70(1):244-8; discussion 251-2.

Dias-Baruffi M, Zhu H, Cho M, Karmakar S, McEver RP, Cummings RD, 2003. Dimeric galectin-1 induces surface exposure of phosphatidylserine and phagocytic recognition of leukocytes without inducing apoptosis. *J Biol Chem*;278(42):41282-93.

Disney-Hogg L, Cornish AJ, Sud A, Law PJ, Kinnersley B, Jacobs DI, Ostrom QT, Labreche K, Eckel-Passow JE, Armstrong GN, Claus EB, Il'yasova D, Schildkraut J, Barnholtz-Sloan JS, Olson SH, Bernstein JL, Lai RK, Schoemaker MJ, Simon M, Hoffmann P, Nöthen MM, Jöckel KH, Chanock S, Rajaraman P, Johansen C, Jenkins RB, Melin BS, Wrensch MR, Sanson M, Bondy ML, Houlston RS, 2018. Impact of atopy on risk of glioma: a Mendelian randomisation study. *BMC Med*;16(1):42. DOI: 10.1186/s12916-018-1027-5. Epub 2018 Mar 15.

Dix AR, Brooks WH, Roszman TL, Morford LA, 1999. Immune defects observed in patients with primary malignant brain tumors. *J Neuroimmunol*;100(1-2):216-32.

Do JS, Visperas A, Sanogo YO, Bechtel JJ, Dvorina N, Kim S, Jang E, Stohlman SA, Shen B, Fairchild RL, Baldwin WM III, Vignali DA, Min B, 2016. An IL-27/Lag3 axis enhances Foxp3⁺ regulatory T cell-suppressive function and therapeutic efficacy. *Mucosal Immunol*;9(1):137-45.

Dogra P, Rancan C, Ma W, Toth M, Senda T, Carpenter DJ, Kubota M, Matsumoto R, Thapa P, Szabo PA, Li Poon MM, Li J, Arakawa-Hoyt J, Shen Y, Fong L, Lanier LL, Farber DL, 2020. Tissue determinants of human NK cell development, function, and residence. *Cell*;180(4):749-763.e13. DOI: 10.1016/j.cell.2020.01.022. Epub 2020 Feb 13.

Dong H, Zhu G, Tamada K, Chen L, 1999. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med*;5(12):1365-9.

Douagi I, Colucci F, Di Santo JP, Cumano A, 2002. Identification of the earliest prethymic bipotent T/NK progenitor in murine fetal liver. *Blood*;99(2):463-71.

Douglass AD, Vale RD, 2005. Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. *Cell*;121(6):937-50.

Dubinski D, Wölfer J, Hasselblatt M, Schneider-Hohendorf T, Bogdahn U, Stummer W, Wiendl H, Grauer OM, 2016. CD4+ T effector memory cell dysfunction is associated with the accumulation of granulocytic myeloid-derived suppressor cells in glioblastoma patients. *Neuro Oncol*;18(6):807-18.

Dubois LG, Campanati L, Righy C, D'Andrea-Meira I, Spohr TC, Porto-Carreiro I, Pereira CM, Balça-Silva J, Kahn SA, DosSantos MF, Oliveira Mde A, Ximenes-da-Silva A, Lopes MC, Faveret E, Gasparetto EL, Moura-Neto V, 2014. Gliomas and the vascular fragility of the blood brain barrier. *Front Cell Neurosci*;8:418. DOI: 10.3389/fncel.2014.00418. Epub 2014 Dec 12.

Durgin JS, Henderson F Jr, Nasrallah MP, Mohan S, Wang S, Lacey SF, Melenhorst JJ, Desai AS, Lee JYK, Maus MV, June CH, Brem S, O'Connor RS, Binder Z, O'Rourke DM, 2021. Case report: prolonged survival following EGFRvIII CAR T cell treatment for recurrent glioblastoma. *Front Oncol*;11:669071. DOI: 10.3389/fonc.2021.669071. Epub 2021 May 7.

Ebert RH, Florey HW, 1939. The extravascular development of the monocyte observed in vivo. *Br J Exp Pathol*;20(4):342-56.

Efird JT, Friedman GD, Sidney S, Klatsky A, Habel LA, Udaltsova NV, Van den Eeden S, Nelson LM, 2004. The risk for malignant primary adult-onset glioma in a large, multiethnic, managed-care cohort: cigarette smoking and other lifestyle behaviors. *J Neurooncol*;68(1):57-69.

Engelhardt B, Ransohoff RM, 2012. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. *Trends Immunol*;33(12):579-89.

Enciso-Mora V, Hosking FJ, Di Stefano AL, Zelenika D, Shete S, Broderick P, Idbaih A, Delattre JY, Hoang-Xuan K, Marie Y, Labussière M, Alentorn A, Ciccarino P, Rossetto M, Armstrong G, Liu Y, Gousias K, Schramm J, Lau C, Hepworth SJ, Schoemaker M, Strauch K, Müller-Nurasyid M, Schreiber S, Franke A, Moebus S, Eisele L, Swerdlow A, Simon M, Bondy M, Lathrop M, Sanson M, Houlston RS, 2013. Low penetrance susceptibility to glioma is caused by the TP53 variant rs78378222. *Br J Cancer*;108(10):2178-85.

Erhart F, Buchroithner J, Reitermaier R, Fischhuber K, Klingenbrunner S, Sloma I, Hibsh D, Kozol R, Efroni S, Ricken G, Wöhrer A, Haberler C, Hainfellner J, Kruppl G, Felzmann T, Dohnal AM, Marosi C, Visus C, 2018. Immunological analysis of phase II glioblastoma dendritic cell vaccine (Audencel) trial: immune system characteristics influence outcome and Audencel up-regulates Th1-related immunovariabiles. *Acta Neuropathol Commun*;6(1):135. DOI: 10.1186/s40478-018-0621-2. Epub 2018 Dec 5.

Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG, 2000. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med*;343(19):1350-4. Erratum in: *N Engl J Med*;343(23):1740.

Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, Cianfarani F, Odorisio T, Traidl-Hoffmann C, Behrendt H, Durham SR, Schmidt-Weber CB, Cavani A, 2009. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest*;119(12):3573-85.

Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM, 2000. A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature*;405(6782):85-90.

Fadul CE, Fisher JL, Gui J, Hampton TH, Côté AL, Ernstoff MS, 2011. Immune modulation effects of concomitant temozolomide and radiation therapy on peripheral blood mononuclear cells in patients with glioblastoma multiforme. *Neuro Oncol*;13(4):393-400. DOI: 10.1093/neuonc/noq204. Epub 2011 Feb 20.

Farber DL, Yudanin NA, Restifo NP, 2014. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol*;14(1):24-35.

Feuk-Lagerstedt E, Jordan ET, Leffler H, Dahlgren C, Karlsson A, 1999. Identification of CD66a and CD66b as the major galectin-3 receptor candidates in human neutrophils. *J Immunol*;163(10):5592-8.

Fife BT, Guleria I, Gubbels Bupp M, Eagar TN, Tang Q, Bour-Jordan H, Yagita H, Azuma M, Sayegh MH, Bluestone JA, 2006. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. *J Exp Med*;203(12):2737-47.

Fife BT, Pauken KE, Eagar TN, Obu T, Wu J, Tang Q, Azuma M, Krummel MF, Bluestone JA, 2009. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nat Immunol*;10(11):1185-92.

Figuerola JM, Carter BS, 2018. Detection of glioblastoma in biofluids. *J Neurosurg*;129(2):334-40.

Filipp D, Ballek O, Manning J, 2012. Lck, membrane microdomains, and TCR triggering machinery: defining the new rules of engagement. *Front Immunol*;3:155. DOI: 10.3389/fimmu.2012.00155. Epub 2012 Jun 12.

Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR, Cumano A, Geissmann F, 2006. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science*;311(5757):83-7.

Fong B, Jin R, Wang X, Safaee M, Lisiero DN, Yang I, Li G, Liao LM, Prins RM, 2012. Monitoring of regulatory T cell frequencies and expression of CTLA-4 on T cells, before and after DC vaccination, can predict survival in GBM patients. *PLoS One*;7(4):e32614. DOI: 10.1371/journal.pone.0032614. Epub 2012 Apr 2.

Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T, 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*;192(7):1027-34.

French H, Fontes-Villalba A, Maharaj M, Naidoo CSY, Bhatia K, Paterson A, Cook R, Parratt J, 2022. Tumefactive multiple sclerosis versus high grade glioma: A diagnostic dilemma. *Surg Neurol Int*;13:146. DOI: 10.25259/SNI_239_2022. Epub 2022 Apr 15.

Friebel E, Kapolou K, Unger S, Núñez NG, Utz S, Rushing EJ, Regli L, Weller M, Greter M, Tugues S, Neidert MC, Becher B, 2020. Single-cell mapping of human brain cancer reveals tumor-specific instruction of tissue-invading leukocytes. *Cell*;181(7):1626-1642.e20.

Friedberg MH, Glantz MJ, Klempner MS, Cole BF, Perides G, 1998. Specific matrix metalloproteinase profiles in the cerebrospinal fluid correlated with the presence of malignant astrocytomas, brain metastases, and carcinomatous meningitis. *Cancer*;82(5):923-30.

Fu W, Wang W, Li H, Jiao Y, Huo R, Yan Z, Wang J, Wang S, Wang J, Chen D, Cao Y, Zhao J, 2020. Single-cell atlas reveals complexity of the immunosuppressive microenvironment of initial and recurrent glioblastoma. *Front Immunol*;11:835. DOI: 10.3389/fimmu.2020.00835. Epub 2020 May 7.

Fuleihan R, Ramesh N, Loh R, Jabara H, Rosen RS, Chatila T, Fu SM, Stamenkovich I, Geha RS, 1993. Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc Natl Acad Sci USA*;90(6):2170-3.

Furlan G, Minowa T, Hanagata N, Kataoka-Hamai C, Kaizuka Y, 2014. Phosphatase CD45 both positively and negatively regulates T cell receptor phosphorylation in reconstituted membrane protein clusters. *J Biol Chem*;289(41):28514-25.

Gabrilovich DI, Bronte V, Chen SH, Colombo MP, Ochoa A, Ostrand-Rosenberg S, Schreiber H, 2007. The terminology issue for myeloid-derived suppressor cells. *Cancer Res*;67(1):425; author reply 426. DOI: 10.1158/0008-5472.CAN-06-3037.

Gabrusiewicz K, Li X, Wei J, Hashimoto Y, Marisetty AL, Ott M, Wang F, Hawke D, Yu J, Healy LM, Hossain A, Akers JC, Maiti SN, Yamashita S, Shimizu Y, Dunner K, Zal MA, Burks JK, Gumin J, Nwajei F, Rezavanian A, Zhou S, Rao G, Sawaya R, Fuller GN, Huse JT, Antel JP, Li S, Cooper L, Sulman EP, Chen C, Geula C, Kalluri R, Zal T, Heimberger AB, 2018. Glioblastoma stem cell-derived exosomes induce M2 macrophages and PD-L1 expression on human monocytes. *Oncoimmunolog*;7(4):e1412909. DOI: 10.1080/2162402X.2017.1412909. Epub 2018 Jan 16.

Galy A, Travis M, Cen D, Chen B, 1995. Human T, B, natural killer and dendritic cells arise from a common bone marrow progenitor subset. *Immunity*;3(4):459-73.

Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N, 2013. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*;6(269):pl1. Database available at: <http://cbiportal.org>

Garín MI, Chu CC, Golshayan D, Cernuda-Morollón E, Wait R, Lechler RI, 2007. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood*;109(5):2058-65.

Gascoigne NR, Casas J, Brzostek J, Rybakina V, 2011. Initiation of TCR phosphorylation and signal transduction. *Front Immunol*;2:72. DOI: 10.3389/fimmu.2011.00072. Epub 2011 Dec 7.

Geginat J, Paroni M, Maglie S, Alfen JS, Kastirr I, Gruarin P, De Simone M, Pagani M, Abrignani S, 2014. Plasticity of human CD4 T cell subsets. *Front Immunol*;5:630. Epub 2014 Dec 16. DOI 10.3389/fimmu.2014.00630.

Genoud V, Marinari E, Nikolaev SI, Castle JC, Bukur V, Dietrich PY, Okada H, Walker PR, 2018. Responsiveness to anti-PD-1 and anti-CTLA-4 immune checkpoint blockade in SB28 and GL261 mouse glioma models. *Oncoimmunology*;7(12):e1501137. DOI: 10.1080/2162402X.2018.1501137. Epub 2018 Sep 5.

Ghosh S, Huang J, Inkman M, Zhang J, Thotala S, Tikhonova E, Mihecheva N, Frenkel F, Ataullakhanov R, Wang X, DeNardo D, Hallahan D, Thotala D, 2023a. Radiation-induced circulating myeloid-derived suppressor cells induce systemic lymphopenia after chemoradiotherapy in patients with glioblastoma. *Sci Transl Med*;15(680):eabn6758. DOI: 10.1126/scitranslmed.abn6758. Epub 2023 Jan 25.

Ghosh S, Johanns TM, Chheda MG, Liu E, Butt O, Abraham C, Badiyan S, Huang Y, DeNardo D, Kim AH, Hallahan D, Thotala D, Huang J, 2023b. A pilot phase Ib study to evaluate tadalafil to overcome immunosuppression during chemoradiotherapy for IDH-wild-type glioblastoma. *Neurooncol Adv*;5(1):vdad088. DOI: 10.1093/noajnl/vdad088. Epub 2023 Jul 19.

Gieseke F, Kruchen A, Tzaribachev N, Bentzien F, Dominici M, Müller I, 2013. Proinflammatory stimuli induce galectin-9 in human mesenchymal stromal cells to suppress T-cell proliferation. *Eur J Immunol*;43(10):2741-9.

Gil CD, Gullo CE, Olini SM, 2010. Effect of exogenous galectin-1 on leukocyte migration: modulation of cytokine levels and adhesion molecules. *Int J Clin Exp Pathol*;4(1):74-84.

Gill BJ, Pisapia DJ, Malone HR, Goldstein H, Lei L, Sonabend A, Yun J, Samanamud J, Sims JS, Banu M, Dovas A, Teich AF, Sheth SA, McKhann GM, Sisti MB, Bruce JN, Sims PA, Canoll P, 2014. MRI-localized biopsies reveal subtype-specific differences in molecular and cellular composition at the margins of glioblastoma. *Proc Natl Acad Sci U S A*;111(34):12550-5.

Gillespie M, Jassal B, Stephan R, Milacic M, Rothfels K, Senff-Ribeiro A, Griss J, Sevilla C, Matthews L, Gong C, Deng C, Varusai T, Ragueneau E, Haider Y, May B, Shamovsky V, Weiser J, Brunson T, Sanati N, Beckman L, Shao X, Fabregat A, Sidiropoulos K, Murillo J, Viteri G, Cook J, Shorser S, Bader G, Demir E, Sander C, Haw R, Wu G, Stein L, Hermjakob H, D'Eustachio P, 2022. The reactome pathway knowledgebase 2022. *Nucleic Acids Res*;50(D1):D687-D692. Database available at <https://reactome.org>

Gilmour KC, Fujii H, Cranston T, Davies EG, Kinnon C, Gaspar HB, 2001. Defective expression of the interleukin-2/interleukin-15 receptor beta subunit leads to a natural killer cell-deficient form of severe combined immunodeficiency. *Blood*;98(3):877-9.

Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M, 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*;330(6005):841-5.

Giovannone N, Smith LK, Treanor B, Dimitroff CJ, 2018. Galectin-glycan interactions as regulators of B cell immunity. *Front Immunol*;9:2839. DOI: 10.3389/fimmu.2018.02839. Epub 2018 Dec 4.

Gkanatsiou O, 2022. Identification of immunosuppressive mechanisms in glioblastoma multiforme brain tumours. Ph.D thesis. Aston University. Available at <https://research.aston.ac.uk/en/studentTheses/identification-of-immunosuppressive-mechanisms-in-glioblastoma-mu>

Goddery EN, Fain CE, Lipovsky CG, Ayasoufi K, Yokanovich LT, Malo CS, Khadka RH, Tritz ZP, Jin F, Hansen MJ, Johnson AJ, 2021. Microglia and perivascular macrophages act as antigen presenting cells to promote CD8 T cell infiltration of the brain. *Front Immunol*;12:726421. DOI: [10.3389/fimmu.2021.726421](https://doi.org/10.3389/fimmu.2021.726421). Epub 2021 Aug 30.

Goldberg SB, Gettinger SN, Mahajan A, Chiang AC, Herbst RS, Sznol M, Tsiouris AJ, Cohen J, Vortmeyer A, Jilaveanu L, Yu J, Hegde U, Speaker S, Madura M, Ralabate A, Rivera A, Rowen E, Gerrish H, Yao X, Chiang V, Kluger HM, 2016. Pembrolizumab for patients with melanoma or non-small-cell lung cancer and untreated brain metastases: early analysis of a non-randomised, open-label, phase 2 trial. *Lancet Oncol*;17(7):976-83.

Goldberg SB, Schalper KA, Gettinger SN, Mahajan A, Herbst RS, Chiang AC, Lilenbaum R, Wilson FH, Omay SB, Yu JB, Jilaveanu L, Tran T, Pavlik K, Rowen E, Gerrish H, Komlo A, Gupta R, Wyatt H, Ribeiro M, Kluger Y, Zhou G, Wei W, Chiang VL, Kluger HM, 2020. Pembrolizumab for management of patients with NSCLC and brain metastases: long-term results and biomarker analysis from a non-randomised, open-label, phase 2 trial. *Lancet Oncol*;21(5):655-63.

Gonzales J, Kumar A, Conrad CA, Levin VA, 2007. Effect of bevacizumab on radiation necrosis of the brain. *Int J Radiat Oncol Bio Phys*;67(2):323-6.

González-Tablas Pimenta M, Otero Á, Arandia Guzman DA, Pascual-Argente D, Ruíz Martín L, Sousa-Casasnovas P, García-Martin A, Roa Montes de Oca JC, Villaseñor-Ledezma J, Torres Carretero L, Almeida M, Ortiz J, Nieto A, Orfao A, Tabernero MD, 2021. Tumor cell and immune cell profiles in primary human glioblastoma: Impact on patient outcome. *Brain Pathol*;31(2):365-80.

Gooden MJ, Wiersma VR, Samplonius DF, Gerssen J, van Ginkel RJ, Nijman HW, Hirashima M, Niki T, Eggleton P, Helfrich W, Bremer E, 2013. Galectin-9 activates and expands human T-helper 1 cells. *PLoS One*;8(5):e65616. DOI: 10.1371/journal.pone.0065616. Epub 2013 May 31.

Goods BA, Hernandez AL, Lowther DE, Lucca LE, Lerner BA, Gunel M, Raddassi K, Coric V, Hafler DA, Love JC, 2017. Functional differences between PD-1+ and PD-1- CD4+ effector T cells in healthy donors and patients with glioblastoma multiforme. *PLoS One*;12(9):e0181538. DOI: 10.1371/journal.pone.0181538 Epub 2017 Sep 7.

Gordon EJ, Gale NW, Harvey NL, 2008. Expression of the hyaluronan receptor LYVE-1 is not restricted to the lymphatic vasculature; LYVE-1 is also expressed on embryonic blood vessels. *Dev Dyn*;237(7):1901-9.

Gordon-Alonso M, Hirsch T, Wildmann C, van der Bruggen P, 2017. Galectin-3 captures interferon-gamma in the tumor matrix reducing chemokine gradient production and T-cell tumor infiltration. *Nat Commun*;8(1):793. DOI: 10.1038/s41467-017-00925-6. Epub 2017 Oct 6.

Gordower L, Decaestecker C, Kacem Y, Lemmers A, Gusman J, Burchert M, Danguy A, Gabius H, Salmon I, Kiss R, Camby I, 1999. Galectin-3 and galectin-3-binding site expression in human adult astrocytic tumours and related angiogenesis. *Neuropathol Appl Neurobiol*;25(4):319-30.

Gousias K, Markou M, Voulgaris S, Goussia A, Voulgari P, Bai M, Polyzoidis K, Kyritsis A, Alamanos Y, 2009. Descriptive epidemiology of cerebral gliomas in northwest Greece and study of potential predisposing factors, 2005-2007. *Neuroepidemiology*;33(2):89-95.

Greenhall GHB, Rous BA, Robb ML, Brown C, Hardman G, Hilton RM, Neuberger JM, Dark JH, Johnson RJ, Forsythe JLR, Tomlinson LA, Callaghan CJ, Watson CJE, 2023. Organ transplants from deceased donors with primary brain tumors and risk of cancer transmission. *JAMA Surg*;158(5):504-13.

Grégoire H, Roncali L, Rousseau A, Chérel M, Delneste Y, Jeannin P, Hindré F, Garcion E, 2020. Targeting tumor associated macrophages to overcome conventional treatment resistance in glioblastoma. *Front Pharmacol*;11:368. DOI: 10.3389/fphar.2020.00368. Epub 2020 Apr 8.

Guo Q, Shen S, Guan G, Zhu C, Zou C, Cao J, Cheng W, Xu X, Yu J, Lin Z, Wang G, Chen L, Cheng P, Wu A, 2022a. Cancer cell intrinsic TIM-3 induces glioblastoma progression. *iScience*;25(11):105329. DOI: 10.1016/j.isci.2022.105329. Epub 2022 Oct 10.

Guo X, Zhang Y, Jiao H, Miao X, 2022b. The prognostic significance of PD-L1 expression in patients with glioblastoma: a meta-analysis. *Front Oncol*;12:925560. DOI: 10.3389/fonc.2022.925560. Epub 2022 Oct 12.

Gustafson MP, Lin Y, New KC, Bulur PA, O'Neill BP, Gastineau DA, Dietz AB, 2010. Systemic immune suppression in glioblastoma: the interplay between CD14+HLA-DRlo/neg monocytes, tumor factors, and dexamethasone. *Neuro Oncol*;12(7):631-44.

Hahn HP, Pang M, He J, Hernandez JD, Yang RY, Li LY, Wang X, Liu FT, Baum LG, 2004. Galectin-1 induces nuclear translocation of endonuclease G in caspase- and cytochrome c-independent T cell death. *Cell Death Differ*;11(12):1277-86.

Hambleton S, Salem S, Bustamante J, Bigley V, Boisson-Dupuis S, Azevedo J, Fortin A, Haniffa M, Ceron-Gutierrez L, Bacon CM, Menon G, Trouillet C, McDonald D, Carey P, Ginhoux F, Alsina L, Zumwalt TJ, Kong XF, Kumararatne D, Butler K, Hubeau M, Feinberg J, Al-Muhsen S, Cant A, Abel L, Chaussabel D, Doffinger R, Talesnik E, Grumach A, Duarte A, Abarca K, Moraes-Vasconcelos D, Burk D, Berghuis A, Geissmann F, Collin M, Casanova JL, Gros P, 2011. IRF8 mutations and human dendritic-cell immunodeficiency. *N Engl J Med*;365(2):127-38.

Hannier S, Tournier M, Bismuth G, Triebel F, 1998. CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. *J Immunol*;161(8):4058-65.

Hannier S, Triebel F, 1999. The MHC class II ligand lymphocyte activation gene-3 is co-distributed with CD8 and CD3-TCR molecules after their engagement by mAb or peptide-MHC class I complexes. *Int Immunol*;11(11):1745-52.

Hara A, Niwa M, Noguchi K, Kanayama T, Niwa A, Matsuo M, Hatano Y, Tomita H, 2020. Galectin-3 as a next-generation biomarker for detecting early stage of various diseases. *Biomolecules*;10(3):389. DOI: 10.3390/biom10030389. Epub 2020 Mar 3.

Harrison PW, Amode MR, Austine-Orimoloye O, Azov AG, Barba M, Barnes I, Becker A, Bennett R, Berry A, Bhai J, Bhurji SK, Boddu S, Branco Lins PR, Brooks L, Ramaraju SB, Campbell LI, Martinez MC, Charkhchi M, Chougule K, Cockburn A, Davidson C, De Silva NH, Dodiya K, Donaldson S, El Houdaigui B, Naboulsi TE, Fatima R, Giron CG, Genez T, Grigoriadis D, Ghattaoraya GS, Martinez JG, Gurbich TA, Hardy M, Hollis Z, Hourlier T, Hunt T, Kay M, Kaykala V, Le T, Lemos D, Lodha D, Marques-Coelho D, Maslen G, Merino GA, Mirabueno LP, Mushtaq A, Hossain SN, Ogeh DN, Sakthivel MP, Parker A, Perry M, Piližota I, Poppleton D, Prosovetskaia I, Raj S, Pérez-Silva JG, Salam AIA, Saraf S, Saraiva-Agostinho N, Sheppard D, Sinha S, Sipos B, Sitnik V, Stark W, Steed E, Suner MM, Surapaneni L, Sutinen K, Tricomi FF, Urbina-Gómez D, Veidenberg A, Walsh TA, Ware D, Wass E, Willhoft NL, Allen J, Alvarez-Jarreta J, Chakiachvili M, Flint B, Giorgetti S, Haggerty L, Ilsley GR, Keatley J, Loveland JE, Moore B, Mudge JM, Naamati G, Tate J, Trevanion SJ, Winterbottom A, Frankish A, Hunt SE, Cunningham F, Dyer S, Finn RD, Martin FJ, Yates AD, 2024. Ensembl 2024. *Nucleic Acids Res*;52(D1):D891-D899. DOI: 10.1093/nar/gkad1049. Available at: <https://www.ensembl.org/index.html>

Harshyne LA, Nasca BJ, Kenyon LC, Andrews DW, Hooper DC, 2016. Serum exosomes and cytokines promote a T-helper cell type 2 environment in the peripheral blood of glioblastoma patients. *Neuro Oncol*;18(2):206-15.

Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, Zheng XX, Strom TB, Hafler DA, Kuchroo VK, 2009. TIM-3 is expressed on activated human CD4⁺ T cells and regulates Th1 and Th17 cytokines. *Eur J Immunol*;39(9):2492-501.

Haynes BF, Searce RM, Lobach DF, Hensley LL, 1984. Phenotypic characterization and ontogeny of mesodermal-derived and endocrine epithelial components of the human thymic microenvironment. *J Exp Med*;159(4):1149-68.

He J, Baum LG, 2006. Endothelial cell expression of galectin-1 induced by prostate cancer cells inhibits T-cell transendothelial migration. *Lab Invest*;86(6):578-90.

He X, Zhang S, Chen J, Li D, 2019. Increased LGALS3 expression independently predicts shorter overall survival in patients with the proneural subtype of glioblastoma. *Cancer Med*;8(5):2031-40.

Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC, Stupp R, 2005. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*;352(10):997-1003.

Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, Yang DS, Sun W, Qiao W, Hiraoka N, Fuller GN, 2008. Incidence and prognostic impact of FoxP3⁺ regulatory T cells in human gliomas. *Clin Cancer Res*;14(16):5166-72.

Himes BT, Geiger PA, Ayasoufi K, Bhargav AG, Brown DA, Parney IF, 2021. Immune suppression in glioblastoma: current understanding and therapeutic implications. *Front Oncol*;11;770561. Epub 2021 Oct 28. DOI: 10.3389/fonc.2021.770561.

Himes BT, Peterson TE, de Mooij T, Garcia LMC, Jung MY, Uhm S, Yan D, Tyson J, Jin-Lee HJ, Parney D, Abukhadra Y, Gustafson MP, Dietz AB, Johnson AJ, Dong H, Maus RL, Markovic S, Lucien F, Parney IF, 2020. The role of extracellular vesicles and PD-L1 in glioblastoma-mediated immunosuppressive monocyte induction. *Neuro Oncol*;22(7):967-78.

Hochberg F, Toniolo P, Cole P, Salcman M, 1990. Nonoccupational risk indicators of glioblastoma in adults. *J Neurooncol*;8(1):55-60. Erratum in: *J Neurooncol*;9(1):90.

Homma T, Fukushima T, Vaccarella S, Yonekawa Y, Di Patre PL, Franceschi S, Ohgaki H, 2006. Correlation among pathology, genotype, and patient outcomes in glioblastoma. *J Neuropathol Exp Neurol*;65(9):846-54.

Huang J, Liu F, Liu Z, Tang H, Wu H, Gong Q, Chen J, 2017. Immune checkpoint in glioblastoma: promising and challenging. *Front Pharmacol*;8:242. DOI: 10.3389/fphar.2017.00242. Epub 2017 May 9.

Huang YH, Zhu C, Kondo Y, Anderson AC, Gandhi A, Russell A, Dougan SK, Petersen BS, Melum E, Pertel T, Clayton KL, Raab M, Chen Q, Beauchemin N, Yazaki PJ, Pyzik M, Ostrowski MA, Glickman JN, Rudd CE, Ploegh HL, Franke A, Petsko GA, Kuchroo VK, Blumberg RS, 2015. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. *Nature*;517(7534):386-90.

Huard B, Prigent P, Pagès F, Bruniquel D, Triebel F, 1996a. T cell major histocompatibility complex class II molecules down-regulate CD4⁺ T cell clone responses following LAG-3 binding. *Eur J Immunol*;26(5):1180-6.

Huard B, Prigent P, Tournier M, Bruniquel D, Triebel F, 1996b. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. *Eur J Immunol*;25(9):2718-21.

Huard B, Mastrangeli R, Prigent P, Bruniquel D, Donini S, El-Tayar N, Maigret B, Dréano M, Triebel F, 1997. Characterization of the major histocompatibility complex class II binding site on LAG-3 protein. *Proc Natl Acad Sci U S A*;94(11):5744-9.

Huber M, Heink S, Grothe H, Guralnik A, Reinhard K, Elflein K, Hünig T, Mittrücker H-W, Brüstle A, Kamradt T, Lohoff, M, 2009. A Th17-like developmental process leads to CD8⁺ Tc17 cells with reduced cytotoxic activity. *Eur J Immunol*;39(7):1716-25.

Huflejt ME, Leffler H, 2004. Galectin-4 in normal tissues and cancer. *Glycoconj J*;20(4):247-55.

Hung AL, Maxwell R, Theodros D, Belcaid Z, Mathios D, Luksik AS, Kim E, Wu A, Xia Y, Garzon-Muvdi T, Jackson C, Ye X, Tyler B, Selby M, Korman A, Barnhart B, Park SM, Youn JI, Chowdhury T, Park CK, Brem H, Pardoll DM, Lim M, 2018. TIGIT and PD-1 dual checkpoint blockade enhances antitumor immunity and survival in GBM. *Oncoimmunology*;7(8):e1466769. DOI: 10.1080/2162402X.2018. Epub 2018 May 24.

Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczeck RA, 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature*;397(6716):263-6.

Ikemori RY, Machado CM, Furuzawa KM, Nonogaki S, Osinaga E, Umezawa K, de Carvalho MA, Verinaud L, Chammas R, 2014. Galectin-3 up-regulation in hypoxic and nutrient deprived microenvironments promotes cell survival. *PLoS One*;9(11):e111592. DOI: 10.1371/journal.pone.0111592. Epub 2014 Nov 4.

Inagaki Y, Sohma Y, Horie H, Nozawa R, Kadoya T, 2000. Oxidized galectin-1 promotes axonal regeneration in peripheral nerves but does not possess lectin properties. *Eur J Biochem*;267(10):2955-64.

Iorgulescu JB, Gokhale PC, Speranza MC, Eschle BK, Poitras MJ, Wilkens MK, Soroko KM, Chhoeu C, Knott A, Gao Y, Lim-Fat MJ, Baker GJ, Bonal DM, Nguyen QD, Grant GRL, Ligon KL, Sorger PK, Chiocca EA, Anderson AC, Kirschmeier PT, Sharpe AH, Freeman GJ, Reardon DA, 2021. Concurrent dexamethasone limits the clinical benefit of immune checkpoint blockade in glioblastoma. *Clin Cancer Res*;27(1):276-87.

Isaguliantis M, Bayurova E, Avdoshina D, Kondrashova A, Chiodi F, Palefsky JM, 2021. Oncogenic effects of HIV-1 proteins, mechanisms behind. *Cancers (Basel)*;13(2):305. DOI: 10.3390/cancers13020305. Epub 2021 Jan 15.

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR, 2006. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*;126(6):1121-33.

Jacob L, de Brito Neto J, Lenck S, Corcy C, Benbelkacem F, Geraldo LH, Xu Y, Thomas JM, El Kamouh MR, Spajer M, Potier MC, Haik S, Kalamarides M, Stankoff B, Lehericy S, Eichmann A, Thomas JL, 2022. Conserved meningeal lymphatic drainage circuits in mice and humans. *J Exp Med*;219(8):e20220035. DOI: 10.1084/jem.20220035. Epub 2022 Jul 1.

Jacquelot N, Tellier J, Nutt SI, Belz Gt, 2021. Tertiary lymphoid structures and B lymphocytes in cancer prognosis and response to immunotherapies. *Oncoimmunology*;10(1):1900508. DOI: 10.1080/2162402X.2021. Epub 2021 Mar 29.

Janes PW, Ley SC, Magee AI, 1999. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J Cell Biol*;147(2):447-61.

Jia W, Kidoya H, Yamakawa D, Naito H, Takakura N, 2013. Galectin-3 accelerates M2 macrophage infiltration and angiogenesis in tumors. *Am J Pathol*;182(5):1821-31.

Jimshelishvili S, Alshareef AT, Papadimitriou K, Bregy A, Shah AH, Graham RM, Ferraro N, Komotar RJ, 2014. Extracranial glioblastoma in transplant recipients. *J Cancer Res Clin Oncol*;140(5):801-7.

Johnson DR, Fogh SE, Giannini C, Kaufmann TJ, Raghunathan A, Theodosopoulos PV, Clarke JL, 2015. Case-based review: newly diagnosed glioblastoma. *Neuro-Oncology Practice*;2(3):106-21.

Jokonya L, Musara A, Esene IN, Kabulo KDM, Kabeya CM, Kalangu KKN, 2018. Prevalence of human immunodeficiency virus infection in brain glioma patients: Is the virus protective from gliomas? *Surg Neurol Int*;9:103. DOI: 10.4103/sni.sni_423_17. Epub 2018 May 24.

Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, Wong JC, Satkunarajah M, Schwenker M, Chapman JM, Gyenes G, Vali B, Hycza MD, Yue FY, Kovacs C, Sassi A, Loutfy M, Halpenny R, Persad D, Spotts G, Hecht FM, Chun TW, McCune JM, Kaul R, Rini JM, Nixon DF, Ostrowski MA, 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med*;205(12):2763-79.

Jung MY, Aibaidula A, Brown DA, Himes BT, Cumba Garcia LM, Parney IF, 2022. Superinduction of immunosuppressive glioblastoma extracellular vesicles by IFN- γ through PD-L1 and IDO1. *Neurooncol Adv*;4(1):vdac017. DOI: 10.1093/noajnl/vdac017. Epub 2022 Feb 15.

Kaffes I, Szulzewsky F, Chen Z, Herting CJ, Gabanic B, Velázquez Vega JE, Shelton J, Switchenko JM, Ross JL, McSwain LF, Huse JT, Westermarck B, Nelander S, Forsberg-Nilsson K, Uhrbom L, Maturi NP, Cimino PJ, Holland EC, Kettenmann H, Brennan CW, Brat DJ, Hambardzumyan D, 2019. Human mesenchymal glioblastomas are characterized by an increased immune cell presence compared to proneural and classical tumors. *Oncoimmunology*;8(11):e1655360. DOI: 10.1080/2162402X.2019. Epub 2019 Aug 22.

Kaneko S, Nakatani Y, Takezaki T, Hide T, Yamashita D, Ohtsu N, Ohnishi T, Terasaka S, Houkin K, Kondo T, 2015. Ceacam1L modulates STAT3 signaling to control the proliferation of glioblastoma-initiating cells. *Cancer Res*;75(19):4224-34.

Kaplan S, Novikov I, Modan B, 1997. Nutritional factors in the etiology of brain tumors: potential role of nitrosamines, fat, and cholesterol. *Am J Epidemiol*;146(10):832-41.

Kaur M, Kaur T, Kamboj SS, Singh J, 2016. Roles of alectin-7 in cancer. *Asian Pac J Cancer Prev*;17(2):455-61.

Kaur S, Singh J, Kaur M, 2023. Multifaceted role of galectin-4 in cancer: A systematic review. *Eur J Clin Invest*;53(7):e13987. DOI: 10.1111/eci.13987. Epub 2023 Mar 28.

Kawamura S, Onai N, Miya F, Sato T, Tsunoda T, Kurabayashi K, Yotsumoto S, Kuroda S, Takenaka K, Akashi K, Ohteki T, 2017. Identification of a human clonogenic progenitor with strict monocyte differentiation potential: a counterpart of

mouse cMoPs. *Immunity*;46(5):835-848.e4. doi: 10.1016/j.immuni.2017.04.019. Epub 2017 May 16.

Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, Sharpe AH, 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med*;203(4):883-95.

Keskin DB, Anandappa AJ, Sun J, Tirosh I, Mathewson ND, Li S, Oliveira G, Giobbie-Hurder A, Felt K, Gjini E, Shukla SA, Hu Z, Li L, Le PM, Allesøe RL, Richman AR, Kowalczyk MS, Abdelrahman S, Geduldig JE, Charbonneau S, Pelton K, Iorgulescu JB, Elagina L, Zhang W, Olive O, McCluskey C, Olsen LR, Stevens J, Lane WJ, Salazar AM, Daley H, Wen PY, Chiocca EA, Harden M, Lennon NJ, Gabriel S, Getz G, Lander ES, Regev A, Ritz J, Neuberg D, Rodig SJ, Ligon KL, Suvà ML, Wucherpennig KW, Hacohen N, Fritsch EF, Livak KJ, Ott PA, Wu CJ, Reardon DA, 2019. Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature*;565(7738):234-9.

Kim SJ, Hwang JA, Ro JY, Lee YS, Chun KH, 2013. Galectin-7 is epigenetically-regulated tumor suppressor in gastric cancer. *Oncotarget*;4(9):1461-71.

Kim JE, Patel MA, Mangraviti A, Kim ES, Theodros D, Velarde E, Liu A, Sankey EW, Tam A, Xu H, Mathios D, Jackson CM, Harris-Bookman S, Garzon-Muvdi T, Sheu M, Martin AM, Tyler BM, Tran PT, Ye X, Olivi A, Taube JM, Burger PC, Drake CG, Brem H, Pardoll DM, Lim M, 2017. Combination therapy with anti-PD-1, anti-TIM-3, and focal radiation results in regression of murine gliomas. *Clin Cancer Res*;23(1):124-36.

Kitahara CM, Linet MS, Brenner AV, Wang SS, Melin BS, Wang Z, Inskip PD, Freeman LE, Braganza MZ, Carreón T, Feychting M, Gaziano JM, Peters U, Purdue MP, Ruder AM, Sesso HD, Shu XO, Waters MA, White E, Zheng W, Hoover RN, Fraumeni JF Jr, Chatterjee N, Yeager M, Chanock SJ, Hartge P, Rajaraman P, 2014. Personal history of diabetes, genetic susceptibility to diabetes, and risk of brain glioma: a pooled analysis of observational studies. *Cancer Epidemiol Biomarkers Prev*;23(1):47-54.

Kivisäkk P, Imitola J, Rasmussen S, Elyaman W, Zhu B, Ransohoff RM, Khoury SJ, 2009. Localizing central nervous system immune surveillance: meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. *Ann Neurol*;65(4):457-69.

Kleihues P, Schäuble B, zur Hausen A, Estève J, Ohgaki H, 1997. Tumors associated with p53 germline mutations: a synopsis of 91 families. *Am J Pathol*;150(1):1-13.

Klein F, Diskin R, Scheid JF, Gaebler C, Mouquet H, Georgiev IS, Pancera M, Zhou T, Incesu RB, Fu BZ, Gnanapragasam PN, Oliveira TY, Seaman MS, Kwong PD, Bjorkman PJ, Nussenzweig MC, 2013. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell*;153(1):126-38.

- Klein L, Kyewski B, Allen PM, Hogquist KA, 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol*;14(6):377-391.
- Kmieciak J, Poli A, Brons NH, Waha A, Eide GE, Enger PØ, Zimmer J, Chekenya M, 2013. Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level. *J Neuroimmunol*;264(1-2):71-83.
- Knudsen AM, Rudkjøbing SJ, Sørensen MD, Dahlrot RH, Kristensen BW, 2021. Expression and prognostic value of the immune checkpoints galectin-9 and PD-L1 in glioblastomas. *J Neuropathol Exp Neurol*;80(6):541-51.
- Kogawa Y, Nakajima K, Sasaguri K, Hamada N, Kawasaki H, Sato S, Kadoya T, Horie H, 2011. Oxidized galectin-1 reduces lipopolysaccharide-induced increase of proinflammatory cytokine mRNA in cultured macrophages. *Clin Cosmet Investig Dent*;3:1-8.
- Korin B, Ben-Shaanan TL, Schiller M, Dubovik T, Azulay-Debby H, Boshnak NT, Koren T, Rolls A, 2017. High-dimensional, single-cell characterization of the brain's immune compartment. *Nat Neurosci*;20(9):1300-9.
- Koshy M, Villano JL, Dolecek TA, Howard A, Mahmood U, Chmura SJ, Weichselbaum RR, McCarthy BJ, 2012. Improved survival time trends for glioblastoma using the SEER 17 population-based registries. *J Neurooncol*;107(1):207-12.
- Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, Hussell T, Feldmann M, Udalova IA, 2011. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol*;12(3):231-8.
- Krautter F, Recio C, Hussain MT, Lezama DR, Maione F, Chimen M, Iqbal AJ, 2020. Characterisation of endogenous Galectin-1 and -9 expression in monocyte and macrophage subsets under resting and inflammatory conditions. *Biomed Pharmacother*;130:110595. DOI: 10.1016/j.biopha.2020.110595. Epub 2020 Aug 6.
- Kräutler NJ, Suan D, Butt D, Bourne K, Hermes JR, Chan TD, Sundling C, Kaplan W, Schofield P, Jackson J, Basten A, Christ D, Brink R, 2017. Differentiation of germinal center B cells into plasma cells is initiated by high-affinity antigen and completed by Tfh cells. *J Exp Med*;214(5):1259-67.
- Kuhnt D, Becker A, Ganslandt O, Bauer M, Buchfelder M, Nimsky C, 2011. Correlation of the extent of tumor volume resection and patient survival in surgery of glioblastoma multiforme with high-field intraoperative MRI guidance. *Neuro Oncol*;13(12):1339-48. Erratum in: *Neuro Oncol*;16(10):1429.
- Kular L, Pakradouni J, Kitabgi P, Laurent M, Martinerie C, 2011. The CCN family: A new class of inflammation modulators? *Biochimie*;93(3):377-88.

Kunzmann S, Seher A, Kramer BW, Schenk R, Schütze N, Jakob F, Sebald W, Speer CP, 2008. Connective tissue growth factor does not affect transforming growth factor-beta 1-induced Smad3 phosphorylation and T lymphocyte proliferation inhibition. *Int Arch Allergy Immunol*;147(2):152-60.

Kusmartsev S, Nagaraj S, Gabrilovich DI, 2005. Tumor-associated CD8⁺ T cell tolerance induced by bone marrow-derived immature myeloid cells. *J Immunol*;175(7):4583-92.

Kvisten M, Mikkelsen VE, Stensjøen AL, Solheim O, Van Der Want J, Torp SH, 2019. Microglia and macrophages in human glioblastomas: A morphological and immunohistochemical study. *Mol Clin Oncol*;11(1):31-6.

La M, Cao TV, Cerchiaro G, Chilton K, Hirabayashi J, Kasai K, Oliani SM, Chernajovsky Y, Perretti M, 2003. A novel biological activity for galectin-1: inhibition of leukocyte-endothelial cell interactions in experimental inflammation. *Am J Pathol*;163(4):1505-15.

Labrie M, Vladoiu MC, Grosset AA, Gaboury L, St-Pierre Y, 2014. Expression and functions of galectin-7 in ovarian cancer. *Oncotarget*;5(17):7705-21.

Lancaster JN, Li Y, Ehrlich LIR, 2018. Chemokine-mediated choreography of thymocyte development and selection. *Trends Immunol*;39(2):86-98.

Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ, 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol*;2(3):261-8.

Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS, 2011. Peripheral education of the immune system by colonic commensal microbiota. *Nature*;478(7368):250-4.

Lee M, Wrensch M, Miike R, 1997. Dietary and tobacco risk factors for adult onset glioma in the San Francisco Bay Area (California, USA). *Cancer Causes Control*;8(1):13-24.

Lee KH, Holdorf AD, Dustin ML, Chan AC, Allen PM, Shaw AS, 2002. T cell receptor signaling precedes immunological synapse formation. *Science*;295(5559):1539-42.

Lee MJ, Woo MY, Chwae YJ, Kwon MH, Kim K, Park S, 2012. Down-regulation of interleukin-2 production by CD4(+) T cells expressing TIM-3 through suppression of NFAT dephosphorylation and AP-1 transcription. *Immunobiology*;217(10):986-95.

Lee J, Breton G, Oliveira TY, Zhou YJ, Aljoufi A, Pühr S, Cameron MJ, Sékaly RP, Nussenzweig MC, Liu K, 2015. Restricted dendritic cell and monocyte progenitors in human cord blood and bone marrow. *J Exp Med*;212(3):385-99.

Lee KS, Lee K, Yun S, Moon S, Park Y, Han JH, Kim CY, Lee HS, Choe G, 2018a. Prognostic relevance of programmed cell death ligand 1 expression in glioblastoma. *J Neurooncol*;136(3):453-61.

Lee E, Yong RL, Paddison P, Zhu J, 2018b. Comparison of glioblastoma (GBM) molecular classification methods. *Semin Cancer Biol*;53:201-11.

Lee AH, Sun L, Mochizuki AY, Reynoso JG, Orpilla J, Chow F, Kienzler JC, Everson RG, Nathanson DA, Bensinger SJ, Liao LM, Cloughesy T, Hugo W, Prins RM, 2021. Neoadjuvant PD-1 blockade induces T cell and cDC1 activation but fails to overcome the immunosuppressive tumor associated macrophages in recurrent glioblastoma. *Nat Commun*;12(1):6938. DOI: 10.1038/s41467-021-26940-2. Epub 2021 Nov 26.

Lehrer S, Rheinstein PH, Rosenzweig KE, 2019. Allergy may confer better survival on patients with gliomas. *Clin Neurol Neurosurg*;177:63-7.

Le Mercier M, Lefranc F, Mijatovic T, Debeir O, Haibe-Kains B, Bontempi G, Decaestecker C, Kiss R, Mathieu V, 2008. Evidence of galectin-1 involvement in glioma chemoresistance. *Toxicol Appl Pharmacol*;229(2):172-83.

Le Mercier M, Fortin S, Mathieu V, Kiss R, Lefranc F, 2010. Galectins and gliomas. *Brain Pathol*;20(1):17-27.

Leung SY, Wong MP, Chung LP, Chan AS, Yuen ST, 1997. Monocyte chemoattractant protein-1 expression and macrophage infiltration in gliomas. *Acta Neuropathol*;93(5):518-27.

Li B, Li J, Devlin BH, Markert ML, 2011. Thymic microenvironment reconstitution after postnatal human thymus transplantation. *Clin Immunol*;140(3):244-59.

Li H, Wu K, Tao K, Chen L, Zheng Q, Lu X, Liu J, Shi L, Liu C, Wang G, Zou W, 2012. Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. *Hepatology*;56(4):1342-51.

Li J, Liu X, Duan Y, Liu Y, Wang H, Lian S, Zhuang G, Fan Y, 2017a. Combined blockade of T cell immunoglobulin and mucin domain 3 and carcinoembryonic antigen-related cell adhesion molecule 1 results in durable therapeutic efficacy in mice with intracranial gliomas. *Med Sci Monit*;23:3593-602.

Li G, Wang Z, Zhang C, Liu X, Cai J, Wang Z, Hu H, Wu F, Bao Z, Liu Y, Zhao L, Liang T, Yang F, Huang R, Zhang W, Jiang T, 2017b. Molecular and clinical characterization of TIM-3 in glioma through 1,024 samples. *Oncoimmunology*;6(8):e1328339. DOI: 10.1080/2162402X.2017.1328339. Epub 2017 Jul 28.

Li J, Liu X, Duan Y, Wang H, Su W, Wang Y, Zhuang G, Fan Y, 2018. Abnormal expression of circulating and tumor-infiltrating carcinoembryonic antigen-related cell adhesion molecule 1 in patients with glioma. *Oncol Lett*;15(3):3496-503.

Lidström T, Cumming J, Gaur R, Frängsmyr L, Pateras IS, Mickert MJ, Franklin O, Forsell MNE, Arnberg N, Dongre M, Patthey C, Öhlund D, 2023. Extracellular

galectin 4 drives immune evasion and promotes T-cell apoptosis in pancreatic cancer. *Cancer Immunol Res*;11(1):72-92.

Lim M, Weller M, Idbaih A, Steinbach J, Finocchiaro G, Raval RR, Ansstas G, Baehring J, Taylor JW, Honnorat J, Petrecca K, De Vos F, Wick A, Sumrall A, Sahebjam S, Mellinghoff IK, Kinoshita M, Roberts M, Slepets R, Warad D, Leung D, Lee M, Reardon DA, Omuro A, 2022. Phase III trial of chemoradiotherapy with temozolomide plus nivolumab or placebo for newly diagnosed glioblastoma with methylated MGMT promoter. *Neuro Oncol*;24(11):1935-49.

Lin P, Jiang H, Zhao YJ, Pang JS, Liao W, He Y, Lin ZY, Yang H, 2021. Increased infiltration of CD8 T cells in recurrent glioblastoma patients is a useful biomarker for assessing the response to combined bevacizumab and lomustine therapy. *Int Immunopharmacol*;97:107826. DOI: 10.1016/j.intimp.2021.107826. Epub 2021 Jun 4.

Ling C, Sandor M, Fabry Z, 2003. In situ processing and distribution of intracerebrally injected OVA in the CNS. *J Neuroimmunol*;141(1-2):90-8.

Linos E, Raine T, Alonso A, Michaud D, 2007. Atopy and risk of brain tumors: a meta-analysis. *J Natl Cancer Inst*;99(20):1544-50.

Linterman MA, Rigby RJ, Wong R, Silva D, Withers D, Anderson G, Verma NK, Brink R, Hutloff A, Goodnow CC, Vinuesa CG, 2009. Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. *Immunity*;30(2):228-41.

Liu FT, Hsu DK, Zuberi RI, Kuwabara I, Chi EY, Henderson WR Jr, 1995. Expression and function of galectin-3, a beta-galactoside-binding lectin, in human monocytes and macrophages. *Am J Pathol*;147(4):1016-28.

Liu Y, Carlsson R, Ambjørn M, Hasan M, Badn W, Darabi A, Siesjö P, Issazadeh-Navikas S, 2013. PD-L1 expression by neurons nearby tumors indicates better prognosis in glioblastoma patients. *J Neurosci*;33(35):14231-45.

Liu Z, Han H, He X, Li S, Wu C, Yu C, Wang S, 2016. Expression of the galectin-9-Tim-3 pathway in glioma tissues is associated with the clinical manifestations of glioma. *Oncol Lett*;11(3):1829-34.

Lohr J, Ratliff T, Huppertz A, Ge Y, Dictus C, Ahmadi R, Grau S, Hiraoka N, Eckstein V, Ecker RC, Korff T, von Deimling A, Unterberg A, Beckhove P, Herold-Mende C, 2011. Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF- β . *Clin Cancer Res*;17(13):4296-308.

London F, Cambron B, Jacobs S, Delrée P, Gustin T, 2020. Glioblastoma in a fingolimod-treated multiple sclerosis patient: Causal or coincidental association? *Mult Scler Relat Disord*;41:102012. DOI: 10.1016/j.msard.2020.102012. Epub 2020 Feb 15.

Louis DN, von Deimling A, Chung RY, Rubio MP, Whaley JM, Eibl RH, Ohgaki H, Wiestler OD, Thor AD, Seizinger BR, 1993. Comparative study of p53 gene and protein alterations in human astrocytic tumors. *J Neuropathol Exp Neurol*;52(1):31-8.

Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P, 2007. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol*;114(2):97-109. Erratum in: *Acta Neuropathol*;114(5):547.

Louis DN, Perry A, Burger P, Ellison DW, Reifenberger G, von Deimling A, Aldape K, Brat D, Collins VP, Eberhart C, Figarella-Branger D, Fuller GN, Giangaspero F, Giannini C, Hawkins C, Kleihues P, Korshunov A, Kros JM, Beatriz Lopes M, Ng HK, Ohgaki H, Paulus W, Pietsch T, Rosenblum M, Rushing E, Soylemezoglu F, Wiestler O, Wesseling P, International Society Of Neuropathology—Haarlem, 2014. International Society Of Neuropathology--Haarlem consensus guidelines for nervous system tumor classification and grading. *Brain Pathol*;24(5):429-35.

Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P, Ellison DW, 2016. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol*;131(6):803-20.

Louis DN, Perry A, Wesseling P, Brat DJ, Cree IA, Figarella-Branger D, Hawkins C, Ng HK, Pfister SM, Reifenberger G, Soffietti R, von Deimling A, Ellison DW, 2021. The 2021 WHO classification of tumors of the central nervous system: a summary. *Neuro-Oncol*;23(8):1231-51.

Loussouarn D, Oliver L, Salaud C, Samarut E, Bourgade R, Bérout C, Morenton E, Heymann D, Vallette FM, 2023. Spatial distribution of immune cells in primary and recurrent glioblastoma: a small case study. *Cancers (Basel)*;15(12):3256. DOI: 10.3390/cancers15123256. Epub 2023 Jun 20.

Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, Derecki NC, Castle D, Mandell JW, Lee KS, Harris TH, Kipnis J, 2015. Structural and functional features of central nervous system lymphatic vessels. *Nature*;523(7560):337-41.

Louveau A, Herz J, Alme MN, Salvador AF, Dong MQ, Viar KE, Herod SG, Knopp J, Setliff JC, Lupi AL, Da Mesquita S, Frost EL, Gaultier A, Harris TH, Cao R, Hu S, Lukens JR, Smirnov I, Overall CC, Oliver G, Kipnis J, 2018. CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature. *Nat Neurosci*;21(10):1380-91.

Lu KH, Michel J, Kilian M, Aslan K, Qi H, Kehl N, Jung S, Sanghvi K, Lindner K, Zhang XW, Green EW, Poschke I, Ratliff M, Bunse T, Sahm F, von Deimling A, Wick W, Platten M, Bunse L, 2022. T cell receptor dynamic and transcriptional determinants of T cell expansion in glioma-infiltrating T cells. *Neurooncol Adv*;4(1):vdac140. DOI: 10.1093/noajnl/vdac140. Epub 2022 Aug 31.

Lun M, Lok E, Gautam S, Wu E, Wong ET, 2011. The natural history of extracranial metastasis from glioblastoma multiforme. *J Neurooncol*;105(2):261-73.

- Luo Z, Ji Y, Tian D, Zhang Y, Chang S, Yang C, Zhou H, Chen ZK, 2018. Galectin-7 promotes proliferation and Th1/2 cells polarization toward Th1 in activated CD4⁺ T cells by inhibiting the TGFβ/Smad3 pathway. *Mol Immunol*;101:80-5.
- Ma X, Liu Y, Liu Y, Alexandrov LB, Edmonson MN, Gawad C, Zhou X, Li Y, Rusch MC, Easton J, Huether R, Gonzalez-Pena V, Wilkinson MR, Hermida LC, Davis S, Sioson E, Pounds S, Cao X, Ries RE, Wang Z, Chen X, Dong L, Diskin SJ, Smith MA, Guidry Auvil JM, Meltzer PS, Lau CC, Perlman EJ, Maris JM, Meshinchi S, Hunger SP, Gerhard DS, Zhang J, 2018. Pan-cancer genome and transcriptome analyses of 1,699 paediatric leukaemias and solid tumours. *Nature*;555(7696):371-6.
- MacLennan IC, 1994. Germinal centres. *Ann Rev Immunol*;12(1):117-39.
- Maeda TK, Sugiura D, Okazaki IM, Maruhashi T, Okazaki T, 2019. Atypical motifs in the cytoplasmic region of the inhibitory immune co-receptor LAG-3 inhibit T cell activation. *J Biol Chem*;294(15):6017-26.
- Maggs L, Cattaneo G, Dal AE, Moghaddam AS, Ferrone S, 2021. CAR T cell-based immunotherapy for the treatment of glioblastoma. *Front Neurosci*;15:662064. DOI: 10.3389/fnins.2021.662064. Epub 2021 May 25.
- Magnaldo T, Fowles D, Darmon M, 1998. Galectin-7, a marker of all types of stratified epithelia. *Differentiation*;63(3):159-68.
- Malik RK, Ghurye RR, Lawrence-Watt DJ, Stewart HJ, 2009. Galectin-1 stimulates monocyte chemotaxis via the p44/42 MAP kinase pathway and a pertussis toxin-sensitive pathway. *Glycobiology*;19(12):1402-7.
- Malmer B, Haraldsson S, Einarsdottir E, Lindgren P, Holmberg D, 2005. Homozygosity mapping of familial glioma in Northern Sweden. *Acta Oncol*;44(2):114-9.
- Malmer BS, Feychting M, Lönn S, Lindström S, Grönberg H, Ahlbom A, Schwartzbaum J, Auvinen A, Collatz-Christensen H, Johansen C, Kiuru A, Mudie N, Salminen T, Schoemaker MJ, Swerdlow AJ, Henriksson R, 2007. Genetic variation in p53 and ATM haplotypes and risk of glioma and meningioma. *J Neurooncol*;82(3):229-37.
- Malmström A, Grönberg BH, Marosi C, Stupp R, Frappaz D, Schultz H, Abacioglu U, Tavelin B, Lhermitte B, Hegi ME, Rosell J, Henriksson R; Nordic Clinical Brain Tumour Study Group (NCBTSG), 2012. Temozolomide versus standard 6-week radiotherapy versus hypofractionated radiotherapy in patients older than 60 years with glioblastoma: the Nordic randomised, phase 3 trial. *Lancet Oncol*;13(9):916-26.
- Malo CS, Huggins MA, Goddery EN, Tolcher HMA, Renner DN, Jin F, Hansen MJ, Pease LR, Pavelko KD, Johnson AJ, 2018. Non-equivalent antigen presenting capabilities of dendritic cells and macrophages in generating brain-infiltrating CD8⁺ T cell responses. *Nat Commun*;9(1):633. DOI: 10.1038/s41467-018-03037-x. Epub 2018 Feb 12.

Mandal S, Curtis L, Pind M, Murphy LC, Watson PH, 2007. S100A7 (psoriasin) influences immune response genes in human breast cancer. *Exp Cell Res*;313(14):3016-25.

Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT, 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*;441(7090):231-4.

Mansour AA, Raucci F, Sevim M, Saviano A, Begum J, Zhi Z, Pezhman L, Tull S, Maione F, Iqbal AJ, 2022. Galectin-9 supports primary T cell transendothelial migration in a glycan and integrin dependent manner. *Biomed Pharmacother*;151:113171. DOI: 10.1016/j.biopha.2022.113171. Epub 2022 May 25.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M, 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*;25(12):677-86.

Marchetti L, Engelhardt B, 2020. Immune cell trafficking across the blood-brain barrier in the absence and presence of neuroinflammation. *Vasc Biol*;2(1):H1-H18. DOI: 10.1530/VB-19-0033. Epub 2020 Mar 20.

Martinez-Lage M, Lynch TM, Bi Y, Cocito C, Way GP, Pal S, Haller J, Yan RE, Ziober A, Nguyen A, Kandpal M, O'Rourke DM, Greenfield JP, Greene CS, Davuluri RV, Dahmane N, 2019. Immune landscapes associated with different glioblastoma molecular subtypes. *Acta Neuropathol Commun*;7(1):203. doi: 10.1186/s40478-019-0803-6. Epub 2019 Nov 29.

Maruhashi T, Okazaki IM, Sugiura D, Takahashi S, Maeda TK, Shimizu K, Okazaki T, 2018. LAG-3 inhibits the activation of CD4⁺ T cells that recognize stable pMHCII through its conformation-dependent recognition of pMHCII. *Nat Immunol*;19(12):1415-1426.

Marzagalli M, Ebelt ND, Manuel ER, 2019. Unraveling the crosstalk between melanoma and immune cells in the tumor microenvironment. *Semin Cancer Biol*;59:236-50.

Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG, 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*;427(6972):355-60.

Mauldin IS, Jo J, Wages NA, Yogendran LV, Mahmutovic A, Young SJ, Lopes MB, Slingluff CL Jr, Erickson LD, Fadul CE, 2021. Proliferating CD8⁺ T cell infiltrates are associated with improved survival in glioblastoma. *Cells*;10(12):3378. DOI: 10.3390/cells10123378. Epub 2021 Dec 1.

Mauris J, Shaikhibrahim Z, Cao Z, Panjwani NA, Argueso P, 2011. Characterization of new galectin-3 binding-partners in corneal epithelium. *Invest Ophthalmol Vis Sci*;52(14):310.

Medvedovic J, Ebert A, Tagoh H, Busslinger M, 2011. Pax5: a master regulator of B cell development and leukemogenesis. *Adv Immunol*;111:179-206.

Mendez Valdez MJ, Lu VM, Kim E, Rivas SR, Govindarajan V, Ivan M, Komotar R, Nath A, Heiss JD, Shah AH, 2022. Glioblastoma multiforme in patients with human immunodeficiency virus: an integrated review and analysis. *J Neurooncol*;159(3):571-9.

Meng FW, Liu FS, Liu WH, Li L, Jie LL, 2020. Formation of new lymphatic vessels in glioma: An immunohistochemical analysis. *Neuropathology*;40(3):215-23.

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM, 2000. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol*;164(12):6166-73.

Minegishi Y, Coustan-Smith E, Wang YH, Cooper MD, Campana D, Conley ME, 1998. Mutations in the human $\lambda 5/14.1$ gene result in B cell deficiency and agammaglobulinemia. *J Exp Med*;187(1):71-7.

Mischel PS, Cloughesy TF, 2003. Targeted molecular therapy of GBM. *Brain Pathol*;13(1):52-61.

Mitsdoerffer M, Aly L, Barz M, Engleitner T, Sie C, Delbridge C, Lepennetier G, Öllinger R, Pfaller M, Wiestler B, Rad R, Meyer B, Knier B, Schmidt-Graf F, Gempt J, Korn T, 2022. The glioblastoma multiforme tumor site promotes the commitment of tumor-infiltrating lymphocytes to the T_H17 lineage in humans. *Proc Natl Acad Sci U S A*;119(34):e2206208119. DOI: 10.1073/pnas.2206208119. Epub 2022 Aug 15.

Mohme M, Schliffke S, Maire CL, Rüniger A, Glau L, Mende KC, Matschke J, Gehbauer C, Akyüz N, Zapf S, Holz M, Schaper M, Martens T, Schmidt NO, Peine S, Westphal M, Binder M, Tolosa E, Lamszus K, 2018. Immunophenotyping of newly diagnosed and recurrent glioblastoma defines distinct immune exhaustion profiles in peripheral and tumor-infiltrating lymphocytes. *Clin Cancer Res*;24(17):4187-200.

Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE, 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*;68(5):869-77.

Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, Freeman GJ, Kuchroo VK, 2002. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature*;415(6871):536-41.

Montgomery S, Hassan A, Bahmanyar S, Brus O, Hussein O, Hiyoshi A, Hillert J, Olsson T, Fall K, 2013. Mortality following a brain tumour diagnosis in patients with multiple sclerosis. *BMJ Open*;3(11):e003622. DOI: 10.1136/bmjopen-2013-003622. Epub 2013 Nov 11.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL, 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*;136(7):2348-57.

Mosmann TR, Sad S, 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today*;17(3):138-46.

Mosser DM, Edwards JP, 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*;8(12):958-69. Erratum in: *Nat Rev Immunol*.2010 Jun;10(6):460.

Mouta Carreira C, Nasser SM, di Tomaso E, Padera TP, Boucher Y, Tomarev SI, Jain RK, 2001. LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. *Cancer Res*;61(22):8079-84.

Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, De Baetselier P, Van Ginderachter JA, 2008. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood*;111(8):4233-44.

Mukherjee R, Kanti Barman P, Kumar Thatoi P, Tripathy R, Kumar Das B, Ravindran B, 2015. Non-classical monocytes display inflammatory features: validation in sepsis and systemic lupus erythematosus. *Sci Rep*;5:13886. DOI: 10.1038/srep13886. Epub 2015 Sep 11.

Muntjewerff EM, Meesters LD, van den Bogaart G, 2020. Antigen cross-presentation by macrophages. *Front Immunol*;11:1276. DOI: 10.3389/fimmu.2020.01276. Epub 2020 Jul 8.

Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T, 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell*;102(5):553-63.

Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdts S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA, 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*;41(1):14-20.

Nadler LM, Korsmeyer SJ, Anderson KC, Boyd AW, Slaughenhaupt B, Park E, Jensen J, Coral F, Mayer RJ, Sallan SE, Ritz J, Schlossman SF, 1984. B cell origin of non-T cell acute lymphoblastic leukemia. A model for discrete stages of neoplastic and normal pre-B cell differentiation. *J Clin Invest*;74(2):332-40.

Nambiar DK, Aguilera T, Cao H, Kwok S, Kong C, Bloomstein J, Wang Z, Rangan VS, Jiang D, von Eyben R, Liang R, Agarwal S, Colevas AD, Korman A, Allen CT, Uppaluri R, Koong AC, Giaccia A, Le QT, 2019. Galectin-1-driven T cell exclusion in the tumor endothelium promotes immunotherapy resistance. *J Clin Invest*;129(12):5553-67.

Nasser MW, Qamri Z, Deol YS, Ravi J, Powell CA, Trikha P, Schwendener RA, Bai XF, Shilo K, Zou X, Leone G, Wolf R, Yuspa SH, Ganju RK, 2011. S100A7 enhances

mammary tumorigenesis through upregulation of inflammatory pathways. *Cancer Res*;72(3):604-15.

Nduom EK, Wei J, Yaghi NK, Huang N, Kong LY, Gabrusiewicz K, Ling X, Zhou S, Ivan C, Chen JQ, Burks JK, Fuller GN, Calin GA, Conrad CA, Creasy C, Ritthipichai K, Radvanyi L, Heimberger AB, 2016. PD-L1 expression and prognostic impact in glioblastoma. *Neuro Oncol*;18(2):195-205.

Neder L, Marie SK, Carlotti CG Jr, Gabbai AA, Rosemberg S, Malheiros SM, Siqueira RP, Oba-Shinjo SM, Uno M, Aguiar PH, Miura F, Chammas R, Colli BO, Silva WA Jr, Zago MA, 2004. Galectin-3 as an immunohistochemical tool to distinguish pilocytic astrocytomas from diffuse astrocytomas, and glioblastomas from anaplastic oligodendrogliomas. *Brain Pathol*;14(4):399-405.

Nevalainen T, Autio A, Hurme M, 2022. Composition of the infiltrating immune cells in the brain of healthy individuals: effect of aging. *Immun Ageing*;19(1):45. DOI: 10.1186/s12979-022-00302-y. Epub 2022 Oct 8.

Newcombe AA, Gibson BES, Keeshan K, 2018. Harnessing the potential of epigenetic therapies for childhood acute myeloid leukemia. *Exp Hematol*;63:1-11.

Ni TT, 2018. Diabetes mellitus might be a protective factor of glioma. *J Cancer Res Ther*;14(Supplement):S644-7.

Ni X, Wu W, Sun X, Ma J, Yu Z, He X, Cheng J, Xu P, Liu H, Shang T, Xi S, Wang J, Zhang J, Chen Z, 2022. Interrogating glioma-M2 macrophage interactions identifies Gal-9/Tim-3 as a viable target against *PTEN*-null glioblastoma. *Sci Adv*;8(27):eabl5165. DOI: 10.1126/sciadv.abl5165. Epub 2022 Jul 8.

Nio-Kobayashi J, Itabashi T, 2021. Galectins and their ligand glycoconjugates in the central nervous system under physiological and pathological conditions. *Front Neuroanat*;15:767330. DOI: 10.3389/fnana.2021.767330. Epub 2021 Oct 15.

Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, Sasayama S, Mizoguchi A, Hiai H, Minato N, Honjo T, 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science*;291(5502):319-22.

Nishio S, Morioka T, Inamura T, Takeshita I, Fukui M, Sasaki M, Nakamura K, Wakisaka S, 1998. Radiation-induced brain tumours: potential late complications of radiation therapy for brain tumours. *Acta Neurochir (Wien)*;140(8):763-70.

Nobusawa S, Watanabe T, Kleihues P, Ohgaki H, 2009. IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. *Clin Cancer Res*;15(19):6002-7.

Norling LV, Sampaio AL, Cooper D, Perretti M, 2008. Inhibitory control of endothelial galectin-1 on in vitro and in vivo lymphocyte trafficking. *FASEB J*;22(3):682-90.

Novak R, Dabelic S, Dumic J, 2012. Galectin-1 and galectin-3 expression profiles in classically and alternatively activated human macrophages. *Biochim Biophys Acta*;1820(9):1383-90.

Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, Burkhard C, Schüler D, Probst-Hensch NM, Maiorka PC, Baeza N, Pisani P, Yonekawa Y, Yasargil MG, Lütolf UM, Kleihues P, 2004. Genetic pathways to glioblastoma: a population-based study. *Cancer Res*;64(19):6892-9.

Ohgaki H, Kleihues P, 2007. Genetic pathways to primary and secondary glioblastoma. *Am J Pathol*;170(5):1445-53.

Ohgaki H, Kleihues P, 2013. The definition of primary and secondary glioblastoma. *Clin Cancer Res*;19(4):764-72.

Okazaki T, Okazaki IM, Wang J, Sugiura D, Nakaki F, Yoshida T, Kato Y, Fagarasan S, Muramatsu M, Eto T, Hioki K, Honjo T, 2011. PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. *J Exp Med*;208(2):395-407.

Olate-Briones A, Escalona E, Salazar C, Herrada MJ, Liu C, Herrada AA, Escobedo N, 2022. The meningeal lymphatic vasculature in neuroinflammation. *FASEB J*;36(5):e22276. DOI: 10.1096/fj.202101574RR. Epub 2022 May.

Omuro A, Brandes AA, Carpentier AF, Idbaih A, Reardon DA, Cloughesy T, Sumrall A, Baehring J, van den Bent M, Bähr O, Lombardi G, Mulholland P, Tabatabai G, Lassen U, Sepulveda JM, Khasraw M, Vauleon E, Muragaki Y, Di Giacomo AM, Butowski N, Roth P, Qian X, Fu AZ, Liu Y, Potter V, Chalamandaris AG, Tatsuoka K, Lim M, Weller M, 2023. Radiotherapy combined with nivolumab or temozolomide for newly diagnosed glioblastoma with unmethylated MGMT promoter: An international randomized phase III trial. *Neuro Oncol*;25(1):123-34.

O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrisette JJD, Martinez-Lage M, Brem S, Maloney E, Shen A, Isaacs R, Mohan S, Plesa G, Lacey SF, Navenot JM, Zheng Z, Levine BL, Okada H, June CH, Brogdon JL, Maus MV, 2017. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med*;9(399):eaaa0984. DOI: 10.1126/scitranslmed.aaa0984. Epub 2017 Jul 19.

Orrego E, Castaneda CA, Castillo M, Bernabe LA, Casavilca S, Chakravarti A, Meng W, Garcia-Corrochano P, Villa-Robles MR, Zevallos R, Mejia O, Deza P, Belmar-Lopez C, Ojeda L, 2018. Distribution of tumor-infiltrating immune cells in glioblastoma. *CNS Oncol*;7(4):CNS21. DOI: 10.2217/cns-2017-0037. Epub 2018 Oct 9.

Ostrom QT, Gittleman H, Farah P, Ondracek A, Chen Y, Wolinsky Y, Stroup NE, Kruchko C, Barnholtz-Sloan JS, 2013. CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006-2010. *Neuro Oncol*;15(Suppl 2):ii1-56. Erratum in: *Neuro Oncol*;16(5):760.

Ostrom QT, Gittleman H, Fulop J, Liu M, Blanda R, Kromer C, Wolinsky Y, Kruchko C, Barnholtz-Sloan JS, 2015. CBTRUS Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2008-2012. *Neuro Oncol*;17(Suppl 4):iv1-iv62.

Otáhal P, Angelisová P, Hrdinka M, Brdicka T, Novák P, Drbal K, Horejsí V, 2010. A new type of membrane raft-like microdomains and their possible involvement in TCR signaling. *J Immunol*;184(7):3689-96.

Paclik D, Danese S, Berndt U, Wiedenmann B, Dignass A, Sturm A, 2008. Galectin-4 controls intestinal inflammation by selective regulation of peripheral and mucosal T cell apoptosis and cell cycle. *PLoS One*;3(7):e2629. DOI: 10.1371/journal.pone.0002629. Epub 2008 Jul 9.

Paclik D, Werner L, Guckelberger O, Wiedenmann B, Sturm A, 2011. Galectins distinctively regulate central monocyte and macrophage function. *Cell Immunol*;271(1):97-103.

Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, Linsley PS, Thompson CB, Riley JL, 2005. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol*;25(21):9543-53.

Pasquier B, Pasquier D, N'Golet A, Panh MH, Couderc P, 1980. Extraneural metastases of astrocytomas and glioblastomas: clinicopathological study of two cases and review of literature. *Cancer*;45(1):112-25.

Passlick B, Flieger D, Ziegler-Heitbrock HW, 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*;74(7):2527-34.

Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, Bigley V, Flavell RA, Gilroy DW, Asquith B, Macallan D, Yona S, 2017. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J Exp Med*;214(7):1913-23.

Pathan M, Fonseka P, Chitti SV, Kang T, Sanwlani R, Van Deun J, Hendrix A, Mathivanan S, 2019. Vesiclepedia 2019: a compendium of RNA, proteins, lipids and metabolites in extracellular vesicles. *Nucleic Acids Res*;47(D1):D516-D519. Database available at <http://microvesicles.org>

Patsoukis N, Li L, Sari D, Petkova V, Boussiotis VA, 2013. PD-1 increases PTEN phosphatase activity while decreasing PTEN protein stability by inhibiting casein kinase 2. *Mol Cell Biol*;33(16):3091-8.

Pavri R, Nussenzweig MC, 2011. AID targeting in antibody diversity. *Adv Immunol*;110:1-26.

Pawelec G, 2017. Immunosenescence and cancer. *Biogerontology*;18(4):717-721.

Paunu N, Lahermo P, Onkamo P, Ollikainen V, Rantala I, Helén P, Simola KO, Kere J, Haapasalo H, 2002. A novel low-penetrance locus for familial glioma at 15q23-q26.3. *Cancer Res*;62(13):3798-802.

Peck A, Mellins ED, 2010. Plasticity of T-cell phenotype and function: the T helper type 17 example. *Immunology*;129(2):147-53.

Pera A, Campos C, López N, Hassouneh F, Alonso C, Tarazona R, Solana R, 2015. Immunosenescence: Implications for response to infection and vaccination in older people. *Maturitas*;82(1):50-5.

Perillo NL, Pace KE, Seilhamer JJ, Baum LG, 1995. Apoptosis of T cells mediated by galectin-1. *Nature*;378(6558):736-9.

Perry VH, Hume DA, Gordon S, 1985. Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience*;15(2):313-26.

Phan TG, Paus D, Chan TD, Turner ML, Nutt SL, Basten A, Brink R, 2006. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J Exp Med*. 2006 Oct 30;203(11):2419-24.

Plantone D, Renna R, Sbardella E, Koudriavtseva T, 2015. Concurrence of multiple sclerosis and brain tumors. *Front Neurol*;6:40. DOI: 10.3389/fneur.2015.00040. Epub 2015 Mar 4.

Qian J, Wang C, Wang B, Yang J, Wang Y, Luo F, Xu J, Zhao C, Liu R, Chu Y. The IFN- γ /PD-L1 axis between T cells and tumor microenvironment: hints for glioma anti-PD-1/PD-L1 therapy. *J Neuroinflammation*;15(1):290. DOI: 10.1186/s12974-018-1330-2. Epub 2018 Oct 17.

Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, Baker J, Jeffery LE, Kaur S, Briggs Z, Hou TZ, Futter CE, Anderson G, Walker LS, Sansom DM, 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*;332(6029):600-3.

Raber PL, Thevenot P, Sierra R, Wyczzechowska D, Halle D, Ramirez ME, Ochoa AC, Fletcher M, Velasco C, Wilk A, Reiss K, Rodriguez PC, 2014. Subpopulations of myeloid-derived suppressor cells impair T cell responses through independent nitric oxide-related pathways. *Int J Cancer*;134(12):2853-64.

Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KGC, Dörner T, Hiepe F, 2006. Competence and competition: the challenges of becoming a long-lived plasma cell. *Nat Rev Immunol*;6(10):741-50.

Rahimi Koshkaki H, Minasi S, Ugolini A, Trevisi G, Napoletano C, Zizzari IG, Gessi M, Giangaspero F, Mangiola A, Nuti M, Buttarelli FR, Rughetti A, 2020. Immunohistochemical characterization of immune infiltrate in tumor microenvironment of glioblastoma. *J Pers Med*;10(3):112. DOI: 10.3390/jpm10030112. Epub 2020 Sep 3.

Rangachari M, Zhu C, Sakuishi K, Xiao S, Karman J, Chen A, Angin M, Wakeham A, Greenfield EA, Sobel RA, Okada H, McKinnon PJ, Mak TW, Addo MM, Anderson AC, Kuchroo VK, 2012. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3–mediated cell death and exhaustion. *Nat Med*;18(9):1394-400.

Raychaudhuri B, Rayman P, Ireland J, Ko J, Rini B, Borden EC, Garcia J, Vogelbaum MA, Finke J, 2011. Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. *Neuro Oncol*;13(6):591-9.

Reardon DA, Brandes AA, Omuro A, Mulholland P, Lim M, Wick A, Baehring J, Ahluwalia MS, Roth P, Bähr O, Phuphanich S, Sepulveda JM, De Souza P, Sahebjam S, Carleton M, Tatsuoka K, Taitt C, Wirtes R, Sampson J, Weller M, 2020. Effect of nivolumab vs bevacizumab in patients with recurrent glioblastoma: the CheckMate 143 phase 3 randomized clinical trial. *JAMA Oncol*;6(7):1003-10.

Rempel SA, Dudas S, Ge S, Gutiérrez JA, 2000. Identification and localization of the cytokine SDF1 and its receptor, CXCR4 chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. *Clin Cancer Res*;6(1):102-11.

Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, Catalan N, Forveille M, Dufourcq-Lapelouise R, Gennery A, Tezcan I, Ersoy F, Kayserili H, Ugazio AG, Brousse N, Muramatsu M, Notarangelo LD, Kinoshita K, Honjo T, Fischer A, Durandy A, 2000. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell*;102(5):565-75.

Ricklefs FL, Alayo Q, Krenzlin H, Mahmoud AB, Speranza MC, Nakashima H, Hayes JL, Lee K, Balaj L, Passaro C, Rooj AK, Krasemann S, Carter BS, Chen CC, Steed T, Treiber J, Rodig S, Yang K, Nakano I, Lee H, Weissleder R, Breakefield XO, Godlewski J, Westphal M, Lamszus K, Freeman GJ, Bronisz A, Lawler SE, Chiocca EA, 2018. Immune evasion mediated by PD-L1 on glioblastoma-derived extracellular vesicles. *Sci Adv*;4(3):eaar2766. DOI: 10.1126/sciadv.aar2766. Epub 2018 Mar 7.

Rivest S. Regulation of innate immune responses in the brain. *Nat Rev Immunol*. 2009 Jun;9(6):429-39.

Roco JA, Mesin L, Binder SC, Nefzger C, Gonzalez-Figueroa P, Canete PF, Ellyard J, Shen Q, Robert PA, Cappello J, Vohra H, Zhang Y, Nowosad CR, Schiepers A, Corcoran LM, Toellner KM, Polo JM, Meyer-Hermann M, Victora GD, Vinuesa CG, 2019. Class-switch recombination occurs infrequently in germinal centers. *Immunity*;51(2):337-350.e7. DOI: 10.1016/j.immuni.2019.07.001. Epub 2019 Jul 30.

Rong Y, Durden DL, Van Meir EG, Brat DJ, 2006. Pseudopalisades in glioblastoma: familiar morphological feature links pathology, hypoxia and angiogenesis. *J Neuropathol Exp Neurol*;65(6):529-39.

Rosales C, Uribe-Querol E. Phagocytosis: a fundamental process in immunity. *Biomed Res Int*;2017:9042851. DOI: 10.1155/2017/9042851. Epub 2017 Jun 12.

Rudjord-Levann AM, Ye Z, Hafkenscheid L, Horn S, Wiegertjes R, Nielsen MAI, Song M, Mathiesen CBK, Stoop J, Stowell S, Straten PT, Leffler H, Vakhrushev SY, Dabelsteen S, Olsen JV, Wandall HH, 2023. Galectin-1 induces a tumor-associated macrophage phenotype and upregulates indoleamine 2,3-dioxygenase-1. *iScience*;26(7):106984. DOI: 10.1016/j.isci.2023.106984. Epub 2023 May 27.

Sad S, Marcotte R, Mosmann TR, 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity*;2(3):271-9.

Sadetzki S, Bruchim R, Oberman B, Armstrong GN, Lau CC, Claus EB, Barnholtz-Sloan JS, Il'yasova D, Schildkraut J, Johansen C, Houlston RS, Shete S, Amos CI, Bernstein JL, Olson SH, Jenkins RB, Lachance D, Vick NA, Merrell R, Wrensch M, Davis FG, McCarthy BJ, Lai R, Melin BS, Bondy ML; Gliogene Consortium, 2013. Description of selected characteristics of familial glioma patients - results from the Gliogene Consortium. *Eur J Cancer*;49(6):1335-45.

Sakhdari A, Mujib S, Vali B, Yue FY, MacParland S, Clayton K, Jones RB, Liu J, Lee EY, Benko E, Kovacs C, Gommerman J, Kaul R, Ostrowski MA, 2012. Tim-3 negatively regulates cytotoxicity in exhausted CD8+ T cells in HIV infection. *PLoS One*;7(7):e40146. DOI: 10.1371/journal.pone.0040146. Epub 2012 Jul 5.

Salama AD, Chitnis T, Imitola J, Ansari MJ, Akiba H, Tushima F, Azuma M, Yagita H, Sayegh MH, Khoury SJ, 2003. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med*;198(1):71-8.

Salvati M, Frati A, Caroli E, Russo N, Polli FM, Domenicucci M, Delfini R, 2003. Glioblastoma in kidney transplant recipients. Report of five cases. *J Neurooncol*;63(1):33-7.

Sano H, Hsu DK, Yu L, Apgar JR, Kuwabara I, Yamanaka T, Hirashima M, Liu FT, 2000. Human galectin-3 is a novel chemoattractant for monocytes and macrophages. *J Immunol*;165(4):2156-64.

Sanz E, Muñoz-A N, Monserrat J, Van-Den-Rym A, Escoll P, Ranz I, Alvarez-Mon M, de-la-Hera A, 2010. Ordering human CD34+CD10-CD19+ pre/pro-B-cell and CD19- common lymphoid progenitor stages in two pro-B-cell development pathways. *Proc Natl Acad Sci U S A*;107(13):5925-30.

Satelli A, Rao PS, Thirumala S, Rao US, 2011. Galectin-4 functions as a tumor suppressor of human colorectal cancer. *Int J Cancer*;129(4):799-809.

Sayour EJ, McLendon P, McLendon R, De Leon G, Reynolds R, Kresak J, Sampson JH, Mitchell DA, 2015. Increased proportion of FoxP3+ regulatory T cells in tumor infiltrating lymphocytes is associated with tumor recurrence and reduced survival in patients with glioblastoma. *Cancer Immunol Immunother*;64(4):419-27.

Scherer H, 1940. Cerebral astrocytomas and their derivatives. *Am J Cancer*;40(2):159-98.

Schlichtner S, Yasinska IM, Lall GS, Berger SM, Ruggiero S, Cholewa D, Aliu N, Gibbs BF, Fasler-Kan E, Sumbayev VV, 2023. T lymphocytes induce human cancer cells derived from solid malignant tumors to secrete galectin-9 which facilitates immunosuppression in cooperation with other immune checkpoint proteins. *J Immunother Cancer*;11(1):e005714. DOI: 10.1136/jitc-2022-005714. Epub 2023 Jan.

Schiff D, O'Neill B, Wijdicks E, Antin JH, Wen PY, 2001. Gliomas arising in organ transplant recipients: an unrecognized complication of transplantation? *Neurology*;57(8):1486-8.

Schwartzbaum J, Ahlbom A, Malmer B, Lönn S, Brookes AJ, Doss H, Debinski W, Henriksson R, Feychting M, 2005. Polymorphisms associated with asthma are inversely related to glioblastoma multiforme. *Cancer Res*;65(14):6459-65.

Schwartzbaum J, Ding B, Johannesen TB, Osnes LT, Karavodin L, Ahlbom A, Feychting M, Grimsrud TK, 2012. Association between prediagnostic IgE levels and risk of glioma. *J Natl Cancer Inst*;104(16):1251-9.

Schwartzbaum J, Edlinger M, Zigmont V, Stattin P, Rempala GA, Nagel G, Hammar N, Ulmer H, Föger B, Walldius G, Manjer J, Malmström H, Feychting M, 2017. Associations between prediagnostic blood glucose levels, diabetes, and glioma. *Sci Rep*;7(1):1436. DOI: 10.1038/s41598-017-01553-2. Epub 2017 May 3.

Seidler S, Zimmermann HW, Bartneck M, Trautwein C, Tacke F, 2010. Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. *BMC Immunol*;11:30. doi: 10.1186/1471-2172-11-30. Epub 2010 Jun 21.

Seliger C, Ricci C, Meier CR, Bodmer M, Jick SS, Bogdahn U, Hau P, Leitzmann MF, 2016. Diabetes, use of antidiabetic drugs, and the risk of glioma. *Neuro Oncol*;18(3):340-9.

Sharim J, Tashjian R, Golzy N, Pouratian N. Glioblastoma following treatment with fingolimod for relapsing-remitting multiple sclerosis. *J Clin Neurosci*;30:166-8.

Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, Qiu Y, Jussif JM, Carter LL, Wood CR, Chaudhary D, 2004. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett*;574(1-3):37-41.

Shi C, Pamer EG, 2011. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol*;11(11):762-74.

Shimato S, Maier LM, Maier R, Bruce JN, Anderson RC, Anderson DE, 2012. Profound tumor-specific Th2 bias in patients with malignant glioma. *BMC Cancer*;12:561. DOI: 10.1186/1471-2407-12-561. Epub 2012 Nov 27.

Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, Charron J, Datta M, Young F, Stall AM, Alt FW, 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*;68(5):855-67.

Shinnakasu R, Inoue T, Kometani K, Moriyama S, Adachi Y, Nakayama M, Takahashi Y, Fukuyama H, Okada T, Kurosaki T, 2016. Regulated selection of germinal-center cells into the memory B cell compartment. *Nat Immunol*;17(7):861-9.

Shinohara T, Taniwaki M, Ishida Y, Kawaichi M, Honjo T, 1994. Structure and chromosomal localization of the human PD-1 gene (PDCD1). *Genomics*;23(3):704-6.

Shiromizu CM, Jancic CC, 2018. $\gamma\delta$ T lymphocytes: an effector cell in autoimmunity and infection. *Front Immunol*;9:2389. DOI:10.3389/fimmu.2018.02389. Epub 2018 Oct 16.

Sierra A, de Castro F, Del Río-Hortega J, Rafael Iglesias-Rozas J, Garrosa M, Kettenmann H, 2016. The "Big-Bang" for modern glial biology: Translation and comments on Pío del Río-Hortega 1919 series of papers on microglia. *Glia*;64(11):1801-40.

Sierra Morales F, Wright RB, Novo JE, Arvanitis LD, Stefoski D, Koralnik IJ, 2017. Glioblastoma in natalizumab-treated multiple sclerosis patients. *Ann Clin Transl Neurol*;4(7):512-6.

Simons K, Ikonen E, 1997. Functional rafts in cell membranes. *Nature*;387(6633):569-72.

Singer A, Adoro S, Park JH, 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol*;8(10):788-801.

Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO, 2008. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*;10(12):1470-6.

Smith DR, Hardman JM, Earle KM, 1969. Metastasizing neuroectodermal tumors of the central nervous system. *J Neurosurg*;31(1):50-8.

Smith KG, Light A, Nossal GJ, Tarlinton DM, 1997. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *EMBO J*;16(11):2996-3006.

Smyth LCD, Rustenhoven J, Scotter EL, Schweder P, Faull RLM, Park TIH, Dragunow M, 2018. Markers for human brain pericytes and smooth muscle cells. *J Chem Neuroanat*;92:48-60.

Song L, Tang JW, Owusu L, Sun MZ, Wu J, Zhang J, 2014. Galectin-3 in cancer. *Clin Chim Acta*;431:185-91.

Sørensen MD, Dahlrot RH, Boldt HB, Hansen S, Kristensen BW, 2018. Tumour-associated microglia/macrophages predict poor prognosis in high-grade gliomas and correlate with an aggressive tumour subtype. *Neuropathol Appl Neurobiol*;44(2):185-206.

Stelzer G, Rosen N, Plaschkes I, Zimmerman S, Twik M, Fishilevich S, Stein TI, Nudel R, Lieder I, Mazor Y, Kaplan S, Dahary D, Warshawsky D, Guan-Golan Y, Kohn A, Rappaport N, Safran M, Lancet D, 2016. The GeneCards Suite: From gene data mining to disease genome sequence analyses. *Curr Protoc Bioinformatics*;54:1.30.1-1.30.33. Database available at www.genecards.org

Stillman BN, Hsu DK, Pang M, Brewer CF, Johnson P, Liu FT, Baum LG, 2006. Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J Immunol*;176(2):778-89.

Stowell SR, Karmakar S, Stowell CJ, Dias-Baruffi M, McEver RP, Cummings RD, 2007. Human galectin-1, -2, and -4 induce surface exposure of phosphatidylserine in activated human neutrophils but not in activated T cells. *Blood*;109(1):219-27.

Stowell SR, Qian Y, Karmakar S, Koyama NS, Dias-Baruffi M, Leffler H, McEver RP, Cummings RD, 2008. Differential roles of galectin-1 and galectin-3 in regulating leukocyte viability and cytokine secretion. *J Immunol*;180(5):3091-102.

Stowell SR, Karmakar S, Arthur CM, Ju T, Rodrigues LC, Riul TB, Dias-Baruffi M, Miner J, McEver RP, Cummings RD, 2009. Galectin-1 induces reversible phosphatidylserine exposure at the plasma membrane. *Mol Biol Cell*;20(5):1408-18.

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group, 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*;352(10):987-96.

Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG, Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group, 2009. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol*;10(5):459-66.

Su TT, Rawlings DJ, 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *J Immunol*;168(5):2101-10.

Sullivan BM, Juedes A, Szabo SJ, von Herrath M, Glimcher LH, 2003. Antigen-driven effector CD8 T cell function regulated by T-bet. *Proc Natl Acad Sci U S A*;100(26):15818-23.

Sun L, Kienzler JC, Reynoso JG, Lee A, Shiuan E, Li S, Kim J, Ding L, Monteleone AJ, Owens GC, Phillips JJ, Everson RG, Nathanson D, Cloughesy TF, Li G, Liao LM, Hugo W, Kim W, Prins RM, 2023. Immune checkpoint blockade induces distinct alterations in the microenvironments of primary and metastatic brain tumors. *J Clin Invest*;133(17):e169314. DOI: 10.1172/JCI169314. Epub 2023 Sep 1.

Sutter PA, Crocker SJ, 2022. Glia as antigen-presenting cells in the central nervous system. *Curr Opin Neurobiol*;77:102646. DOI: 10.1016/j.conb.2022.102646. Epub 2022 Nov 10.

Suttles J, Stout RD, 2009. Macrophage CD40 signaling: a pivotal regulator of disease protection and pathogenesis. *Semin Immunol*;21(5):257-64.

Swallow MM, Wallin JJ, Sha WC, 1999. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNF α . *Immunity*;11(4):423-32.

Szulzewsky F, Pelz A, Feng X, Synowitz M, Markovic D, Langmann T, Holtman IR, Wang X, Eggen BJ, Boddeke HW, Hambardzumyan D, Wolf SA, Kettenmann H, 2015. Glioma-associated microglia/macrophages display an expression profile different from M1 and M2 polarization and highly express Gpnmb and Spp1. *PLoS One*;10(2):e0116644. DOI: 10.1371/journal.pone.0116644. Epub 2015 Feb 6.

Taggart D, Andreou T, Scott KJ, Williams J, Rippas N, Brownlie RJ, Ilett EJ, Salmond RJ, Melcher A, Longer M, 2018. Anti-PD-1/anti-CTLA-4 efficacy in melanoma brain metastases depends on extracranial disease and augmentation of CD8⁺ T cell trafficking. *Proc Natl Acad Sci U S A*;115(7):E1540-9.

Takashima Y, Kawaguchi A, Kanayama T, Hayano A, Yamanaka R, 2018. Correlation between lower balance of Th2 helper T-cells and expression of PD-L1/PD-1 axis genes enables prognostic prediction in patients with glioblastoma. *Oncotarget*;9(27):19065-78.

Tan Y, Zheng Y, Xu D, Sun Z, Yang H, Yin Q, 2021. Galectin-3: a key player in microglia-mediated neuroinflammation and Alzheimer's disease. *Cell Biosci*;11(1):78. DOI: 10.1186/s13578-021-00592-7. Epub 2021 Apr 27.

Tawbi HA, Forsyth PA, Algazi A, Hamid O, Hodi FS, Moschos SJ, Khushalani NI, Lewis K, Lao CD, Postow MA, Atkins MB, Ernstoff MS, Reardon DA, Puzanov I, Kudchadkar RR, Thomas RP, Tarhini A, Pavlick AC, Jiang J, Avila A, Demelo S, Margolin K, 2018. Combined nivolumab and ipilimumab in melanoma metastatic to the brain. *N Engl J Med*;379(8):722-30.

Thiault N, Darrigues J, Adoue V, Gros M, Binet B, Peral C, Leobon B, Fazilleau N, Joffre OP, Robey EA, van Meerwijk JP, Romagnoli P, 2015. Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors. *Nat Immunol*;16(6):628-34.

Thiemann S, Baum LG, 2016. Galectins and immune responses-just how do they do those things they do? *Annu Rev Immunol*;34:243-64.

Thijssen VL, Hulsmans S, Griffioen AW, 2008. The galectin profile of the endothelium: altered expression and localization in activated and tumor endothelial cells. *Am J Pathol*;172(2):545-53.

Thomas AA, Fisher JL, Rahme GJ, Hampton TH, Baron U, Olek S, Schwachula T, Rhodes CH, Gui J, Tafe LJ, Tsongalis GJ, Lefferts JA, Wishart H, Kleen J, Miller M, Whipple CA, de Abreu FB, Ernstoff MS, Fadul CE, 2015. Regulatory T cells are not a strong predictor of survival for patients with glioblastoma. *Neuro Oncol*;17(6):801-9.

Thome JJ, Yudanin N, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawala T, Kato T, Lerner H, Shen Y, Farber DL, 2014. Spatial map of human T cell compartmentalization and maintenance over decades of life. *Cell*;159(4):814-28.

Thome JJ, Bickham KL, Ohmura Y, Kubota M, Matsuoka N, Gordon C, Granot T, Griesemer A, Lerner H, Kato T, Farber DL, 2016a. Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. *Nat Med*;22(1):72-7.

Thome JJ, Grinshpun B, Kumar BV, Kubota M, Ohmura Y, Lerner H, Sempowski GD, Shen Y, Farber DL, 2016b. Longterm maintenance of human naive T cells through *in situ* homeostasis in lymphoid tissue sites. *Sci Immunol*;1(6):eaah6506. DOI: 10.1126/sciimmunol.aah6506. Epub 2016 Dec 2.

Tian T, Liang R, Erel-Akbaba G, Saad L, Obeid PJ, Gao J, Chiocci EA, Weissleder R, Tannous BA, 2022. Immune checkpoint inhibition in GBM primed with radiation by engineered extracellular vesicles. *ACS Nano*;16(2):1940-53.

Tian X, Zhang A, Qiu C, Wang W, Yang Y, Qiu C, Liu A, Zhu L, Yuan S, Hu H, Wang W, Wei Q, Zhang X, Xu J, 2015. The upregulation of LAG-3 on T cells defines a subpopulation with functional exhaustion and correlates with disease progression in HIV-infected subjects. *J Immunol*;194(8):3873-82.

Tiegs SL, Russell DM, Nemazee D, 1993. Receptor editing in self-reactive bone marrow B cells. *J Exp Med*;177(4):1009-20.

Toscano MA, Bianco GA, Ilarregui JM, Croci DO, Correale J, Hernandez JD, Zwirner NW, Poirier F, Riley EM, Baum LG, Rabinovich GA, 2007. Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol*;8(8):825-34.

Toussaint LG 3rd, Nilson AE, Goble JM, Ballman KV, James CD, Lefranc F, Kiss R, Uhm JH, 2012. Galectin-1, a gene preferentially expressed at the tumor margin, promotes glioblastoma cell invasion. *Mol Cancer*;11:32. doi: 10.1186/1476-4598-11-32. Epub 2012 May 14.

Tracey BM, Feizi T, Abbott WM, Carruthers RA, Green BN, Lawson AM, 1992. Subunit molecular mass assignment of 14,654 Da to the soluble beta-galactoside-binding lectin from bovine heart muscle and demonstration of intramolecular disulfide bonding associated with oxidative inactivation. *J Biol Chem*;267(15):10342-7.

Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, Hercend T, 1990. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med*;171(5):1393-405.

Tritz ZP, Orozco RC, Malo CS, Ayasoufi K, Fain CE, Khadka RH, Goddery EN, Yokanovich LT, Settell ML, Hansen MJ, Jin F, Pavelko KD, Pease LR, Johnson AJ, 2020. Conditional silencing of H-2D^b class I molecule expression modulates the protective and pathogenic kinetics of virus-antigen-specific CD8 T cell responses during Theiler's virus infection. *J Immunol*;205(5):1228-38.

Tritz ZP, Ayasoufi K, Johnson AJ, 2021. Anti-PD-1 checkpoint blockade monotherapy in the orthotopic GL261 glioma model: the devil is in the detail. *Neurooncol Adv*;3(1):vdab066. DOI: 10.1093/noajnl/vdab066. Epub 2021 May 14.

Tsai CH, Tzeng SF, Chao TK, Tsai CY, Yang YC, Lee MT, Hwang JJ, Chou YC, Tsai MH, Cha TL, Hsiao PW, 2016. Metastatic progression of prostate cancer is mediated by autonomous binding of galectin-4-O-glycan to cancer cells. *Cancer Res*;76(19):5756-67. Erratum in *Cancer Res*;77(10):2772.

Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, Shalabi A, Shin T, Pardoll DM, Tsuchiya H, 2001. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med*;193(7):839-46.

UniProt Consortium, 2023. UniProt: the Universal Protein Knowledgebase in 2023. *Nucleic Acids Res*;51(D1):D523-D531. Database available at: www.uniprot.org

Valle E, Guiney DG, 2005. Characterization of Salmonella-induced cell death in human macrophage-like THP-1 cells. *Infect Immunol* 73(5):2835-40.

van der Vos KE, Abels ER, Zhang X, Lai C, Carrizosa E, Oakley D, Prabhakar S, Mardini O, Crommentuijn MH, Skog J, Krichevsky AM, Stemmer-Rachamimov A, Mempel TR, El Khoury J, Hickman SE, Breakefield XO, 2016. Directly visualized glioblastoma-derived extracellular vesicles transfer RNA to microglia/macrophages in the brain. *Neuro Oncol*;18(1):58-69.

Van Woensel M, Mathivet T, Wauthoz N, Rosière R, Garg AD, Agostinis P, Mathieu V, Kiss R, Lefranc F, Boon L, Belmans J, Van Gool SW, Gerhardt H, Amighi K, De Vleeschouwer S, 2017. Sensitization of glioblastoma tumor micro-environment to chemo- and immunotherapy by Galectin-1 intranasal knock-down strategy. *Sci Rep*;7(1):1217. DOI: 10.1038/s41598-017-01279-1. Epub 2017 Apr 27.

Vannemreddy PS, Fowler M, Polin RS, Todd JR, Nanda A, 2000. Glioblastoma multiforme in a case of acquired immunodeficiency syndrome: investigation a possible oncogenic influence of human immunodeficiency virus on glial cells. Case report and review of the literature. *J Neurosurg*;92(1):161-4.

Veglia F, Perego M, Gabrilovich D, 2018. Myeloid-derived suppressor cells coming of age. *Nat Immunol*;19(2):108-19.

Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, Martin B, Wilhelm C, Stockinger B, 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*;9(12):1341-6.

Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, Getz G, Perou CM, Hayes DN, Cancer Genome Atlas Research Network, 2010. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*;17(1):98-11.

Verschoor CP, Johnstone J, Millar J, Parsons R, Lelic A, Loeb M, Bramson JL, Bowdish DM, 2014. Alterations to the frequency and function of peripheral blood monocytes and associations with chronic disease in the advanced-age, frail elderly. *PLoS One*;9(8):e104522. DOI: 10.1371/journal.pone.0104522. Epub 2014 Aug 8.

Verschuere T, Van Woensel M, Fieuws S, Lefranc F, Mathieu V, Kiss R, Van Gool SW, De Vleeschouwer S, 2013. Altered galectin-1 serum levels in patients diagnosed with high-grade glioma. *J Neurooncol*;115(1):9-17.

Verschuere T, Toelen J, Maes W, Poirier F, Boon L, Tousseyn T, Mathivet T, Gerhardt H, Mathieu V, Kiss R, Lefranc F, Van Gool SW, De Vleeschouwer S, 2014. Glioma-derived galectin-1 regulates innate and adaptive antitumor immunity. *Int J Cancer*;134(4):873-84.

Virga J, Szivos L, Hortobágyi T, Chalsaraei MK, Zahuczky G, Steiner L, Tóth J, Reményi-Puskár J, Bognár L, Klekner A, 2019. Extracellular matrix differences in glioblastoma patients with different prognoses. *Oncol Lett*;17(1):797-806.

Wainwright DA, Chang AL, Dey M, Balyasnikova IV, Kim CK, Tobias A, Cheng Y, Kim JW, Qiao J, Zhang L, Han Y, Lesniak MS, 2014. Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors. *Clin Cancer Res*;20(20):5290-301.

Waisman A, Johann L, 2018. Antigen-presenting cell diversity for T cell reactivation in central nervous system autoimmunity. *J Mol Med (Berl)*;96(12):1279-92.

Wang YH, Nomura J, Faye-Petersen OM, Cooper MD, 1998. Surrogate light chain production during B cell differentiation: differential intracellular versus cell surface expression. *J Immunol*;161(3):1132-9.

Wang N, Jain RK, Batchelor TT, 2017. New directions in anti-angiogenic therapy for glioblastoma. *Neurotherapeutics*;14(2):321-32.

Wang T, Gao T, Niu X, Xing X, Yang Y, Liu Y, Mao Q, 2018. Clinical characteristics and prognostic analysis of glioma in human immunodeficiency virus-infected patients. *World Neurosurg*;114:e218-e223.

Wang H, Song X, Huang Q, Xu T, Yun D, Wang Y, Hu L, Yan Y, Chen H, Lu D, Chen J, 2019. LGALS3 promotes treatment resistance in glioblastoma and is associated with tumor risk and prognosis. *Cancer Epidemiol Biomarkers Prev*;28(4):760-9.

Wang Y, Sun Y, Tang J, Zhou W, Liu X, Bi Y, Zhang ZJ, 2019. Does diabetes decrease the risk of glioma? A systematic review and meta-analysis of observational studies. *Ann Epidemiol*;30:22-9.e3. DOI: 10.1016/j.annepidem.2018.11.010. Epub 2018 Nov 26.

Wang M, Cai Y, Peng Y, Xu B, Hui W, Jiang Y, 2020. Exosomal LGALS9 in the cerebrospinal fluid of glioblastoma patients suppressed dendritic cell antigen presentation and cytotoxic T-cell immunity. *Cell Death Dis*;11(10):896. DOI: 10.1038/s41419-020-03042-3. Epub 2020 Oct 22.

Wegner RE, Abel S, Horne ZD, Hasan S, Verma V, Ranjan T, Williamson RW, Karlovits SM, 2019. National trends in radiation dose escalation for glioblastoma. *Radiat Oncol J*;37(1):13-21.

Wei J, Barr J, Kong LY, Wang Y, Wu A, Sharma AK, Gumin J, Henry V, Colman H, Priebe W, Sawaya R, Lang FF, Heimberger AB, 2010. Glioblastoma cancer-initiating cells inhibit T-cell proliferation and effector responses by the signal transducers and activators of transcription 3 pathway. *Mol Cancer Ther*;9(1):67-78.

Weinmann D, Kenn M, Schmidt S, Schmidt K, Walzer SM, Kubista B, Windhager R, Schreiner W, Toegel S, Gabius HJ, 2018. Galectin-8 induces functional disease markers in human osteoarthritis and cooperates with galectins-1 and -3. *Cell Mol Life Sci*;75(22):4187-205.

Wells WJ, Parkman R, Smogorzewska E, Barr M, 1998. Neonatal thymectomy: does it affect immune function? *J Thorac Cardiovasc Surg*;115(5):1041-6.

Wells JE, Howlett M, Cole CH, Kees UR, 2014. Deregulated expression of connective tissue growth factor (CTGF/CCN2) is linked to poor outcome in human cancer. *Int J Cancer*;137(3):504-11.

Wen PY, Weller M, Lee EQ, Alexander BM, Barnholtz-Sloan JS, Barthel FP, Batchelor TT, Bindra RS, Chang SM, Chiocca EA, Cloughesy TF, DeGroot JF, Galanis E, Gilbert MR, Hegi ME, Horbinski C, Huang RY, Lassman AB, Le Rhun E, Lim M, Mehta MP, Mellinger IK, Minniti G, Nathanson D, Platten M, Preusser M, Roth P, Sanson M, Schiff D, Short SC, Taphoorn MJB, Tonn JC, Tsang J, Verhaak RGW, von Deimling A, Wick W, Zadeh G, Reardon DA, Aldape KD, van den Bent MJ, 2020. Glioblastoma in adults: a Society for Neuro-Oncology (SNO) and European Society of Neuro-Oncology (EANO) consensus review on current management and future directions. *Neuro Oncol*;22(8):1073-113.

Wen P, 2021. Surgical nivolumab and ipilimumab for recurrent GBM. In: *Clinicaltrials.gov* [online]. 2021 [cited 2023 Sep 22]. Available at: <https://www.clinicaltrials.gov/study/NCT04606316>.

Wiemels JL, Wiencke JK, Patoka J, Moghadassi M, Chew T, McMillan A, Miike R, Barger G, Wrensch M, 2004. Reduced immunoglobulin E and allergy among adults with glioma compared with controls. *Cancer Res*;64(22):8468-73.

Wilson TA, Karajannis MA, Harter DH, 2014. Glioblastoma multiforme: State of the art and future therapeutics. *Surg Neurol Int*;5:64. DOI:10.4103/2152-7806.132138. Epub 2014 May 8.

Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S, 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*;322(5899):271-5.

Wing K, Sakaguchi S, 2010. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol*;11(1):7-13.

Wing JB, Sakaguchi S, 2012. Multiple treg suppressive modules and their adaptability. *Front Immunol*;3:178. DOI: 10.3389/fimmu.2012.00178. Epub 2012 Jun 29.

Wong ET, Huberman M, Lu XQ, Mahadevan A, 2008. Bevacizumab reverses cerebral radiation necrosis. *J Clin Oncol*;26(34):5649-50.

Workman CJ, Dugger KJ, Vignali DA, 2002. Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. *J Immunol*;169(10):5392-5.

Workman CJ, Vignali DA, 2003. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. *Eur J Immunol*;33(4):970-9.

Workman CJ, Cauley LS, Kim IJ, Blackman MA, Vignali DA, 2004. Lymphocyte activation gene 3 (CD223) regulates the size of the expanding T cell population following activation in vivo. *J Immunol*;172(9):5450-55.

Workman CJ, Vignali DA, 2005. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J Immunol*;174(2):688-95.

Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, Elsamadicy AA, Cui X, Koyama S, Jackson C, Hansen LJ, Johanns TM, Sanchez-Perez L, Chandramohan V, Yu YA, Bigner DD, Giles A, Healy P, Dranoff G, Weinhold KJ, Dunn GP, Fecci PE, 2018. T-cell exhaustion signatures vary with tumor type and are severe in glioblastoma. *Clin Cancer Res*;24(17):4175-86.

Wrensch M, Minn Y, Chew T, Bondy M, Berger MS, 2002. Epidemiology of primary brain tumors: current concepts and review of the literature. *Neuro Oncol*;4(4):278-99.

Wrensch M, Jenkins RB, Chang JS, Yeh RF, Xiao Y, Decker PA, Ballman KV, Berger M, Buckner JC, Chang S, Giannini C, Halder C, Kollmeyer TM, Kosel ML, LaChance DH, McCoy L, O'Neill BP, Patoka J, Pico AR, Prados M, Quesenberry C, Rice T, Rynearson AL, Smirnov I, Tihan T, Wiemels J, Yang P, Wiencke JK, 2009. Variants in the CDKN2B and RTEL1 regions are associated with high-grade glioma susceptibility. *Nat Genet*;41(8):905-8.

Wu TH, Li YY, Wu TL, Chang JWC, Chou WC, Hsieh LL, Chen JR, Yeh KY, 2014. Culture supernatants of different colon cancer cell lines induce specific phenotype switching and functional alteration of THP-1 cells. *Cell Immunol*;290(1):107-15.

Wu S, Li X, Hong F, Chen Q, Yu Y, Guo S, Xie Y, Xiao N, Kong X, Mo W, Wang Z, Chen S, Zeng F, 2023. Integrative analysis of single-cell transcriptomics reveals age-associated immune landscape of glioblastoma. *Front Immunol*;14:1028775. DOI: 10.3389/fimmu.2023.1028775. Epub 2023 Jan 24.

Xie D, Yin D, Wang HJ, Liu GT, Elashoff R, Black K, Koeffler HP, 2004. Levels of expression of CYR61 and CTGF are prognostic for tumor progression and survival of individuals with gliomas. *Clin Cancer Res*;10(6):2072-81.

Xing Y, Hogquist KA, 2012. T-cell tolerance: central and peripheral. *Cold Spring Harb Perspect Biol*;4(6):a006957. DOI: 10.1101/cshperspect.a006957. Epub 2012 Jun 1.

Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, De Nardo D, Gohel TD, Emde M, Schmidleithner L, Ganesan H, Nino-Castro A, Mallmann MR, Labzin L, Theis H, Kraut M, Beyer M, Latz E, Freeman TC, Ulas T, Schultze JL, 2014. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity*;40(2):274-88.

Yamaguchi T, Wing JB, Sakaguchi S, 2011. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. *Semin Immunol*;23(6):424-30.

Yamaguchi T, Hiromasa K, Kabashima-Kubo R, Yoshioka M, Nakamura M, 2013. Galectin-7, induced by cis-urocanic acid and ultraviolet B irradiation, down-modulates cytokine production by T lymphocytes. *Exp Dermatol*;22(12):840-2.

- Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, Watowich SS, Tian Q, Jetten AM, Dong C, 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity*;28(1):29-39.
- Yang I, Tihan T, Han SJ, Wrensch MR, Wiencke J, Sughrue ME, Parsa AT, 2010. CD8⁺ T-cell infiltrate in newly diagnosed glioblastoma is associated with long-term survival. *J Clin Neurosci*;17(11):1381-5.
- Yang M, Guo W, Yang C, Tang J, Huang Q, Feng S, Jiang A, Xu X, Jiang G, 2017. Mobile phone use and glioma risk: A systematic review and meta-analysis. *PLoS One*;12(5):e0175136. DOI: 10.1371/journal.pone.0175136. Epub 2017 May 4.
- Yang F, He Z, Duan H, Zhang D, Li J, Yang H, Dorsey JF, Zou W, Nabavizadeh SA, Bagley SJ, Abdullah K, Brem S, Zhang L, Xu X, Byrne KT, Vonderheide RH, Gong Y, Fan Y, 2021. Synergistic immunotherapy of glioblastoma by dual targeting of IL-6 and CD40. *Nat Commun*;12(1):3424. DOI: 10.1038/s41467-021-23832-3. Epub 2021 Jun 8.
- Yekula A, Yekula A, Muralidharan K, Kang K, Carter BS, Balaj L, 2020. Extracellular vesicles in glioblastoma tumor microenvironment. *Front Immunol*;10:3137. doi: 10.3389/fimmu.2019.03137. Epub 2020 Jan 21.
- Yel L, Minegishi Y, Coustan-Smith E, Buckley RH, Trübel H, Pachman LM, Kitchingman GR, Campana D, Rohrer J, Conley ME, 1996. Mutations in the mu heavy-chain gene in patients with agammaglobulinemia. *N Engl J Med*;335(20):1486-93.
- Yin D, Chen W, O'Kelly J, Lu D, Ham M, Doan NB, Xie D, Wang C, Vadgama J, Said JW, Black KL, Koeffler HP, 2010. Connective tissue growth factor associated with oncogenic activities and drug resistance in glioblastoma multiforme. *Int J Cancer*;127(10):2257-67.
- Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T, 2012. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med*;209(6):1201-17.
- Yu J, Freud AG, Caligiuri MA, 2013. Location and cellular stages of natural killer cell development. *Trends Immunol*;34(12):573-82. DOI: 10.1016/j.it.2013.07.005. Epub 2013 Sep 19.
- Yu Y, Ma X, Gong R, Zhu J, Wei L, Yao J, 2018. Recent advances in CD8⁺ regulatory T cell research. *Oncol Lett*;15(6):8187-94.
- Yu D, Ye L, 2018. A portrait of CXCR5⁺ follicular cytotoxic CD8⁺ T cells. *Trends Immunol*;39(12):965-79.

Yuan F, Ming H, Wang Y, Yang Y, Yi L, Li T, Ma H, Tong L, Zhang L, Liu P, Li J, Lin Y, Yu S, Ren B, Yang X, 2020. Molecular and clinical characterization of galectin-9 in glioma through 1,027 samples. *J Cell Physiol*;235(5):4326-34.

Zagzag D, Salnikow K, Chiriboga L, Yee H, Lan L, Ali MA, Garcia R, Demaria S, Newcomb EW, 2005. Downregulation of major histocompatibility complex antigens in invading glioma cells: stealth invasion of the brain. *Lab Invest*;85(3):328-41.

Zeiner PS, Preusse C, Golebiewska A, Zinke J, Iriondo A, Muller A, Kaoma T, Filipowski K, Müller-Eschner M, Bernatz S, Blank AE, Baumgarten P, Ilina E, Grote A, Hansmann ML, Verhoff MA, Franz K, Feuerhake F, Steinbach JP, Wischhusen J, Stenzel W, Niclou SP, Harter PN, Mittelbronn M, 2019. Distribution and prognostic impact of microglia/macrophage subpopulations in gliomas. *Brain Pathol*;29(4):513-29.

Zeng H, Yang Z, Xu N, Liu B, Fu Z, Lian C, Guo H, 2017. Connective tissue growth factor promotes temozolomide resistance in glioblastoma through TGF- β 1-dependent activation of Smad/ERK signaling. *Cell Death Dis.*;8(6):e2885. DOI: 10.1038/cddis.2017.248. Epub 2017 Jun 15.

Zhang J, Stevens MF, Bradshaw TD, 2012. Temozolomide: mechanisms of action, repair and resistance. *Curr Mol Pharmacol*;5(1):102-14.

Zhang J, Sai K, Wang XL, Ye SQ, Liang LJ, Zhou Y, Chen ZJ, Hu WM, Liu JM, 2020. Tim-3 expression and MGMT methylation status association with survival in glioblastoma. *Front Pharmacol*;11:584652. DOI: 10.3389/fphar.2020.584652. Epub 2020 Sep 15.

Zhang IY, Liu S, Zhang L, Liang R, Fang Q, Zhao J, Ren L, Medina EF, Filippov A, Bonjoc KJ, Chaudhry A, Dayyani M, Bild AH, Badie B, 2023. RAGE ablation attenuates glioma progression and enhances tumor immune responses by suppressing galectin-3 expression. *Neuro Oncol*;25(5):886-98.

Zhao J, Chen AX, Gartrell RD, Silverman AM, Aparicio L, Chu T, Bordbar D, Shan D, Samanamud J, Mahajan A, Filip I, Orenbuch R, Goetz M, Yamaguchi JT, Cloney M, Horbinski C, Lukas RV, Raizer J, Rae AI, Yuan J, Canoll P, Bruce JN, Saenger YM, Sims P, Iwamoto FM, Sonabend AM, Rabadan R, 2019. Immune and genomic correlates of response to anti-PD-1 immunotherapy in glioblastoma. *Nat Med*;25(3):462-9.

Zhao G, Yu H, Ding L, Wang W, Wang H, Hu Y, Qin L, Deng G, Xie B, Li G, Qi L, 2022. microRNA-27a-3p delivered by extracellular vesicles from glioblastoma cells induces M2 macrophage polarization via the EZH1/KDM3A/CTGF axis. *Cell Death Discov*;8(1):260. DOI: 10.1038/s41420-022-01035-z. Epub 2022 May 14.

Zhou W, Ke SQ, Huang Z, Flavahan W, Fang X, Paul J, Wu L, Sloan AE, McLendon RE, Li X, Rich JN, Bao S, 2015. Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. *Nat Cell Biol*;17(2):170-82.

Zhou F, Shi Q, Fan X, Yu R, Wu Z, Wang B, Tian W, Yu T, Pan M, You Y, Wang Y, 2022. Diverse macrophages constituted the glioma microenvironment and influenced by PTEN status. *Front Immunol*;13:841404.

Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK, 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol*;6(12):1245-52.

Zhu X, Fujita M, Snyder LA, Okada H, 2011. Systemic delivery of neutralizing antibody targeting CCL2 for glioma therapy. *J Neurooncol*;104(1):83-92.