Investigating the inhibitory effects of secreted proteins on macrophage in glioblastoma

Investigating the inhibitory effects of secreted proteins on macrophage in Glioblastoma

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DECLARATION

This work or any part thereof has not previously been presented in any form to the University or any other body whether for the purposes of assessment, publication or for any other purpose (unless otherwise indicated). Save for any express acknowledgements, references and/or bibliographies cited in the work, I confirm that the intellectual content of the work is the result of my own efforts and of other people.

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Abstract

Cancer is one of the major causes for the mortality in the whole world and among the cancers, glioblastoma is the most common and aggressive form of malignant brain tumours, especially in the adults. The complex tumour microenvironment of glioblastoma contributes to immune suppression and inhibition of T cell activity through secreted immuno-suppressive proteins and cytokines, dysfunction of immune cells and upregulation of immune checkpoint molecules. Macrophages are the most populous non neoplastic cells in glioblastoma microenvironment, which is a part of the innate immune response and are involved in immune suppression, tumour growth, invasion, and metastasis.

The glioblastoma secretions to the microenvironment were studied using conditioned media and the day 3 with higher protein concentration were considered for studying the phagocytic activity and antigen presentation marker expression. The proteins present in the conditioned medium which were derived from these glioblastoma cell lines exert an inhibitory effect on macrophage phagocytosis. The transition from monocyte to macrophages and analysed the antigen presentation markers on monocytes and macrophages and antigen presentation marker HLA-DR had downregulated and the co-stimulatory molecule CD86 got upregulated in the presence of conditioned media suggests

secretions from glioblastoma have inhibitory effect on monocytes, macrophages and the LPS stimulated M1 macrophages.

Previous studies using mass spectrometry had revealed that out of 59 glioblastoma samples, 58 showed Galectin-1 as commonly secreted protein and galectin-1 was accumulated near T cells which were present around the blood vessels in glioblastoma samples. The galectin-1 secretion was regulated by IFN-ϒ, which might be from activated T cells. Along with this, the PDL-1 and galectin-9, the immune checkpoint molecules expressed on human brain endothelial cells were studied in the presence of conditioned media and IFN-ϒ. The PDL-1 was upregulated on IFN-ϒ treated conditioned media, and galectin-9 upregulated upon IFN-ϒ and the conditioned media. The immune checkpoint molecules upregulation from IFN-ϒ and conditioned media gives insight to the immune suppression in glioblastoma.

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+/-CTRL	Positive/negative control
Akt	Protein kinase B
APCs	Antigen Presenting Cells
ARG1	Arginase 1
BBB	blood brain barrier
BCA	Bicinchoninic acid
CDKN2A/B	Cyclin-dependent kinase inhibitor 2A/B
CM	Conditioned media
CNS	Central Nervous System
CTLA 4	cytotoxic T-lymphocyte associated protein 4
DCs	Dendritic cells
EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial Mesenchymal Transition
FasL	Fas Ligand
FGFR	Fibroblast growth factor receptor
GAMs	Glioma Associated Macrophages/Microglia
GB	Glioblastoma
HIF	Hypoxia Inducible Factor
HLA-DR	Human Leukocyte Antigen DR isotype
IDH mut/wt	Isocitrate Dehydrogenase mutant/wildtype
IFN-ϒ	Interferon ϒ
iNOS	inducible nitric oxide synthase
LAG-3	Lymphocyte Activation Gene 3
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases

MCP-1………………………………………………… Monocyte chemoattractant protein 1

MDSCs…………………………………………………… Myeloid derived suppressor cells

MGMT……………………………………………… methylguanine-DNA methyltransferase

MHC……………………………………………………Major Histocompatibility Complex

MIF…………………………………………… Macrophage migration inhibitory factor

MMP……………………………………………………………… Matrix metalloproteinases

mTOR……………………………………………………… mammalian target of rapamycin

NK cells………………………………………………………………………Natural Killer cells

PAMP……………………………………………… pathogen-associated molecular pattern

PD-1………………………………………………………………Programmed Death receptor

PDGFB…………………………………………… Platelet Derived Growth Factor Subunit B

PDL-1………………………………………………………………Programmed Death Ligand

PI3K………………………………………………………………phosphoinositide 3-kinase

PMA…………………………………………………… phorbol 12-myristate 13-acetate

PRR……………………………………………………………… pattern recognition receptor

PTEN…………………………………………………… Phosphatase and TENsin homolog

PTN………………………………………………………………………………… Pleiotrophin

Raf/MEK/ERK…………Raf/ Mitogen-activated protein kinase/ extracellular-signal-regulated kinase

Ras…………………………………………………………………………………… rat sarcoma

RB1…………………………………………………………………………… Retinoblastoma 1

ROS………………………………………………………………………Reactive Oxygen Species

SIRPα……………………………………………………………… Signal regulatory protein α

STAT3………………………………… Signal transducer and activator of transcription 3

STIP1…………………………………………………… Stress-induced phosphoprotein 1

TAMs……………………………………………………… tumour associated macrophages

TCRs………………………………………………………………………………T cell Receptors

TERT……………………………………………………… Telomerase reverse transcriptase

TGF-β……………………………………………………………………Transformation Growth Factor-β

TIGIT………………………………………….……….. T cell immunoglobulin and ITIM domain

TILs………………………………………………..……………….…. tumour infiltrating lymphocytes

TIM-3……………………….……T cell immunoglobulin and mucin-domain containing-3

TNF……………………………..…………………………………….………….Tumour necrosis factor

VEGF……………………………………………………………Vascular Endothelial Growth factor

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1.Introduction

1.1 Glioblastoma

Glioblastoma (GB) is a common and aggressive cancer having higher malignancy rate among the tumours affecting central nervous system with poor survival rate. (Angom et al., 2023, Teraiya et al., 2023). GB had commonly found among adults with symptoms varying from patient to patient but commonly showing symptoms includes headache, vomiting, and difficulties in vision (McKinnon et al., 2021). GB shows resistance to existing treatment options with high microvascular proliferation and pseudo palisading necrosis. WHO has categorised as grade IV tumour due to its complexity (Guo et al., 2023).

GB has a heterogenous population of cells in its tumour microenvironment and the blood brain barrier protects the tumour from prevailing therapeutic options leading to the resistance and cause poor survival rates (Wu et al., 2021). The average overall survival rate among GB patients is less than 15 months and the occurrence rate was reported as 3-4 persons per 100,000 people (Grochans et al., 2022). The overall survival rate is exceptionally low in older patients aged above 70 than the young ones (Iza et al., 2023). GB is generally found among men than women. One of major cause for GB is ionizing radiation and it is known that GB can occur by hereditary (An et al., 2023).

Glioblastoma is classified into isocitrate dehydrogenase wild type (IDH wt) and mutant (IDH mut) type, where IDH is an enzyme involved in Kreb's cycle for the cellular metabolism and the mutation causes synthesis of D-2-hydroxyglutarate instead of α-ketoglutarate leads to tumour. This IDH wt and IDH mut again differentiated into three subtypes called classical, mesenchymal and proneural (Han et al., 2020, Murugan & Alzahrani, 2022, Hoogstrate et al., 2023,). In 2021, in the fifth edition of the International Standards of Classification of brain tumours by WHO, brain tumours were classified into 3 types based on IDH mutant and wild type which are astrocytoma, oligodendroglioma, and glioblastoma (wild type) (Murugan & Alzahrani, 2022).

Almost 90% of the glioblastomas are arising from glial cells in brain due to abnormalities in genetic levels and signalling pathways, whereas 10% are formed from low grade tumours (Reuss, 2023). GB has commonly developed in the frontal, parietal, occipital and temporal lobes in the brain whereas it is uncommon to find GB in the cerebellum and spinal cord in adults compared to young people, where the GB is found in the cerebellum (TAMIMI et al., 2017).

1.1.a Glioblastoma Biology

Brain is the well organised organ, with a complex architecture, comprised of highly specialized cells and linked each other for regulating various biological functions in the human body. Brain consists of neurons and non-neuronal cells that includes glial cells such as astrocytes, microglia, and oligodendrocytes (Jiang & Nardelli, 2016, Quan et al., 2022). The glial cells play a crucial role in maintaining the stability in the brain by providing the support for neurons and immunity to the developing neurons and any dysfunctioning in the glial cell causes neurodegenerative diseases (Tamimi and Huweid, 2017). The one of the tumours originated from the glial cells in the brain are glioblastoma, which is one of the lethal among cancers with high intratumoral heterogeneity and plasticity with high chances of recurrence (Gimple et al., 2019). Glioblastoma originates by the mutations of the oncogenes including *EGFR* and deletions of tumour suppressor genes *CDKN2A, CDKN2B* and *PTEN* in astrocytes, one of the glial cells achieve the tumour characteristics. Also, the neuronal stem cells and oligodendritic precursor cells, which would be deposited in the brain during the embryonic development get aberrations in the oncogenes and tumour suppressor genes and leads to glioblastoma (Zhang et al., 2023, Loras et al., 2023). The GB cells which derived from the astrocytes makes the changes in extra cellular matrix and blood vessels leads to the migration and infiltration of the tumour in surrounding areas of the brain. This changes in the neoplastic tissue environment would change the morphology, metabolism, molecular biology of the astrocytes and leads to immune suppression (Virtuoso et al., 2021). The GB cells have high plasticity and pleomorphic ability with abnormalities in the nucleus than the normal astrocytes leading to brisk mitotic activity that causes rapid proliferation. The pseudopallisading tumour cells supports the formation of necrosis in the tumour core, which would cause the infiltration to the neighbouring normal brain tissues and blood vessel formation (Walker et al., 2011).

In GB, the modifications in the metabolism by various genetic and environmental factors leads to the uncontrolled cell growth and inhibition of apoptosis. The alterations in the carbohydrate, proteins, lipids, and nucleic acid metabolism are commonly observed by the upregulation of PI3K/Akt/mTOR signalling pathways, enhanced glycolytic flux, reprograming in glutamine and arginine metabolism, changes in the lipid storage and modifications in the de novo synthesis and nucleotide salvage pathways (Verdugo et al., 2022, An et al., 2023). The metabolic growth pathways- MAPK, Ras, HIF, EGFR, FGFR were changed or upregulated in the GB causes enhanced cell growth by damages in DNA repair mechanism, autophagy, regulates angiogenesis and immune checkpoints (Zhou & Wahl, 2019).

1.1.b Molecular Biology of Glioblastoma

The aggressiveness of the tumour growth is due its heterogeneity and the aberrantly expressed genes. The DNA methylation and histone modification in the cancer cells causes drug resistance and could lead to mutations in IDH gene, which is the major mutation in GB (Ensenyat-Mendez et al., 2021). The genetic changes in the genes such as *EGFR, PTEN, LOH 10q, RB1*, and *TP53* in various levels could be observed in glioblastoma patients (Fig.1a). Other genes which change in GBM are receptor kinase (RTK), *MGMT, TERT,* and *FGFR1* leads to the poor survival of patients (Li et al., 2016, Hasanzadeh & Niknejad, 2021).

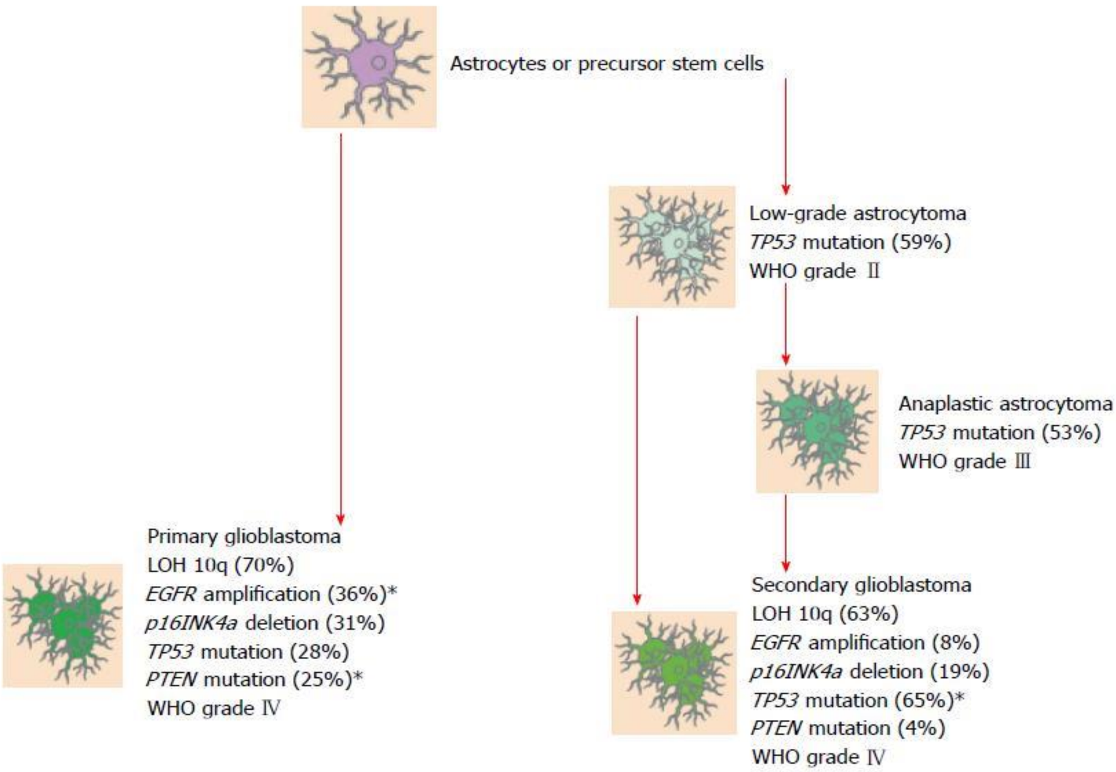


Fig1.a Genetic mutations implicated in the development of all types of brain tumour evolved from astrocytes or neuronal stem cells leads from low grade to malignant gliomas (*Adapted from Joshi, S.K. (2015*).

Previous studies had shown that, the high plasticity between cell types in brain such as astrocyte, mesenchymal, oligodentritic progenitor, and neuronal progenitor-like cells gets genetic aberrations leads to the heterogeneous environment in the glioblastoma causing an inhibitory effect against the existing therapeutics (Pine et al., 2020). The constantly changing and aggressive environment from the genetic changes affects the cells mainly, the cell-to-cell interaction and the cellular metabolism causing rapid tumour growth, invasion, metastasis, and drug resistance (Sharma et al., 2023).

1.1.c Glioblastoma Tumour Microenvironment

The findings from Uneda et al., suggests that the inter and intra tumoral heterogenicity of GB consists of tumour cells, non-tumour cells, GB stem cells, vascular pericytes, endothelial cell, microglia and macrophages and different types of other immunes cells which are recruited through blood make the GB more lethal with poor prognosis (Uneda et al., 2021). This tumour microenvironment have various non cellular components secreted by the heterogeneous population of cells for the cellular communication to the surrounding niche in microenvironment via soluble factors including cytokines, chemokines, factors for rearrangement of matrix and various growth factors through complex signalling process (Sharma et al., 2023). The microenvironment provides nutritional nourishment supporting the tumour growth (Hu et al., 2023).

The blood brain barrier (BBB), which is comprised of microvascular endothelial cells, is the separation between the blood brain interstitial fluids which regulates the movement of molecules to the brain due to its small pore, thereby blocking the drug delivery to the target and making the GB treatment complicated (Noorani&delaRosa,2023;Fig.1b).

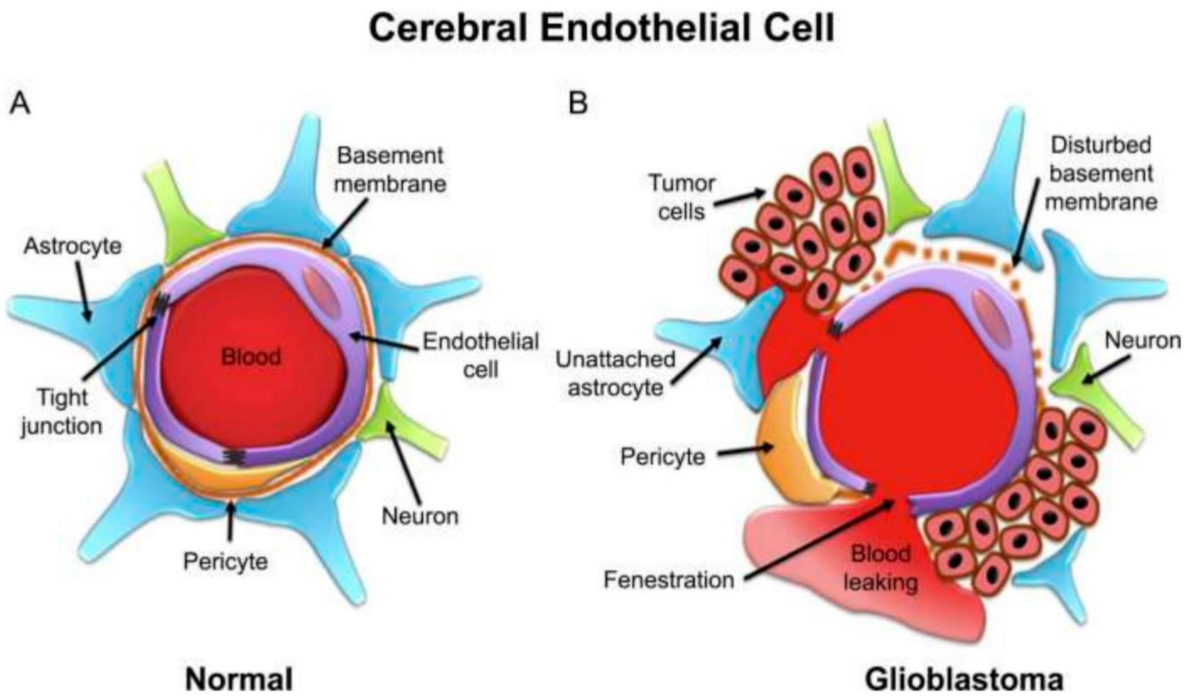


Fig 1.b The BBB disruption in glioblastoma- the blood vessel in cerebrum in normal condition with controlled transportation of molecules and immune cells to the brain. In GB, loss of tightness and leakage of blood to the tumour and surrounding parenchyma of brain (Adapted from Tincu (Iurciuc) et al., 2023).

The BBB in the brain loses its function in GB by the secretion of angiogenic factor VEGF which favours the new vascular tissue formation and the MMP enzyme which degrades the extracellular matrix and basement membrane resulting in GB invasion and migration to the surrounding parenchyma and other areas in the brain. The dysfunctional BBB leads to the chronic inflammation causing loss of anti-tumour immunity in brain (Tripathy et al., 2024). The in vitro studies on GB and BBB have revealed that GB differentiation increases the BBB permeability through downregulation of ATP binding cassette (ABC) transporters and the tight junction proteins, which are upregulated in normal condition, through activation of STAT3, the transcription factor (Digiovanni et al., 2024). The enhanced permeability of BBB makes the tumour growth more rapid thereby it makes the tumour microenvironment more immunosuppressive and drug resistant (Tripathy et al., 2024). The immunologically distinct system in brain makes the GB microenvironment richer with diverse range of immune cells from both the innate and adaptive immune systems where the BBB has been compromised to make the tumour profile colder (Jain et al., 2023).

GB microenvironment consists of the diverse population of cells not limited to tumour cells, but the brain resident and blood recruited immune cells. Due to stimulation from various secretions and factors the microenvironment alters the immune activity of the immune cells resulting in an immune suppressive environment (Jain et al., 2023). The brain resident immune cells and bone marrow derived macrophages which are recruited to the tumour core in the onset of GB, makes these cells as the most abundant non neoplastic cells in the tumour microenvironment (Lin et al., 2023). This population is known as Glioma Associated Macrophages/Microglia (GAMs) which are spread in the perivascular space, meninges, and surrounding ventricles in brain (Lin et al., 2023). The dynamic population of GAMs plays a significant role in GB, where it secretes various cytokine and chemokines for the polarization from anti tumorigenic or proinflammatory M1 to pro tumorigenic or anti-inflammatory M2 resulting thus in a more complex microenvironment (Sharma et al., 2023).

Myeloid derived suppressor cells (MDSCs) are precursors and progenitor cells from spleen which would be differentiated in the bone marrow to granulocytes, macrophages, and dendritic cells with expression of CD33 marker (Gieryng et al., 2017). The MDSCs supresses the cytotoxic activity of both natural killer cells and T cells (both helper and cytotoxic) through the stimulation for the apoptosis of T cells leading to immune suppression in GB. The MDSCs are also increased in the blood as the disease progresses (Gieryng et al., 2017). The natural killer cells are cytotoxic cells rich in perforin and granzyme that have a high potential to kill cancer cells, which would be in less amount due to the immunosuppressive microenvironment of GB (Price et al., 2021; Fig 1c). Dendritic cells (DCs) are one among the antigen presenting cells (APCs) from the innate immune system for activation of T lymphocytes of adaptive immunity by presenting extracellular antigen. These DCs show both anti-tumour and pro tumorigenic activity where the heterogeneity of DCs make more pro tumorigenic in GB microenvironment (Gardam et al., 2023).

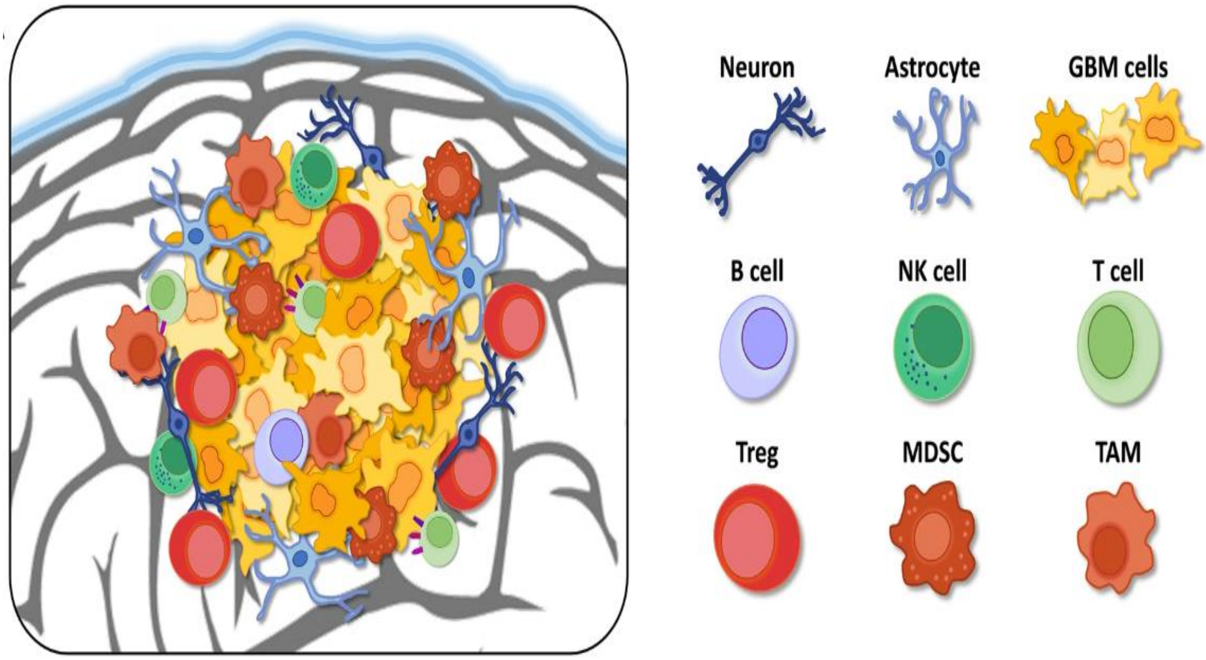


Fig1.c The GB microenvironment with diverse types of cells including tumour cells, brain resident cells, various immune cells – brain resident microglia and macrophages (from bone marrow), Natural killer cells, MDSCs, T cells (cytotoxic, helper and regulatory) and B cells, make more immunosuppressive (*Adapted from Bausart et al., 2022*).

T cells are immune cells having anti-tumour activity which migrates from thymus to lymphoid tissue. T cells are of various subtypes – helper T cells, regulatory T cells, cytotoxic T cells, and memory and innate T cells. The cytotoxic and helper T cells are engaged in immune response in tumour killing whereas regulatory T cells are immunosuppressive nature, thereby these T cells plays a crucial role in GB growth (Wang et al., 2021). In GB, the tumour infiltrating lymphocytes (TILs) expresses various immune checkpoint molecules such as PD-1, CTLA-4, TIM-3, LAG-3 and TIGIT in significantly higher amount than the peripheral blood mononuclear cells, which suggests how the microenvironment becomes immune suppressive (Park et al., 2017). B cells are another set of immune cells which can produce antibodies and have the anti-tumour capability. The B cells serve for both regulatory and anti-tumour responses where its leads to the T cell activation through antigen presentation or complement activation (Hou et al., 2023). The immune suppression of B cells in GB leads to the inhibition of T cell activation (Hou et al., 2024) (Hou et al., 2023).

1.2 Immune system in brain

Immune system in the brain is well organised having both the innate and adaptive cells and organs which would work for the development of neural circuits and protection (Morimoto & Nakajima, 2019). The immune system of CNS has a unique feature where the brain cells are separated from the peripheral immune system except meninges and the tissue resident microglia which act as the baseline parenchymal immune system. This separation protects the brain from severe autoimmune reactions or inflammation (Norris & Kipnis, 2018). The immune cells including brain resident microglia, macrophages, dendritic cells, lymphocytes, and all the other cells involved in immune response are regularly monitored for antigens which might cause disruption to homeostasis. Any changes in the balance between these immune cells and CNS would lead to major problems including neurodegeneration or cancer (Ousman & Kubes, 2012). Blood brain barrier (BBB) is the first line of defence in brain where specialized endothelial cells packed tightly act as physical barrier as well as a selective exchange of molecules for the nutrition and maintenance of brain homeostasis (Lampron et al., 2013). The BBB is separating the CNS from peripheral immune system to avoid the entry of blood recruited immune cells to the system in normal condition whereas it would be modulated with the need or if any disorders in the brain homeostasis (Lampron et al., 2013).

1.2.a Innate immune system in brain

The innate immune system in CNS includes the cells which reside in the brain such as microglia, glial cells, and astrocytes through expression of apoptotic receptors which could clear dead cells through phagocytosis without inducing any inflammation (Griffiths et al., 2009). The brain resident cells display phagocytosis receptors and recognize through cell associated molecular patterns and these cells express complement proteins for the opsonisation of apoptotic cells (Griffiths et al., 2009). The Pattern Recognition Receptors (PRRs) in CNS are identified on microglia, astrocytes, oligodendritic cells, endothelial cells and neurons engaged in clearing any pathogen or any stress to the CNS via various signalling pathways and secreting specialized cytokines which activates phagocytosis and antigen presentation to lymphocytes for the interaction with the CNS and the peripheral immune system (Lampron et al., 2013).

Microglia one of the brain cells which are actively engaged in brain innate immunity, are developed in yolk sac during primitive haematopoiesis and move to the brain making the important brain resident immune cell (Morimoto & Nakajima, 2019). Being a primary and most abundant immune cell in the brain, the microglia play a crucial role in brain development and organisation, thus any dysfunction in the microglia lead to severe neuronal disorders (Zengeler & Lukens, 2021). The microglia and neurons are sustained together by secreting various growth factors for the protection, survival and regeneration of neuronal cells thereby facilitate the equilibrium and optimal function of the CNS. The microglia facilitate phagocytosis and antigen presentation through MHC class I and II molecules for the activation of T lymphocytes (Ousman & Kubes, 2012). The secretion of various cytokines, chemokines and chemoattractant by the microglia during any infection or injury attracts blood recruited immune cells to the brain (Ousman & Kubes, 2012).

Monocytes are the first line defence cells developed in the bone marrow with diverse morphology and surface markers and capable of differentiating into macrophages or dendritic cells based on stimulation (Savinetti et al., 2021). Monocytes are recruited to brain in any disorders through the blood and enter the brain parenchyma by loosening the BBB. The monocytes are plastic cells which connects the innate immunity with the adaptive immunity through recruiting as first among the leukocytes to the brain in any diseases (Zhao et al., 2020). The monocytes are recruited or patrol through blood stream to examine any inflammation or pathological state in the brain and based on stimulation, it will act for either immune response by differentiating to macrophages or dendritic cells, or it will help to regenerate or remodel the inflamed tissue, through the curing (Zhao et al., 2020).

Monocyte derived macrophages exhibit high plasticity and expresses either in anti-inflammatory or pro inflammatory based on the stimulation (Wattananit et al., 2016). According to London et al.,2013, the monocyte derived macrophages are recruited to the brain in need, if the microglia were incapable of inducing immune response in damaged CNS and perform the same work as the microglia does normally (London et al., 2013). Macrophages display a wide range of activation stages as they are highly plastic, and the two major phenotypes displayed by macrophages are classical, pro inflammatory, anti-tumour M1 macrophages and the alternative, anti-inflammatory, pro tumorigenic M2 macrophages (Savinetti et al., 2021).

Dendritic cells are another set of immune cells capable to present antigen for T cell differentiation and activation, which are stimulated by pathogen associated molecular patterns for the antigen specific immunity. Dendritic cells originate from bone marrow as monocytes and later upon stimuli they differentiate to dendritic cells (D'Agostino et al., 2012). Dendritic cells in brain are of immature and mature types where immature types are plasmacytoid that are involved in antigen presentation and inflammation and mature types regulate the genetic changes and functions such as migration with the antigen to lymphoid sites (Colton, 2012). The dendritic cells in the CNS is the blood recruited cells which are not found in stable conditions, whereas it is observed in any diseased conditions (Ismail et al., 2021).

Complement system is a critical effector in clearing pathogens, dead cells and regulating inflammatory immune response by cascade of proteins. In CNS, this complement system has key role in clearing the synapse for the nervous system development (Schartz & Tenner, 2020). The complement components from neurons and glial cells are for the protection from harmful aberrant proteins and cellular debris. The complement cascades regulated by various membrane bound and soluble complement inhibitors and any alterations to these regulatory molecules or signalling would lead to various neurological disorders (Schartz & Tenner, 2020).

1.2.b Adaptive Immune system in Brain

In adaptive immunity, the cells are activated by the innate immune antigen presenting cells which are specific and giving memory to act in subsequent counter attacks by the same antigen (Marshall et al., 2018). The patrolling of this adaptive immune cells are regulated in the CNS, but a minute amount would circulate for the immune surveillance to maintain the homeostatic conditions, any infection or pathological condition would recruit a greater number of adaptive immune cells to the CNS and cerebrospinal fluid (Rickenbach & Gericke, 2022). The key immune cells in adaptive immunity are T cells including CD4+ T helper cells, CD8+ T cells, regulatory T cells, B cells and Natural Killer cells (NK), also a part of innate immune responses. The adaptive immune cells enters into the brain parenchyma when BBB is compromised to facilitate the immune response in any unhealthy conditions (Qin et al., 2020).

T cells in the brain secrete mediators or direct contact with brain parenchyma helps in tissue regeneration and protection (Evans et al., 2019). The major subsets of T cells present in the CNS are CD4+ and CD8+ T cells, tissue resident memory T cells, regulatory T cells, γδ T cells, Th 1 cells and Th 17 cells. These cells play the central role in adaptive immunity as well as inflammatory response and protection by maintaining tissue homeostasis in the brain (Smolders et al., 2018, DeMaio et al., 2022). The T cells enter the CNS through either cerebrospinal fluid, choroid plexus, or subarachnoid space which are controlled by various integrins, selectins and chemokines. CD4+ (includes helper and regulatory) and CD8+ T cells enters the CNS through the antigen presentation by the resident innate immune cells via MHC class I or II. The functional subsets of T cells are chosen either for effector or regulator based on the stimulation (Liblau et al., 2013). The exhaustion or loss of T cell function in brain tumour is due to the inhibition in the secretion of pro inflammatory and antitumour cytokines and chemokines leads to loss of cytotoxic activity of T cells, furthermore, the secretion from tumour leads to the loss of anti-tumour activity. The immune checkpoint molecules such as CTLA, PD-1, TIM-3 and others get highly activated due to stimulation from tumour ceases the immune response against tumour growth (Ravi et al., 2022).

B cells are a type of lymphocytes capable of antibody producing plasma cells, which are present in less amount in the CNS, in both healthy and diseased conditions. The memory B cells develops through the antigen dependent T cell stimulation on the B cell receptor on naïve B cells (Jain & Yong, 2021). In context of infection or any pathological situation, the B cells are produced with antibody secreting plasma cells and regulatory B cells, where B cells have a crucial role in structural and functional neurodevelopment (Lynall et al., 2021, Dwyer et al., 2023). B cells secretes neurotrophins, which supports the neural development and plasticity and produce IL-10 for the immune regulation in the brain (Ortega et al., 2020). According to Candolfi et al., 2011, the B cells are the antigen presentation in a brain tumour rather than the antibody mediated immune response (Candolfi et al., 2011).

Natural killer cells are the lymphocytes presented in both innate and adaptive immunity, where they have the ability for antigen specificity and memory immune responses through a wide variety of phenotypes (Pierce et al., 2020). Natural killer cells have a bridging with the CNS in different neurological conditions. These natural killer cells are highly adhesive and motile in the CNS which could easily pass the BBB in inflammation (Ning et al., 2023). The natural killer cells are present from low grade to high grade type of brain tumours with significant levels and these cells indicate a better survival for the patients (Fares et al., 2023).

1.2.c Immune check point molecules

Immune checkpoint molecules are the receptor-ligand pairs for the regulation of immune response through controlling the length and intensity of immune activity to reduce tissue destruction and manage the self-tolerance. These checkpoint molecules regulate through either inhibition or stimulation of various receptors or ligands on various non-immune cells, T cells in adaptive immunity and other cells in innate immunity (Zhang & Zheng, 2020). The immune checkpoints suppress the immune response in normal conditions to avoid over attack, whereas in cancer, these molecules are over expressed and engage in immune responses causing thus an inhibition. The major immune checkpoints studied on immune cells in tumour microenvironment are Program death receptor 1 (PD-1), Cytotoxic T lymphocyte antigen 4 (CTLA 4), T cell immunoglobulin and Mucin-Domain containing-3 (TIM-3), Lymphocyte activation antigen 3 (LAG-3), B and T lymphocyte attenuator (BTLA), Signal regulatory protein alpha (SIRPα), Sialic acid-binding immunoglobulin – type lectins 7,9,10 & 15 (Siglec), CD200 receptor (CD200R), and T cell immunoglobulin and ITIM domain (TIGIT) (Kanannejad et al., 2023, Guo et al., 2023). The expression of immune checkpoint ligands on glioblastoma cells suppresses the immune responses. The major immune checkpoint molecules expressed on immune cells in glioblastoma are PD-1, CTLA-4, TIM-3 and LAG3, and these molecules are expressed on the surface of activated T cells (Ghouzlani et al., 2021).

PD-1 or Programmed Death protein receptor is expressed on T cells and binds on its ligand PDL-1 or 2, which belongs to B7 protein family. Upon binding, it inhibits and activates various signalling pathways including Akt/PI3K, JAK/STAT3, MAPK, NF-kB, Wnt and Hedgehog causing CD8+ T cell inactivation (Peng & Bao, 2022, Han, Y., Liu, D., & Li, L. ,2020). The antigen presented by the cells through

MHC complex and co-stimulatory molecules activates the T cells and proceeds with the immune response. The cytokines from activated T cells activate PDL-1 on effector cells and activate PD-1 on T cells and carry out the immune regulation. In tumours, these PDL-1 and PD-1 are over expressed, and T cells get exhausted and inactivated or unable to reach the tumour (Alsaab et al., 2017). Downregulation of T cell cytotoxicity in glioblastoma gets through the PDL-1 activation on tumour cells leading to PD-1 activation on microglia through the activation of various Toll like receptor, growth factor receptors and interferon receptors (Litak et al., 2019). In GB, it has been observed that the PDL-1 overexpresses as the intensity of malignancy rises which can predict the intensity and aggressiveness of the disease (Scheffel et al., 2021). The PDL-1 has found majorly on tumour edges than the core revealing its crucial role in facilitating immune suppression and spreading of GB to the surrounding areas of the brain (Scheffel et al., 2021).

Cytotoxic T lymphocyte associated antigen 4 (CTLA-4), the immune checkpoint molecules, regulates the T cell immune response negatively leading to its inhibition in the lymph node. The binding of CD80/CD86 co-stimulatory molecules on antigen presenting cells to CD28 on T cells and T cell receptors to MHC leads to the activation of T cells, whereas the binding co-stimulatory molecules on CTLA-4 on T cells leads to T cell exhaustion (Buchbinder & Desai, 2016). In tumours, the CD28 gets weak, and the CTLA-4 get activated to limit T cell responses by weakening the tumour antigens (Seidel et al., 2018). This CTLA-4 is upregulated in immune cells and includes both conventional T cells as well as regulatory T cells circulating in the tumour microenvironment (Babamohamadi et al., 2024). In GB, the *CTLA-4* gene has mutated and its upregulation in the microenvironment causes enhanced recruitment of macrophages making tumour proliferation, survival, and migration in higher rates through various tumour favouring growth factors and cytokine secretions (Guan et al., 2021).

T cell immunoglobulin and mucin domain containing protein 3 (TIM-3) is expressed on CD4+ and CD8+ T cells for immune inhibition as checkpoint receptor through co-stimulation and co-regulation, especially in cancer (Wolf et al., 2019). The TIM3 binds to the ligand Galetin 9 causing the stimulation for the T cell death. Galectin 9 is a β-galactosidase binding protein which belongs to the galectin family, encoded by the *LGALS9* gene, which plays a major role in tissue repair, maintaining homeostasis and immune regulation (Lv et al., 2023). The TIM3 markers are expressed mainly on IFN-ϒ secreting cells whereas the Galectin 9 is secreted by various immune cells as well as non-immune cells including endothelial cells (Acharya et al., 2020). Galectin 9 and TIM3 interactions are observed in leukaemia, breast cancer, gastric cancer, and glioblastoma through negatively regulating the anti-tumorigenic T cells (Zhang et al., 2024). In GB, the Galectin-9 has been expressed on microvascular proliferative areas with diverse range of cells including GB like stem cells, endothelial cells, myeloid derived suppressor cells, and monocytes. The Galectin 9 from myeloid suppressor cells interacts with TIM3 resulting in an immunosuppressive microenvironment in GB cells (Knudsen et al., 2021).

Inhibitory immune checkpoint Lymphocyte activation gene (LAG-3) is expressed on T cells for immune regulation, to maintain the equilibrium and avoid autoimmune disorders. LAG3 combines with MHC class II on antigen presenting cells and regulates the immune response (Long et al., 2018). LAG3 and PD-1 co expressed together in tumours with high tumour infiltrating lymphocytes, which makes a T cell deficient immune system in the tumour surroundings (Huo et al., 2022). The study from Harris-Bookman et al., 2018, suggests that the LAG-3 in GB has been expressed on initial stages of T cell inhibition (Harris-Bookman et al., 2018).

1.3 Immune suppression in Glioblastoma

The tumour microenvironment in GB is highly complex and diverse with various cells and secreted cytokines causing immune suppression, tumour proliferation and survival to the immune responses (Chen et al., 2020). In CNS, the immune system is highly restricted due to the BBB, absence of conventional lymphatic system, and controlled MHC expression on innate immune cells for antigen presentation. The clearance of tumour associated antigens is the major functions of the immune system and these antigens are unique to cancer. In GB, these antigens belong to mutated and non-mutated autoantigens and specific antigens resulted from genetic abnormalities, where these changes tackle the immune system in responses and influence survival and proliferation (Brown et al., 2018). In GB, the immune cells – including tumour associated macrophages, myeloid derived suppressor cells, regulatory T cells, and the cytokines IL-10 and TGF-β, make an immune suppressive microenvironment that inhibits the effector immune cell activation (Zhou et al., 2023).

The adaptive immune cells population is comparatively lesser in GB, where the B and CD4+ T cells are present more than the CD8+ T cells. Furthermore, the MHC 1 for CD8+ T cell activation is downregulated in IDH mutated GB, causing T cell exhaustion (Lehtipuro et al., 2019). The presence of regulatory T cells reduces effector T cell infiltration, moreover, the vascular endothelial growth factor (VEGF) and hypoxia inducible factor (HIF-α) secreted from GB enhances the PD-L1 expression and regulatory T cell infiltration leading to hypoxia environment, causing the immune suppression in GB microenvironment (Himes et al., 2021). The CD8+ T cells and regulatory T cells maintain a balance to carryout immune activity and regulation, where in GB, the ratio between these two T cells gives from better to poor survival rates. Tumour associated macrophages regulates the T cell infiltration into the GB microenvironment (Tu et al., 2021). The CD4+ T cells in the GB has been corelated with poor prognosis, due to their poor survival rate when the T cells are higher in patients (Woroniecka et al., 2018). The B cells infiltrating into GB are converted to regulatory B cells through the expression of checkpoint molecules such as CD72, CD32 and CD22 leading to inhibition of T cell activation (Hou et al., 2023). The Natural Killer cells expressed in tumour promotes the chemokine CXCR3 and the other cytokines and growth factors shuts the tumour killing effect and leads to resistance. The expression of CD73 and CD155 on NK cells suggests that it contributes to the tumour metastasis and aggressiveness (Fares et al., 2023).

In the onset of tumours, the innate immune system conveys the adaptive immune system through antigens to clear the tumour. The innate immune cells such as macrophages and dendritic cells recognises the tumour and process to eliminate and take a part of antigens to educate adaptive immune system (Kong & Kim, 2022). The dendritic cells, the antigen presenting cell in innate immune system shows downregulation of MHC and costimulatory molecules suggesting that it is immature phenotype in GB that causes T cell inactivation or suppression (Zhou et al., 2022). The chemokines such as CCL17 and CCL22 secreted by mature dendritic cells recruit the regulatory T cells to the tumour microenvironment facilitating immune suppression (Mineharu et al., 2013). In IDH wild type and mutant GBs, monocytes are differentiated into dendritic cells in immature and impaired cells with different phenotypes which are not capable of antigen presentation and co-stimulation for effector T cell activation (Friedrich et al., 2022).

The GB microenvironment comprised of various cells, where having a small count of lymphocytes and the immune cells present in higher amounts are tumour associated macrophages (TAMs). These TAMs consist of brain residing microglia, and bone marrow - monocyte derived macrophages with various phenotypes based on the functional adaptation (Yabo et al., 2023). The microglia are the resident innate immune cells in the CNS, with high plasticity, which are originated from yolk sac of embryonic myeloid progenitor cells and having mononuclear phagocytic activity. The microglia supports the maintenance of the tissue homeostasis in the CNS (Buonfiglioli & Hambardzumyan, 2021). Microglia are present in M1 and M2 phenotypes, where both are found in GB surroundings which would be balanced to favour the tumour growth and invasion through secreting various cytokines, chemokines and activating tumour promoting signalling pathways (Geribaldi-Doldán et al., 2021). Similarly, the bone marrow derived recruited through blood, which differentiated from monocytes, the macrophages, also known as TAMs, shows M1 or M2 phenotypes favouring tumour growth, immune suppression, and angiogenesis (Wang et al., 2022).

1.4 Monocytes and Macrophages in Cancer

Monocytes are the phagocytic cells generated from the bone marrow myeloid precursors which are classified the three categories in human – classical, non-classical and intermediate based on CD14/CD16 expression. Monocytes migrate to the inflamed site and function for differentiating to either macrophages or dendritic cells to perform phagocytosis, antigen presentation and cytokine secretion. In cancer, the monocytes are presented in the tumour site in larger amounts (Chen et al., 2023). In GB, the TAMS includes monocytes, are invaded to the tumour microenvironment for promoting brain metastasis (Friebel et al., 2020). The tumour cells are killed by monocytes through secretion of cytokines and phagocytosis and facilitate antibody mediated cytotoxicity. These monocytes help in lymphocyte recruitment to tumour sites through paracrine signalling and antigen presentation (Olingy et al., 2019). In GB, CCL2, one of the monocytes chemoattractant, and CCL7 secreted by inflamed tissues, to attract monocytes or macrophages, are found to be highly expressed (Caverzán et al., 2023). The co-stimulatory molecules CD80 or CD86, which bind to CD28 on T cells, and the antigen presentation molecule MHC II on monocytes are downregulated in GB causing the inactivation of T cells (Razavi et al., 2016).

The macrophages in the tumours are of three categories including monocyte derived, originate from haematopoietic stem cells in bone marrow, tissue resident macrophages from yolk sac and myeloid suppressor cells from fetal liver. Macrophages are classified into two polarized forms - M1 and M2, and the M2 are having the subtypes M2a, M2b, M2c and M2d (Xu et al., 2022). The M1 macrophages are induced by inflammatory cytokines TNFα, IFN-ϒ and stimulation from bacterial lipopolysaccharide (LPS) in turn releases IL-β, IL-6, ROS, iNOS and IL-12 and these macrophages elicits antibody dependent cellular phagocytosis (Strizova et al., 2023). The M2 macrophages induces IL-4 and IL-13 for tissue repair and releases IL-10 and TGF-β for anti-inflammation. The mannose receptor CD206 and scavenger receptor CD163 are expressed by M2 macrophages for clearing and healing of the tissues (Strizova et al., 2023). The TAMs have a crucial role in proliferation of various types of cancers, through promoting the angiogenesis, extracellular membrane alterations, inflammation, epithelial to mesenchymal transition, and metastasis (Bied et al., 2023). The metastasis associated macrophages supports the secondary tumour growth through various inflammatory mediators and cytokines such as IL-6, IL-1β, and CXCL-8 and the extracellular vesicles transporting the RNAs, lipids, metabolites and proteins favouring the modulation of macrophages for the proliferation (Bied et al., 2023). The macrophages in tumour microenvironment are stimulated to tumour supporting phenotype, where it leads to more intensive tumour, affecting the patients with poor survival (Kowal et al., 2019).

1.4.a Macrophages in Glioblastoma

TAMs include both macrophages and tissue resident microglia which accounts for 50% of the cell population in GB, favouring the tumour proliferation and immune inhibition. The microglia are present in the different areas of the tumour except the brain metastatic core whereas the macrophages are prevalent (Khan et al., 2023). The monocytes differentiated into macrophages (M0) which are later differentiated into M1 or M2 upon stimulation from tumour either pro inflammatory - antitumorigenic or anti-inflammatory - pro tumorigenic (Wu et al., 2022). The M1, the classical macrophages, activated through TLR2/4 immune receptors and secretes pro-inflammatory cytokines includes TNF, IL-β, IL-12, and IL-18 and the molecules for immune stimulation such as CD80/86 co-stimulatory molecules, MHC class II for antigen presentation and CD68 markers. M2 phenotypes are alternatively activated by secreting ARG1, IL-4, IL-10, IL-13, and TGF-β, and through the CD163 and CD204 marker expression (Buonfiglioli & Hambardzumyan, 2021, Wu et al., 2022; Fig. 1d). The microenvironment of GB has heterogeneous population of cells, where both M1 and M2 are present and the M2 among TAMs is shown to be the provider for resistance to conventional therapies, tumour proliferation, immune suppression and poor survival (Andersen et al., 2021).

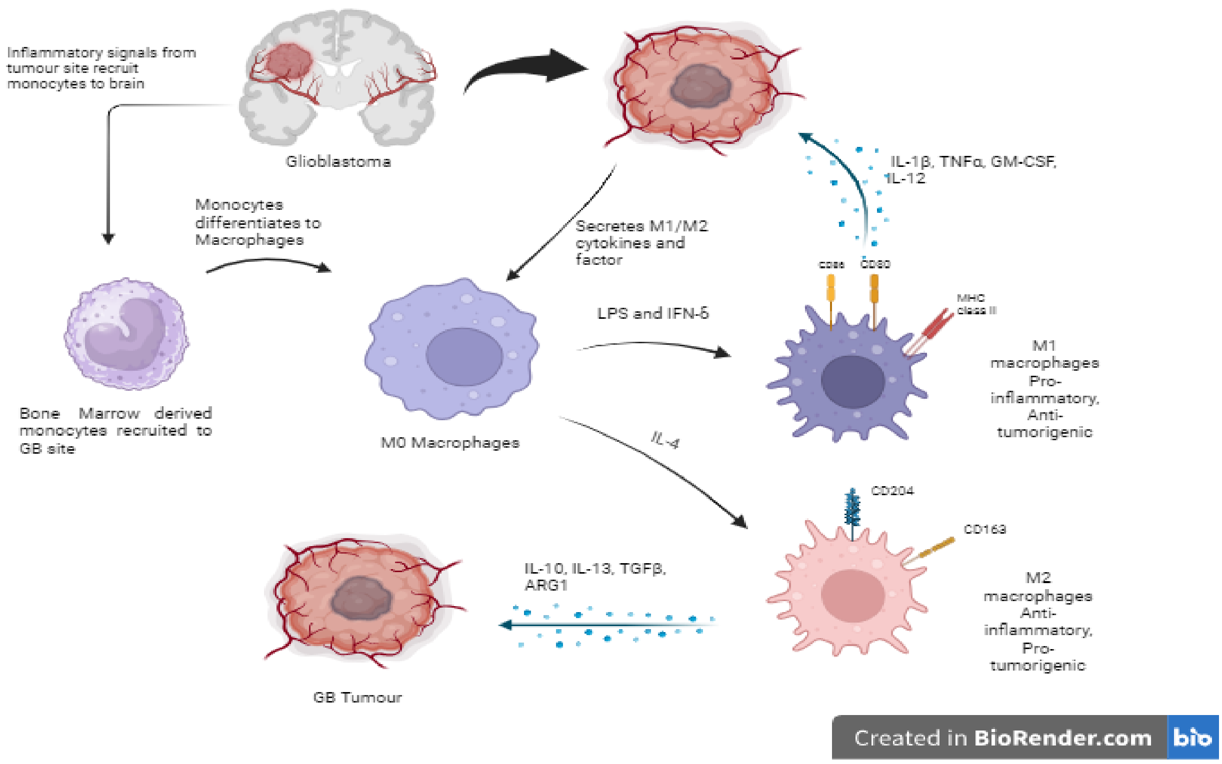


Fig1.d Recruitment of monocytes and differentiation in GB. The signals from tumour recruit monocytes to the site and differentiate into M0 macrophages. Upon release of functional cytokines for re-educating the macrophages to polarise into M1 or M2 phenotype. (Created with BioRender.com).

The monocytes and macrophages, the major innate immune cells execute the first line of defence through pattern recognition receptor (PRRs) binding to pathogen associated molecular patterns (PAMPs) on pathogens or tumours (Chen et al., 2021). Also, the cells can engulf tumours or pathogens through phagocytosis and present them for lymphocyte activation and elimination and memory (Chen et al., 2021). The tumour cells imitate as the normal cells to escape from phagocytosis(Chen et al., 2021). The phagocytosis through the binding of certain receptors and ligands on macrophages and effector cells or pathogens and the membrane of phagocytes (macrophages) protrudes, covers, and binds inside the phagocyte forming phagosome. Once the phagosome has developed, the reactive oxygen species, phospholipase A2, hydrolases, and lysosomes join to the phagosome and kill the cells (Hirayama et al., 2017). The phagocytosis by TAMs on tumour cells are regulated by certain molecule such as phosphatidylserine, calreticulin, CD47, Signal regulatory protein α (SIRPα), PDL-1 and MHC class I which gives signal to phagocytose or not and causes the regulation. In GB, the CD47 or SIRPα upregulated, and the phagocytosis is inhibited (Lecoultre et al., 2020). Wu et al., 2023, reported that bone marrow – monocyte derived macrophages phagocytose GB cells, leads to immunosuppressive TAM, as M2 phenotype, making the activation of immune checkpoint molecules and thus supresses the T cell activation (Wu et al., 2023). In vitro studies on GB showed that the blood recruited macrophages are expressed mostly among TAMs compared to the resident microglia, which are capable of phagocytosis, however, the phagocytosis is regulated or supressed by SIRPα-CD47 signalling (Andoh & Koyama, 2021). The engulfed apoptotic cell products from tumour or bacterial infection are presented on MHC class II on phagocytes for the activation of helper CD4 T cells and on the MHC class I for the activation of CD8 T cells (Germic et al., 2019).

Antigen presentation is a part of immune response and the cancer associated antigens are phagocytosed and presented by MHC molecules on immunocytes making the naïve T cell active. The MHC are of class I and II, where class I presented on all nucleated cells where it shows the intracellular antigen peptides and the class II expressed on all antigen presenting cells showing the peptides of extracellular antigens. The antigen on MHC recognized by the T cell receptors (TCRs) on T cells making a TCR-MHC-antigen complex stimulating and activating the T cells and kills cancer cells (Kallingal et al., 2023). The co-stimulatory molecules associated with MHC complex for antigen presentation is belonging to the B7 superfamily of protein, B7-1 or CD80 and B7-2 or CD86. The co-stimulatory molecules CD80/86 on macrophages are activated upon antigen presentation process where it binds to the ligands on T cells CD28. Another ligand for CD80/86 is CTLA 4 where binding for inhibition and regulates the T cell activation. The CD80/86 are expressed on M1 macrophages whereas in M2, it has been observed as downregulated (Noy & Pollard, 2014). In GB, the brain resident microglia releases MCP-1 and macrophage inflammatory protein-1 for recruiting the macrophages from bone marrow and secretes IL-10 and other anti-inflammatory modulators from tumour polarises the anti-tumour macrophage to immune suppressive phenotype. The GB microenvironment has a suppressive immune system especially from TAMs that lacks phagocytosis and antigen presenting abilities (Adhikaree et al., 2020).

1.4.b Macrophage suppression in GB

The tumour microenvironment facilitates the recruitment of TAMs by various cytokines and the TAMs functions for tissue repair and promotes tumour growth. TAMs produce TGFβ and IL-10 which activates PDL-1 that in turn reduces T activation and recruitment and favours the regulatory T cell to supress anti-tumour responses (Lecoultre et al., 2020). The M2 phenotype of macrophages in GB microenvironment promotes neovascularization that helps in angiogenesis for the proliferation and immune escape (Cui et al., 2018). Along with angiogenesis and immune suppression, these TAMs also support stemness, extra cellular matrix degradation and invasion to brain parenchyma by various factors such as STIP-1, PTN, EGF, PDGFB, MMPs, FasL, MIF and various chemokines (CCL 2,4,5,8) (Andersen et al., 2021). GB expresses CD47, for inhibiting the phagocytosis of macrophages or microglia. Furthermore, *STAT3* is upregulated to secrete tumour favouring cytokines IL-6 and IL-10. In addition, the immune checkpoint molecules such as CTLA-4, PD-1, TIM3 and Fas receptors gets activated either directly or indirectly contributing to immune suppression in GB

(Buonfiglioli & Hambardzumyan, 2021). The excess indolamine 2,3 dioxygenases in the GB microenvironment favours the M2 phenotype change causes downregulation of MHC expression and causes T cell inactivation (Peng et al., 2020).

1.5 Protein Secretion in GB

The proteins secreted from the cells engages in controlling various physiological activities including immune response and regulation, cell signalling and communication, enzymes and growth factors for various signalling pathways that influences the cellular niche. In cancer, the mutations change the gene expression and protein secretion making the tumour proliferative and aggressive leading to metastasis (da Cunha et al., 2019). In GB, the various proteins secreted favours adhesion, growth, and signalling which are upregulated or downregulated to proliferate, invade, and spread at different locations of the brain. The mutations occurring in the oncogenes activate the various pro-oncogenic pathways leading to secretion of proteins. The secretion from GB includes, inflammatory mediators, cytokines, various growth factors, extracellular matrix proteins and its modulating proteins, proteoglycans, and galectins which would contribute to the disease progression (Tarassishin et al., 2014, Almiron Bonnin et al., 2018,). The secretions are of classical and non-classical, where classical pathway is the transportation through endoplasmic reticulum and the non-classical includes transportation through pores, ABC transporters, endosome mediated and Golgi-bypass (Tarassishin et al., 2014, Almiron Bonnin et al., 2018). The secreted proteins act in autocrine, paracrine, and endocrine manner, and as locally and systemically (Ritchie et al., 2021).

1.6 Galectins

Galectins are glycan binding proteins with a carbohydrate recognition domain, having an affinity towards the glycoconjugates with β galactose and these galectins are categorized into three based on its structural variations – prototypical, tandem repeat and chimeric (Ayona et al., 2020). The evolutionarily conserved galectins recognize the N-acetylglucosamine presented on the cell surface glycoconjugate. The classification is based on the number of carbohydrate recognition domain (CRD) and how it is dimerized. The prototype includes galectin-1,2,5,7,10,11,13,14 and 16, the chimera type have galectin-3 and the tandem repeat types are the galectins- 4,6,8,9, and 12, where the galectin -1,2,3,4,7,8,9,10,12,13,14 and 16 were observed in human tissues (Troncoso et al., 2023). Galectins are distributed widely in various tissues and cells and present inside the cell including nucleus and cytosol and also outside the cells (Ayona et al., 2020). The galectins bind to the various glycoconjugates for various physiological and pathological conditions by regulating, signalling, immune responding or suppressing, or proliferating especially in cancer (Nio-

Kobayashi & Itabashi, 2021). Among the galectins, the galectin 1,3,4,8 and 9 are present in the brain cells in CNS (Nio-Kobayashi & Itabashi, 2021). The galectin 1 and 3 are extracellularly expressed which is found from neoplastic to advanced tumour in various cancers supporting the rapid growth, immune inhibition, EMT, metastasis, and stemness through interacting with various receptors and cell surface integrins (Mariño et al., 2023; Fig. 1e).

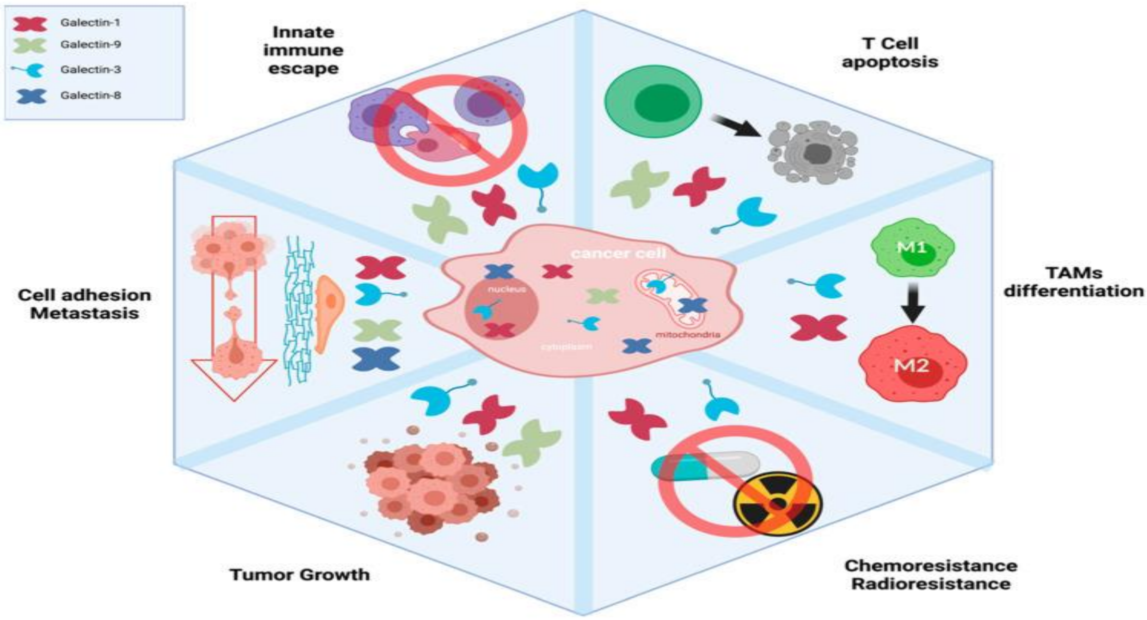


Fig 1.e Galectins involved in cancer - Galectin-1,3,8 and 9. Galectin 1, 3 and 9 from tumour are involved in immune suppression such as innate immune escape, TAM differentiation and T cell death. Galectin 1,3,8 and 9 helps in tumour proliferation, invasion and metastasis and contributes to treatment resistance especially Gal -1 and 3 (Adapted from Ajarrag & St-Pierre, 2021).

1.6.a Galectins in GB

In GB, the galectin-1,3,8 and 9 are expressed which could add to the tumour growth, invasion, immunosuppression, and drug resistance, where these galectin-1 (encoded by *LGALS1* gene) and galectin-3 (encoded by *LGALS3* gene) have a strong relation with malignancy of the tumour (Videla-Richardson et al., 2021). Galectin 1 are the first and most studied among the galectins, which is involved GB growth and development, providing lower survival rates and present in other brain tumours too, where mostly located on the margin of tumours supporting invasion and migration (Ajarrag & St-Pierre, 2021). Galectin 1 secreted from the tumour causes immune suppression by supporting regulatory T cells, cytotoxic T cell apoptosis and secretion of cytokines such as IL-6 and IL-10 (Yu et al., 2023). Galectin-1 attenuates NK cells and dendritic cells, polarizes M1 macrophage to M2 macrophage and rises the immune checkpoint molecule expressions such as PDL-1 and Galectin -9 causing the inhibition of T cell activity (Yu et al., 2023). Like galectin-1, Galectin-3 is another glycan binding protein that favours GB proliferation including immune suppression, invasion and chemoresistance. Galectin-3 alters the antigen recognition by CD8+ T cells through changes in T cell receptor thereby causes immune inhibition (Ajarrag and St-Pierre, 2021). The pro-tumorigenic M2 macrophages are differentiated from monocytes by Galectin-3 and favours the expression of immune checkpoints in GB (Ajarrag and St-Pierre, 2021). Galectin-8 is one among the tandem repeat type where it enhances the adhesion and motility in cancer (Liu et al., 2023). Galectin-8 in GB gets stimulated by hypoxia to favour the stemness by regulating the autophagy and Galectin-8 aids in proliferation and inhibition of apoptosis of tumour cells (Metz et al., 2016, Liu et al., 2023). Galectin 9 is highly expressed in mesenchymal GB and involved in immune inhibition through M2 macrophage activation and T cell inactivation role (Yuan et al., 2019). Galectin 9 contributes to immune checkpoint activation by binding with TIM3 on T cells making exhaustion in T cell activation and infiltration in GB (LIU et al., 2016).

1.6.b Galectin-1 in GB

Galectin-1 had been found mostly on the boarders where the GB and brain meets than the tumour core supporting the tumour spread and movement to surroundings and distant organs. Galectin 1 is associated with immune regulation resulting in immune suppression that favours TAMs expression (Rudjord-Levann et al., 2023). The silencing of brain secreted galectin-1 gene in mice had reduced the tumour favouring macrophage infiltration and the myeloid suppressor cells leading to a better survival (Verschuere et al., 2013). The binding of Galectin-1 on endothelial cells in tumours could contribute to angiogenesis and immune inhibition, where the immune system is regulated by the activation of immune checkpoint molecules which could block T cell activation and the migration to the tumour site (Nambiar et al., 2019). The GB secreted galectin-1 in hypoxia activates Raf/Mek/Erk pathways leading to proliferation of endothelial cells, initiation of blood vessel formation and T cell apoptosis in association with TGF-β secretion resulting in an immunosuppressive microenvironment (Toussaint et al., 2012).

The galectin 1 suppresses the T cell activation as well as the IFN-ϒ from T cells suggesting that the galectin 1 and IFN-ϒ are directly linked through the suppression of galectin-1 by IFN-ϒ (Cedeno-Laurent & Dimitroff, 2012). The T cell migration through endothelial cells is inhibited by galectin-1 through inducing T cell death and reduced endothelial attachment of T cells due to galectin 1 binding (He & Baum, 2006). The endothelial cells in tumour site over expresses the immune inhibitory ligands especially the PDL-1 and Galectin-9, when the Galectin-1 binds and get activated through Jak/STAT pathway, which inhibits the transmigration of T cells to the tumour (Nambiar et al., 2019). The treatment of cancer cells using anti-galectin-1 and anti-PDL-1 or anti-galectin-9 could potentially be a good therapeutic strategy to recruit T cells to the tumour site and to eradicate deadly cancers (Fig. 1f).

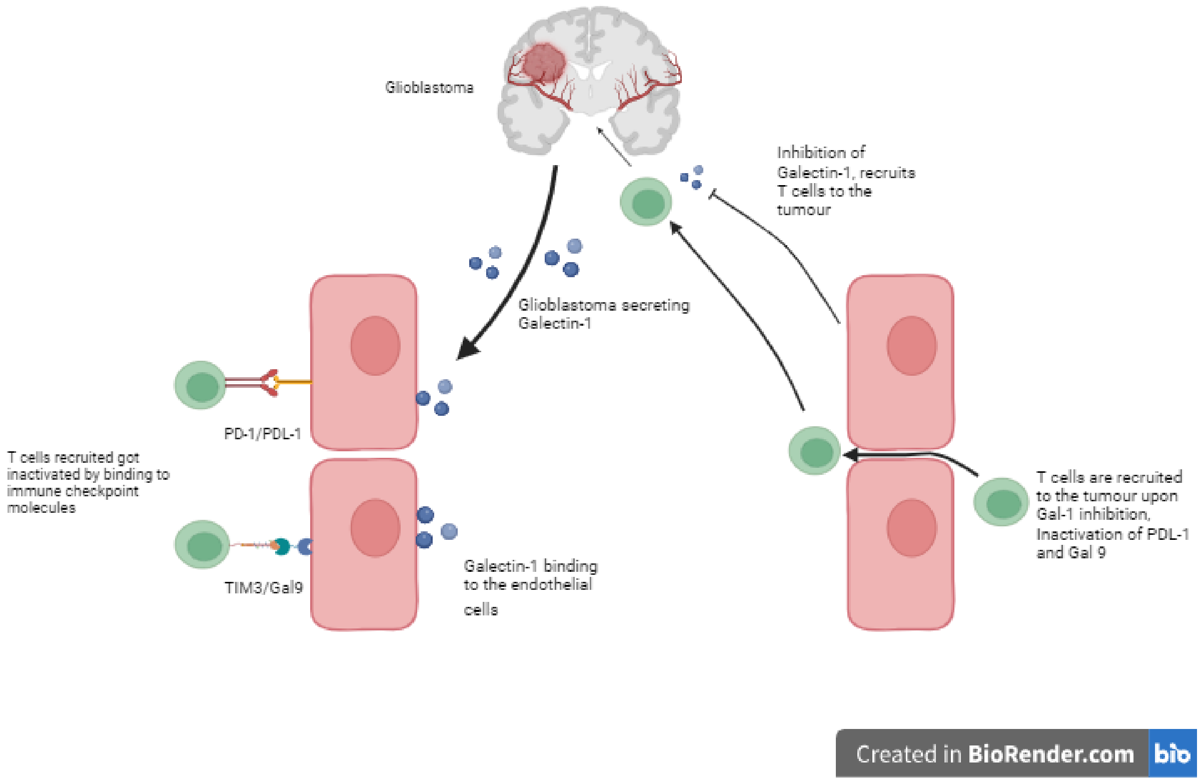


Fig1. f. The secretion of Galectin-1 binds to the blood vessels in glioblastoma and activates PDL-1 and Gal-9 causing immune checkpoint by binding to its receptors PD-1 and TIM3 on T cells. The blockade of Galectin-1 makes the T cell migration to GB and facilitates immune response by inactivating the PDL-1 and Gal-9 (Created with BioRender.com).

The findings described so far provides an understanding of the GB tumour microenvironment, immune suppression, macrophage inhibition and polarization in the microenvironment as well as the role of secreted Galectin-1 in immune suppression and the immune checkpoint molecules in brain endothelial cells. The impact of microenvironment and the secretion from GB makes the tumour more aggressive, immune suppressive and proliferative. The secretion from the tumour regulates the macrophages and its antigen presentation for lymphocyte activation favors in tackling the immune system which might be a therapeutic target for cancer. Understanding the macrophage phenotype and its change in the presence of secretions and how the immune suppression is facilitated holds the key for designing a novel therapeutic strategy.

2.Aim and Objectives

Aim

In glioblastoma, the macrophages are the most abundant immune cells in the microenvironment. The secretions from GB to the microenvironment have an inhibitory effect on macrophages by changing to tumour supporting phenotype, where it either blocks the immune response or supports the tumour growth. The aim of the study was the assessment of inhibitory effects of secreted proteins on macrophages in glioblastoma. In order to achieve this aim, the following objectives were performed:

Objectives

1. Investigate the role of secreted proteins from glioblastoma cells on macrophages.

- The conditioned media from GB cell lines U373 and U251 were taken and analysed for the total protein concentration on different days using BCA assays.
- Using the conditioned media with highest protein concentrations, the phagocytic activity of macrophages was analysed using phagocytosis assay with or without Lipopolysaccharide (LPS) treatment.
- Similarly, the antigen presentation and co-stimulatory marker expression on monocytes and macrophages were observed in the presence of conditioned media using flow cytometry.

2. Measurement of Galectin-1 in GB conditioned media

- Galectin-1 is the protein identified in common from previous studies from our group and studies have shown its potential in immune suppression. Galectin-1 has secreted from GB to suppress the immune response.
- The Galectin-1 concentration from GB conditioned media was estimated using ELISA with or without IFN-ϒ, the interferons from T cells.

3. Assess the expression of PDL-1 and Galectin-9 in human brain endothelial cells in the presence of GB conditioned media, in the presence of IFN-ϒ, both IFN-ϒ and GB conditioned media together using flow cytometry.

3.Materials and Methods

3.1 In vitro Cell cultures

3.1.a Human monocyte primary cells

Human leukaemia monocytic cell lines THP1 and MM6 (ATCC, UK) were cultured in T75 flask with 12 mL RPMI 1640 (Gibco, Thermofischer, UK) with 5% fetal bovine serum (FBS) (Gibco, Thermofischer, UK) and 10mM L-glutamate (Scientific laboratory supplies, UK). Routine observations of the cell cultures within the tissue culture flasks were conducted using a microscope approximately two to three times weekly to assess the cell density, and any alterations in the media’s colour were checked. In instances where the medium's colour changed to yellow, the cells were subcultured. For sub-culturing, cells were first gathered into a pellet through centrifugation. This pellet was then resuspended in 5 mL of fresh serum containing RPMI media. Subsequently, 2×10^5 cells per mL were added into two separate T75 vented tissue culture flasks, each containing 20 mL of the serum-containing media. These flasks were then incubated at 37°C in a cell culture incubator with 5% CO2. Upon reaching confluence, the cells underwent the same subculturing procedure once again. The cells were cultured up to 10 passages and passages between 2 to 8 were used in the studies.

3.1.b Glioblastoma cell lines

Glioblastoma cell lines U373 and U251 (ATCC, UK) were cultured in T75 flask with 12 mL RPMI 1640 with 5% fetal bovine serum (FBS) and 10mM L-glutamate. and subcultured when 80% confluency of cell growth was observed. For the immune checkpoint molecule studies, these GB cells were cultured in DMEM F12 (Gibco, UK) media with 5% FBS and 10mM L-glutamate. Cell in the tissue culture flasks were regularly checked under a microscope (2-3 times a week) for cell density, and changes in the colour of the medium. When a change occurs in the colour of the medium, but density of the cells was found to be low, the media in the tissue culture flask was removed and replaced with fresh serum containing RPMI/DMEM F12 medium. When there occurs a change in the colour of the medium and the cells were confluent, (defined as cells are fully grown with little or no space in between the cells), the cells were sub-cultured. Sub-culturing was done by trypsinizing using 3mL of 0.05% trypsin (Gibco, UK). The cell pellet collected by centrifugation of the trypsinized cells was re-suspended thoroughly in 5 mL of fresh serum containing RPMI/DMEM F12 medium. $2X10^5$ cells per mL was then added to each of two T75 cm2 tissue culture flasks, each containing 20 mL of the serum-containing media and incubated at 37^oC with 5% CO2. When they become confluent again, the same process of subculture was applied.

3.1.c Human brain endothelial cells

Human brain endothelial cell line HBEC 5i (Addexbio, San Diego, California) were grown in gelatine (Gibco, UK) coated T75 flask (prior to adding the media, 3mL of gelatine was added to the flasks and incubated for 30 minutes for the adherence of endothelial cells). The T75 flasks were filled with 20mL of DMEM F12 with 40µg/mL of endothelial cell growth supplements (ECGS) (ScienCell laboratories, USA) and 5% FBS and incubated for 48 hours. The HBEC 5i cells were passaged to 10 passages and the cells between 2 to 8 passages were used for the studies. The cells were trypsinised using 3mL of 0.05% trypsin (Gibco, UK) and subcultured when 80% confluency of cell growth observed. Cell in the tissue culture flasks were regularly checked under a microscope (2-3 times a week) for cell density, and changes in the colour of the medium. When there occurs a change in the colour of the medium and the cells were confluent, (defined as cells are fully grown with little or no space in between the cells), the cells were sub-cultured. Sub-culturing was done by trypsinizing using 3mL of 0.05% trypsin (Gibco, UK). The cell pellet collected by centrifugation of the trypsinized cells was re-suspended thoroughly in 5 mL of fresh serum containing DMEM F12 medium. $2X10^5$ cells per mL was then added to each of two T75 cm2 tissue culture flasks, each containing 20 mL of the serum-containing media and incubated at 37°C with 5% $CO2$

3.2 Phagocytosis assay

3.2. a Conditioned media collection

Glioblastoma cell lines U373 and U251 were taken for conditioned media collection. The cells were revived from -80^{o}C and thawed and seeded to T25 flask and next day, the cells were trypsinised and washed with Dulbecco's Phosphate Buffered Saline (PBS) (Sigma Aldrich, UK) to remove the traces of DMSO (Sigma Aldrich, UK) and then re-seeded to T75 flask with RPMI medium. Once the cells were 80% confluent, cells were trypsinised and seeded 1.8x10^{6} cells into a new T-75 with 10mL of complete RPMI-1640 (seeding density had changed to 1.8X10^{5} cells/mL). The T-75 flasks were kept in the incubator at 37°C 5% CO2 for 24, 48, 72 and 98 hours (for days 1,2,3, and 4). After the incubation, the conditioned media were collected, using a 0.2um filter and 10 mL syringe. The filtered conditioned media were collected and labelled appropriately and stored in -20^{o}C.

3.2, b Protein concentration estimation

The bicinchoninic acid (BCA) assay method had used in the detection of total protein concentration from the GB conditioned media. The procedure for the assay was performed using the given protocol in the kit (Pierce™ BCA Protein Assay Kit, Thermofischer, UK) and the standard protein for the reference was bovine serum albumin (BSA) provided in the kit. 25µL of the standard protein and the GB conditioned media samples were added into each well. The assay was performed on 96 well plate and read the absorbance using the SPECTROstar spectrophotometer at 562 nm. Three technical replicates were performed for each standard and sample.

3.2. c Phagocytosis Assay

A 96 well plate was seeded with $1x10^4$ cells per well and 20ng/mL of PMA (Phorbol 12-myristate 13-acetate) (Sigma, UK) was added for macrophage differentiation. After 24 hours, the cells were washed to remove PMA and then added the conditioned media and normal media in 50% ratio in appropriate wells and LPS (Lipopolysaccharide) (Sigma, UK) were added in 100ng/mL concentration in alternate wells to get cells with or without LPS treated. After 48 hours of incubation, the media were removed and washed. The pHrodo-conjugated *E.coli* (Invitrogen™, Thermofischer, UK) was added in a light free condition by mixing in PBS and added in 200uL to each well. PBS was used as the negative control. The plate was incubated in a non-CO2 incubator for 2 hours. After 2 hours of incubation, images of the cells were taken using the EVOS™ FL Auto 2 Imaging System with the transmitted light and RFP channels. The total cell counts and the cells with fluorescence were counted manually. The formula used to get the phagocytic percentage:

$$\text{Phagocytosis \%} = \frac{\text{Cells with red fluorescence}}{\text{Total number of cells}} \times 100$$

3.3 Flow cytometry

3.3.a. Monocytes in GB Conditioned media

The monocyte cell lines MM6 and THP1 were chosen for the studies and the $3x10^5$ cells/mL were seeded into 6 well plate. The wells were filled with 3mL from normal media, conditioned media from U373 and U251 and both normal media and conditioned media. The cells were then incubated for 24hours in a 5% CO_2 incubator. After incubation, the cells were washed with PBS and transferred into tubes with flow buffer prepared from PBS with 5% of FBS and stained with CD80, D86 and HLA-DR at the dilution 1:30 and added to three conditions normal media, conditioned media from U373 and U251. After 30 minutes of incubation at 4^oC, the washed cells were taken for the flow cytometric analysis using Cytoflex flow cytometry, of CD80,86 and HLA-DR expression (Table 3.3.e).

3.3.b Macrophage Differentiation in Conditioned Media

The monocyte cell lines were seeded at a minimum density of $5x10^5$ cells/mL. The 6 well plate with 3mL of RPMI 1640 media, 1.5mL of RPMI 1640 and 1.5mL of conditioned media from U373 and U251 for making 50% of condition and 3mL of conditioned media from U373 and U251 as taken to get 100% condition. The cells were treated with 20ng/mL PMA and placed in the incubator at 37°C, 5% CO_2 for 24 hours. After incubation, the PMA treated cells were attached to the bottom of the plate. The cells were scraped and media with cells were pipetted to labelled tubes and centrifuged the tubes at 5000rpm in a benchtop microcentrifuge (Heraeus Biofuge pico ultracentrifuge with microlitre rotor 75003328) for 5 minutes and washed with PBS and centrifuged again. After removing the PBS, the flow buffer was added into cell pellets and stained with CD80, CD86 and HLA-DR (Table 3.3.e) at a dilution of 1:30 in separate labelled tubes and incubated for 30minutes at $4^{o}C$. After removing the unbound markers with PBS, the cells were analysed with flow cytometer.

3.3.c M1 polarisation of macrophages in Conditioned Media

The PMA treated cells were washed with PBS to remove PMA to avoid damage to the cells. The Lipopolysaccharide (LPS) at 100ng/mL was added to the wells with the same conditions used for PMA treatment and the cells were added with the same quantity of media. After the incubation, the cells were scraped and washed. The cells were stained in flow buffer (flow buffer was made with PBS with 5% FBS and filtered out with 0.2µm filter) and the expression of CD80, CD86 and HLA-DR (Table 3.3.e) on the LPS treated M1 macrophage was analysed, by measuring the mean fluorescence intensity of the cells using flow cytometer.

3.3.d Immune inhibition molecules on Endothelial cells

The immune checkpoint molecules PD-L1 and Galectin 9, which are engaged in the immune regulation were assessed for their expression in GB conditioned media and IFN-ϒ treated GB conditioned media. Briefly, the brain endothelial cell line HBEC-5i was seeded into the 3mL gelatine treated T75 flask containing DMEM-F12 medium supplemented with 40ug/mL endothelial cell growth supplement (ECGS) and 10% FBS. The GB cell lines U373 and U251 were cultured in DMEM F12 medium were filtered out for the conditioned media (same procedure from page 54). The HBEC-5i cells were seeded to the 6 well plate with 3mLs of DMEM F12 with ECGS as control and the next well with IFN-ϒ (100ng/mL) in 3 mLs of DMEM F12. Other wells were filled with 50% of U373 and 50% DMEM F12 to make up 3mLs. Similarly, U251 conditioned medium were added. The U373 and U251 treated with IFN-ϒ were added to adjacent wells in 1:1 ratio with DMEM F12. After 48 hours of incubation, the cells were scraped and washed in PBS and seeded to tubes with flow buffer. The FITC conjugated anti-PDL-1, and PE conjugated anti-Galectin 9 antibodies (Table 3.3.e) were added with a dilution of 1:30 to the cells and incubated for 30 minutes at 4^{o}C. After the incubation, the cells were washed with PBS and taken for analysing the expression of PDL-1 and Galectin 9 of all the wells by obtaining the mean fluorescence intensity values available from the Cytexpert software used in Cytoflex Beckman Coulter flow cytometer.

3.3.e Table 3.1 Antibodies used in the flow cytometry experiments

Specificity	Clone	Volume (µL)	Supplier	Conjugate
CD80	2D10	3µL	Biolegend, UK	PerCP/Cyanine 5.5
CD86	BU63	3µL	Biolegend, UK	FITC
HLA-DR	Tu39	3µL	Biolegend, UK	PE
CD274 (PDL-1)	MIH2	3µL	Biolegend, UK	FITC
Galectin 9	9M1-3	3µL	Biolegend, UK	PE

3.4 Immunoassay

3.4.a Immunoassay for Galectin 1

The GB cell lines U373 and U251 were treated with 100ng/mL IFN-ϒ and the conditioned media were collected. The enzyme linked immunosorbent assay (ELISA) was performed with the conditioned media from both IFN-ϒ treated and untreated cells as per manufacturer's instructions (Human Galectin-1 Quantikine® ELISA, catalog no. DGAL10, R& D systems). The human Galectin-1 polyclonal antibody coated 96 well plate and the Galectin 1 standard were used to estimate the concentration of Galectin 1 in the GB conditioned media. The standard human Galectin-1 antibody titre ranged from 0ng/mL (Calibrator diluent RD5-24 alone) to 20ng/mL, which was diluted using the Calibrator diluent RD5-24(diluted 1:10). The assay diluent added in 100µL to each well upon mixing the precipitate and followed by adding 100µL of the standard, control (PBS) and the sample to the appropriate wells. After 2 hours of incubation on microplate shaker at room temperature, the wells were washed with the wash buffer. The human Galectin-1 conjugate were added to each well and incubated for 2 hours at the room temperature. Following washing, 200µL of the substrate solution was added and incubated for 30 minutes in the dark. Following incubation 50µL of stop solution was added to each well. The optical density of each well from both the sample and standard was analysed using the SPECTROstar spectrophotometer at 450nm. The wavelength correction was carried out by subtracting the value at 540nm with the value from 450nm. Three technical replicates were performed for each standard and sample.

3.5 Statistical Analysis

The Cytexpert software in Cytoflex Beckman Coulter flow cytometer, Microsoft Excel 360 and GraphPad Prism v.9 were used in data analysis and statistical tests. Student T-test was used for two sample comparison and one-way analysis of variance (One-Way ANOVA) was used for comparing more than two samples.

*, p = <0.05, **, p =<0.01, ***, p = <0.001, **** - p value < 0.0001 *ns* = not significant.

4. Results

4.1 The Phagocytic activity of Macrophages in glioblastoma conditioned media

Glioblastoma (GB) is an aggressive malignant tumour that arises in the central nervous system and remains prevalent among adults (Sumera et al., 2022). GB show very poor survival rates and have very limited treatment options due to the complexity of CNS and the immune system in the brain (Brown et al., 2018). In GB, the immune suppressive environment has been developed leading to the failure of the immune cells in attacking the malignancy and the immune cell in the brain favours the cancer progression (Martinez-Lage et al., 2019). The innate and adaptive immunity play a crucial role in the elimination of cancer, however the failure of innate immune cells in clearing the cancer leads to the loss of adaptive immunity (Yi et al., 2023). Innate immune system is the first line of attack in the body upon infection or malignant cell elimination through phagocytosis by various immune cells such as monocytes, macrophages, and dendritic cells leading to antigen presentation for adaptive immunity (Feng et al., 2019). In tumour microenvironment, the macrophages are the most abundant non neoplastic cells which have critical role in maintaining tumour immunity (Zhou et al., 2020). The macrophages are differentiated into M1 and M2 polarised forms, where M1 involves in the pro-inflammatory and anti-tumour activity and M2 has the anti-inflammatory and tumour promoting ability (Wang et al., 2022). According to Brown et al,2018, the macrophages in the GB tumour is known as tumour associated macrophages (TAMs) which supports the tumour growth with the M2 phenotype (Brown, N.F. *et al.* 2018). The cancer secretes diverse range of proteins which participate in immune suppression, cell signalling, angiogenesis, differentiation, and metastasis (Karagiannis et al., 2010). In GB, the proteins secreted from tumour modulate the tumour microenvironment and thereby leading to the tumour progression, chemoresistance and immune suppression (Iglesia et al., 2022). In order to determine the total protein concentration in glioblastoma cells, the media from day 1 to 4 were filtered out from the two established GB cell lines U373 and U251 with density of $1.8x10^5$ cells in 10mL and used as conditioned media to perform BCA (Bicinchoninic acid assay) assays for protein concentration estimation. The results showed that day 3 had the highest protein concentration for U373 cells (3450±64 μg/mL) whereas a slight reduction in total protein concentration (3360±123 μg/mL) was measured for day 4 compared to day 3 (**Fig 4.1.a**). In U251, the total protein concentration did not vary compared to U373 cell line. From 4 days of conditioned media collection, day 3 showed higher protein concentration than that of day 4 (2790±288 μg/mL; **Fig 4.1.b**). Based on the above results day 3 of conditioned media from both U373 and U251 cell lines was used in further experiments as it had the highest protein concentration compared to other days.

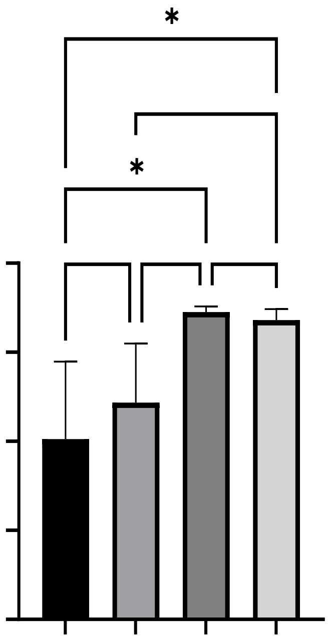


Figure 4.1.a

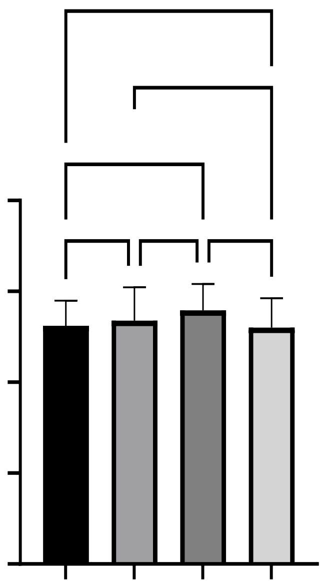


Figure 4.1.b

Fig 4.1. **Total protein concentration of GB conditioned media**. Day 3 showed the highest protein concentration compared to day 1,2 and 4 for both the GB cell lines U373 and U251. Total protein concentration of the GB conditioned media from two cell lines U373 and U251 were estimated using BCA assay with bovine serum albumin as the standard reference. The bar graphs represent the total protein concentration in µg/mL from U373 (a) and U251 (b) with mean and standard deviation 3450±64 µg/mL for U373 cells and 2790±288 µg/mL for U251 cells with significance $P < 0.0012$ (for U373) using GraphPad Prism v.9.

Phagocytosis is one of the tumour elimination strategies by the innate immune cells like macrophages which could be caused aberration in anti-tumour response (Li et al., 2023). In GB, the macrophages are aberrated into tumour associated macrophages with low phagocytic activity especially in the tumour microenvironment (Wu et al., 2023). The conditioned media from day 3 was taken for validating the phagocytosis activity of macrophages. The phagocytosis activity of MM6 cells induced with PMA and LPS were cultured in normal medium, U373 and U251 conditioned media (**Fig 4.1.c.1**) were analysed. The comparison between M0 and M1 phenotype were studied using PMA treated MM6 cells with LPS treatment and without LPS treatment. The normal media was used as the control for further experiments. The cells in the LPS treated normal media and the U373 conditioned media showed (**Fig 4.1.c.2**) almost similar phagocytic activity. The phagocytic activity of MM6 cells in U251 conditioned media had shown lesser than that of the normal media and U373. Along with the LPS treated cells, the MM6 cells grown without LPS were observed. The cells in normal media and conditioned media from U373 and U251 showed a drastic change. The U373 conditioned media grown cells showed reduced percent of phagocytosis (8.3% ±5.35) than the control (13.36%±1.69). Similarly, the U251 showed more reduction (6.22%±4.7) than the U373 and normal media. Overall, a reduction in the phagocytic activity was detected for both the GB cell lines in the presence of conditioned media compared to the control.

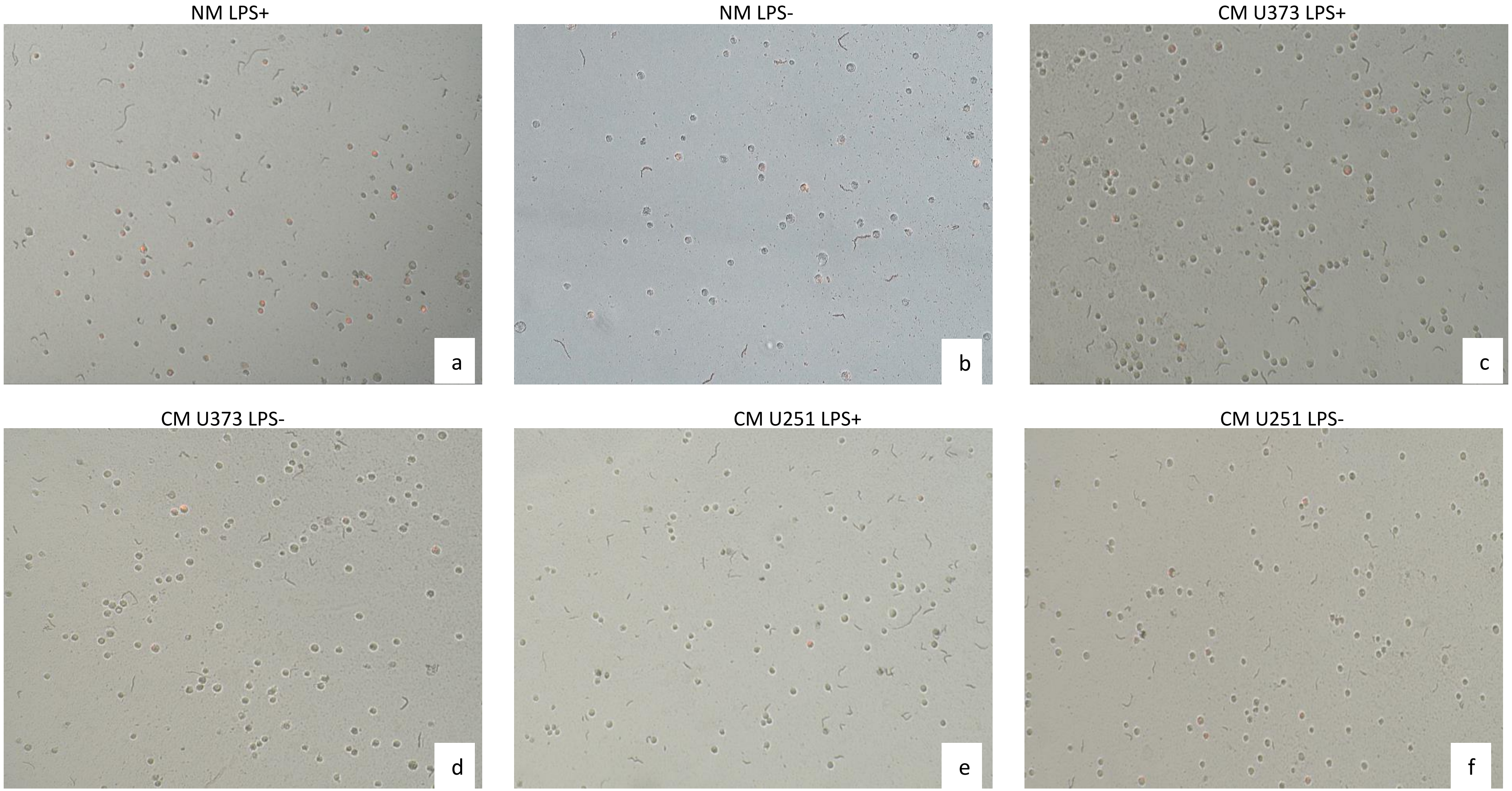


Fig 4.1.c.1, Phagocytic activity of MM6 monocyte cell line in GB conditioned media: The phagocytosis of MM6 cells stimulated with PMA (20ng/mL) and cultured in normal media RPMI 1640 (a & b), 50% of GB cell conditioned media from U373 (c & d) and U251 (e & f) with or without LPS stimulation for M0 or M1 phenotype. The phagocytosed macrophages express the red fluorescence from *E.coli*. All the images are taken in 100x magnification.

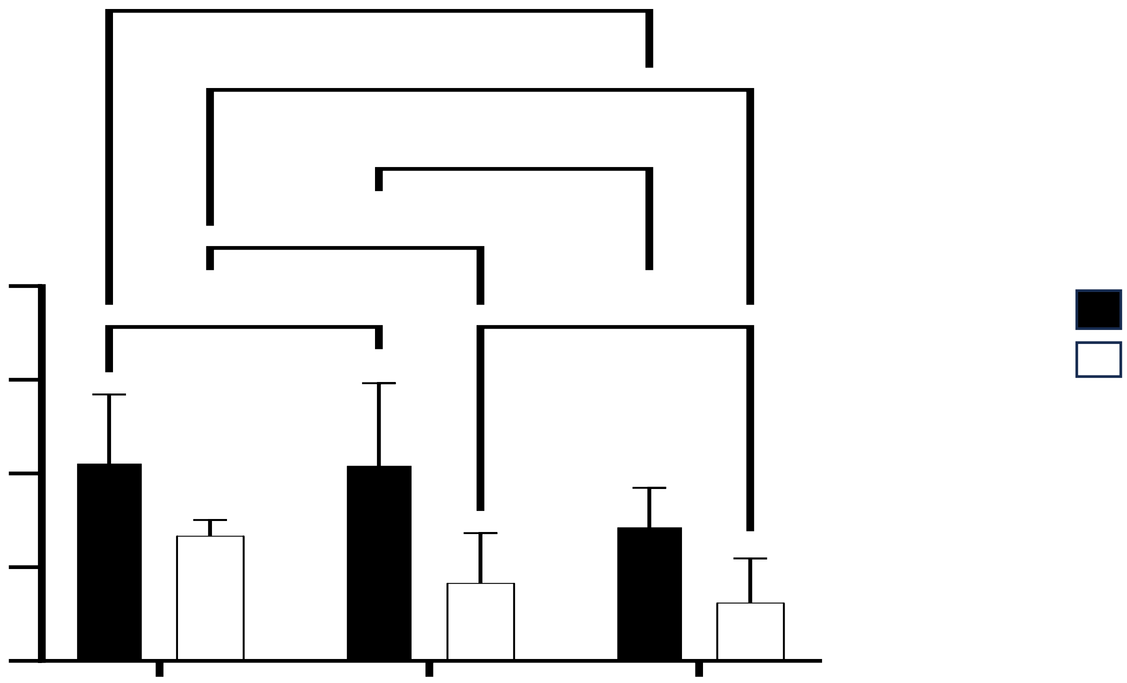


Fig 4.1.c.2, Bar graph for phagocytic activity of MM6 monocyte cell line in GB conditioned media. The bar graph represents the percentage of Phagocytic activity by MM6 cells in M0 or M1 phenotype in normal media and conditioned media with or without LPS treatment with mean and SD. *ns – nonsignificant*.

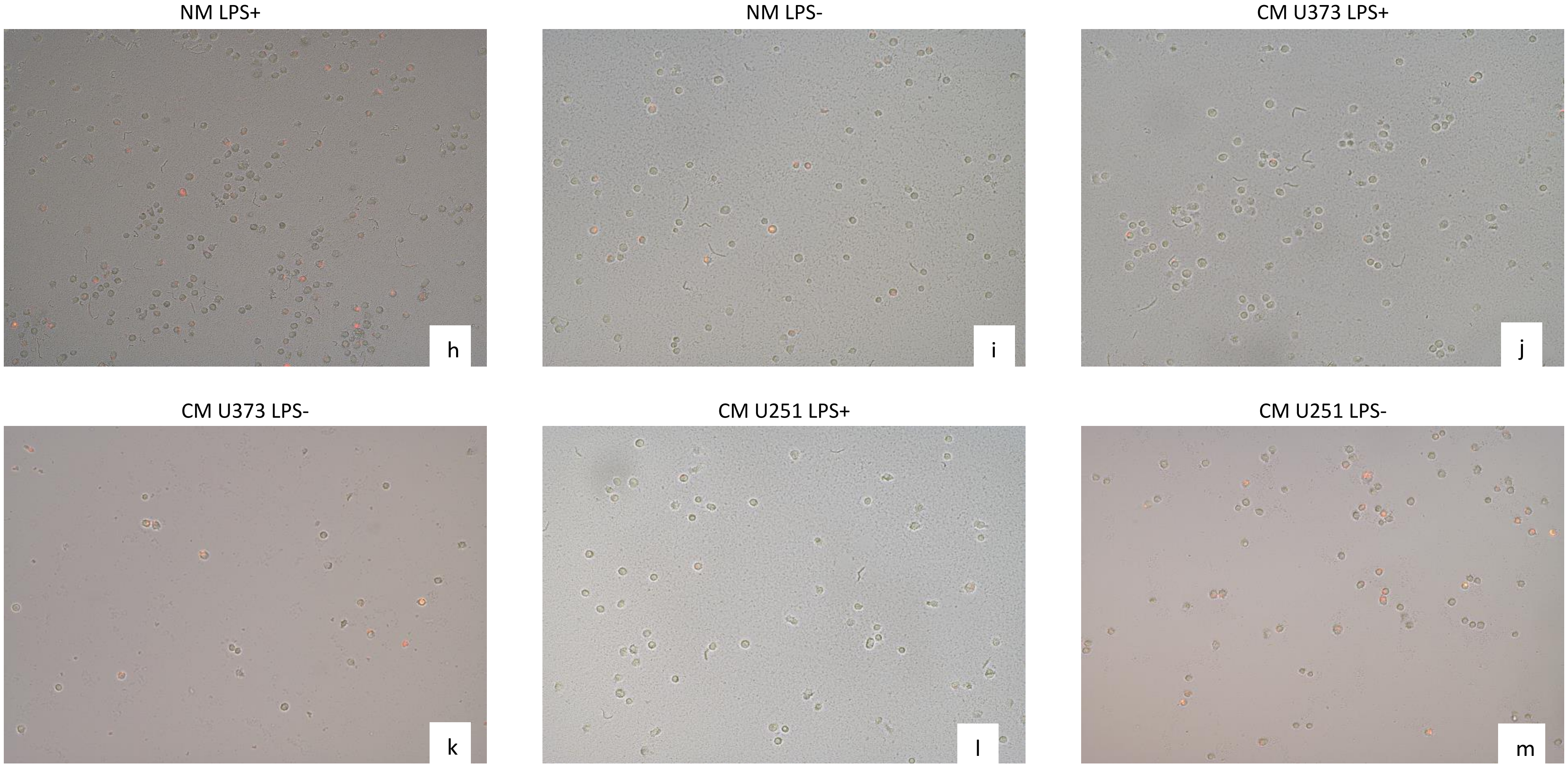


Figure 4.1.d.1, Phagocytic activity of THP1 monocyte cell line in GB conditioned media. THP1 cells were stimulated with PMA and cultured in the normal media (h & i), conditioned media from U373 (j &k) and U251 (l & m) with or without LPS treatment for M0 or M1 phenotypes. The phagocytosed macrophages express the red fluorescence from *E.coli.* All the images are taken in 100x magnification.

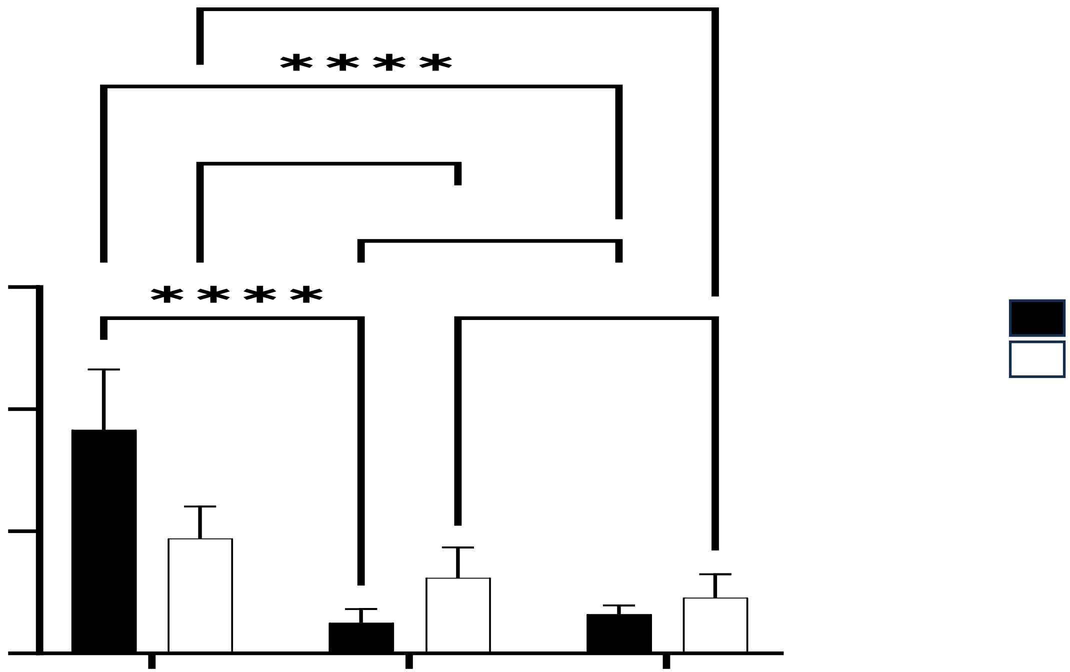


Figure 4.1.d.2, Bar graph illustrates phagocytic activity of THP1 monocyte cell line in GB conditioned media. The bar graph shows the percentage of Phagocytic activity by THP1 cells in normal media and conditioned media with or without LPS treatment with mean and SD. significance ***** - p value < 0.0001 ns – nonsignificant*.

Simultaneously, the THP1 cells were cultured in normal media and conditioned media from U373 and U251 with PMA and with or without LPS stimulation (**Fig 4.1.d**.**1**) for studying the phagocytosis activity. The phagocytosis activity of THP1 cells stimulated with PMA cultured in normal medium with LPS treatment showed higher phagocytic activity (36.6% ± 9.8; **Fig 4.1.d.2**) than the cells cultured in U373 conditioned media (5.04% ± 2.25). Reduced phagocytic activity was detected for the conditioned media from U251 cell lines (6.4% ± 1.45) after LPS treatment compared to the control. There was significant reduction in phagocytosis activity of THP1 cells after treating with GB conditioned media in M1 phenotype. Similarly, the THP1 cells without LPS stimulation showed reduction in the percentage of phagocytosis both in the normal media as well as in the conditioned media. In THP1 cells, the phagocytic activity was decreased in the presence of GB conditioned media, including LPS stimulated and unstimulated cells. The MM6 and THP1 cells without LPS treatment, which is M0 phenotype, in GB conditioned media showed reduction in the percentage of phagocytosis activity compared to the cells in normal medium.

A study by von Roemeling et al (2020) suggests that the macrophages are a bridge to the adaptive immunity through antigen presentation and this antigen presentation of tumour cells to T cells has been exhausted in GB (von Roemeling et al., 2020). To validate the antigen presentation from monocytes and macrophages in GB conditioned media, the CD80 and CD86 co-stimulatory molecule expression as well as the MHC class II expression was analysed by HLA DR and expressional variations were observed using flow cytometry.

4.2 Expression of Antigen presenting markers

4.2.1 The Antigen presentation markers in monocytes

The reduction in the phagocytic acitvity of macrophages gives an insight about the influence of conditioned media and this leads to study the changes on antigen presentation in the presence of GB conditioned media. The blood recruited macrophages were derived from monocytes in the bone marrow and the changes in the monocytes from GB conditioned media, were investigated using THP1 and MM6 cells without any stimulation. The expression of antigen presenting markers for adaptive immune system activation including CD80, CD86 and HLA-DR on monocytes were found to be upregulated in conditioned media from hepatocellular carcinoma (Kuang et al., 2009). The CD80/86 markers are one of the co-stimulatory molecules expressed on the antigen presenting cells which binds to CD28 on T cell for stimulation and activation. Instead, for the immune regulation, CD80/86 binds to Cytotoxic T Lymphocyte Antigen (CTLA) or CD152 on T cells leading to the T cell exhuation and immunosuppression (Ahmed et al., 2022). Understanding the changes of CD80/86 and HLA-DR in the conditioned media it provides an overview of how the GB secreted proteins manipulate the antigen presentation from monocytes causing immune suppression. To estimate the amount of change in monocyte cells, flow cytometry was used. The experiment involved staining of cells using anti- CD80, CD86 and HLA-DR antibody that specifically bind to CD80/86 markers and HLA-DR receptor, following staining, flow cytometry detects and measures the fluorescence emitted by the cells, allowing to assess the level of expression of CD80, CD86 and HLA-DR.

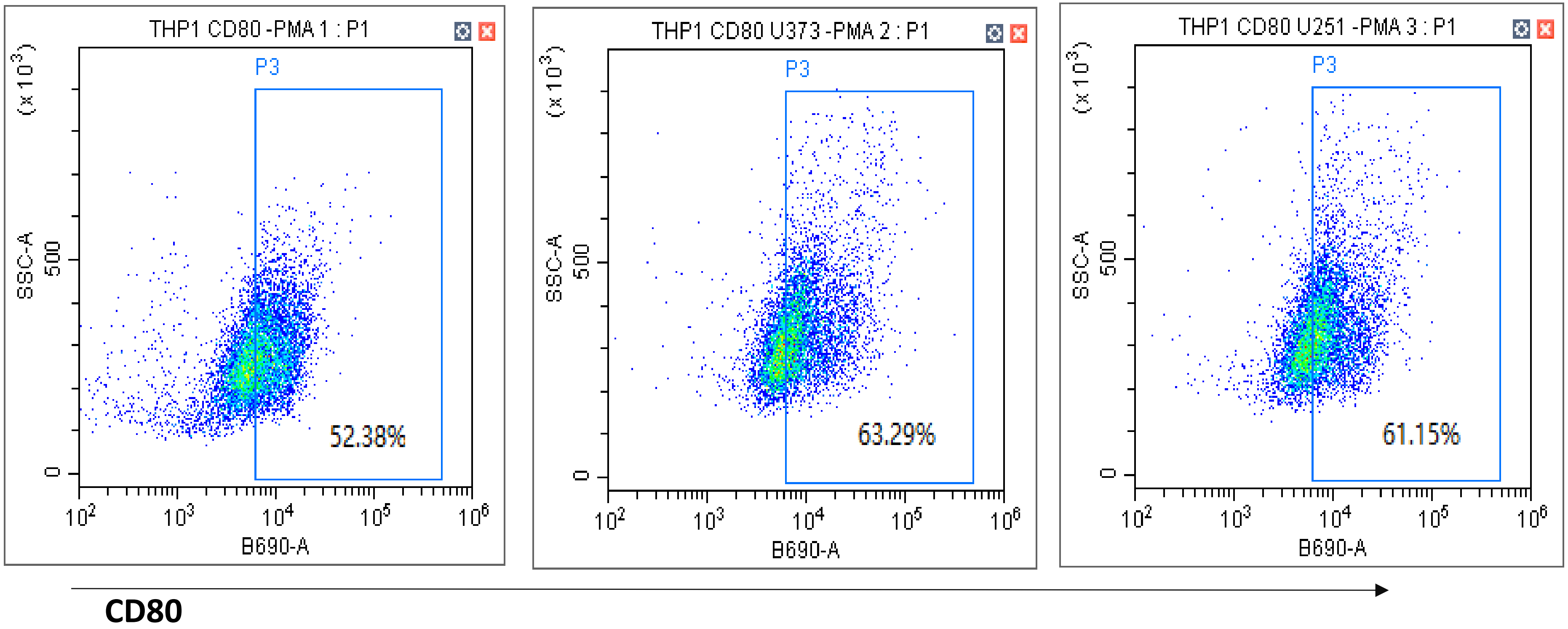
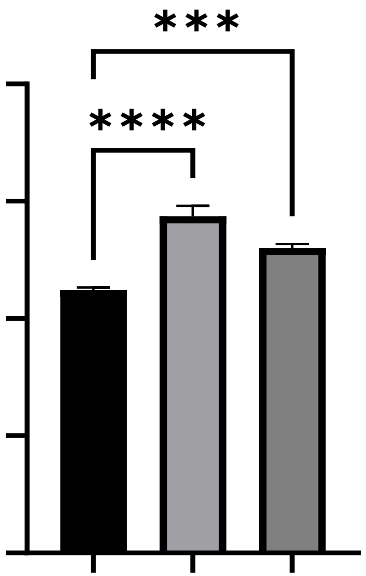


Fig 4.2.1. a. 1 Representative dot plots for the expression of CD80 on monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the THP1 cells cultured in normal media, U373 and U251 conditioned media and stained with PerCP/Cyanine 5.5 conjugated anti CD80. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.



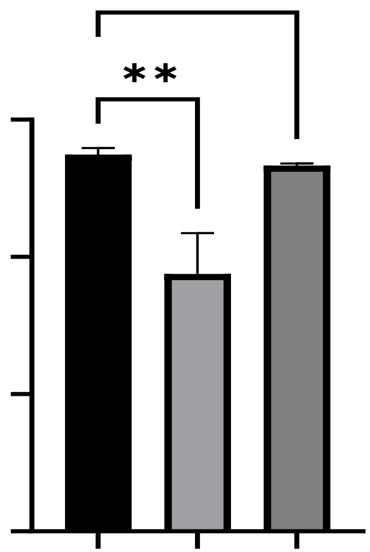


Fig 4.2.1. a.2 Bar graphs represent the MFI of the CD80 expression of monocyte cells THP1 & MM6 cells with mean and SD with significance ***** - p value < 0.0001, ** - p value <0.005, *-p value < 0.05 and ns nonsignificant,* n=3. The normal media is used as the control to analyse the change in the MFI of CD80. N.M= Normal media, C.M= Conditioned media.

THP1 cells were taken in the monocytic stage without any PMA stimulation and allowed to grow in 100% of conditoned medium from U373 and U251 cell lines (**Fig 4.2.1.a**). The dot plots show the drift in percentage of cell population stained with anti-CD80 antibody expression in the normal media and GB conditoned media (**Fig 4.2.1.a.1**). Results revealed that the MFI of CD80 expression in conditioned media for both U373 (MFI- 14365 ± 449) and U251 (MFI-13000 ± 168) demonstrated higher expression when compared to cells in normal media (MFI-11230 ± 98; **Fig 4.2.1.a.2 first graph**). Decreased expression of the CD80 on MM6 monocytic cells (normal media- MFI 13732 ± 232, conditioned media U373 MFI-9382 ± 1484, U251 MFI-13332 ± 77.3) was observed compared to that of THP1 cells (**Fig 4.2.1.a.2 second graph**). Also, a decrease in the expression of PerCP5.5 conjugated anti-CD80 was observed in cells grown in U373 conditioned media, but the cells in conditioned media from U251 showed only a small reduction in CD80 expression than cells from normal media. The CD80 expression on MM6 cells was reduced whereas in THP1 cells, it was increased (**Fig 4.2.1.a.2 graphs**). Furthermore, the expression of the CD86 marker on monocytes in the presence of GB conditioned media was investigated.

The antigen presenting marker CD86, which is another co-stimulatory molecule engaged in T cell activation was found to be expressed on monocyte cells for eliciting theimmune responses (Pinto et al., 2018). Similar to the CD80, CD86 expression on the THP1 and MM6 cells grown in normal media, and 100% of U373 and U251 conditioned media (**Fig 4.2.1.b**) was analysed using FITC conjugated anti-CD86 marker. The dot plots illustrate the changes in percentage of THP1 cells expressing CD86 in GB conditioned media (**Fig 4.2.1.b.1**). The THP1 cells in conditioned media U373 (MFI-5025 ± 155) and U251 (MFI- 5235 ± 149) showed increased CD86 expression compared to normal media (MFI-4366 ± 56) (**Fig 4.2.1.b.2 first graph)**. Along with THP1, reduced CD86 expression in MM6 cells in U373 (MFI-7226 ± 1605) and U251 (MFI-10897 ± 3894) conditioned media was observed (**Fig 4.2.1.b.2 second graph)**, compared to normal media (MFI-8500 ± 1567). The FITC conjugated anti-CD86 expression was higher in the THP1 in the GB conditioned media, whereas MM6 expression was reduced in the presence of conditioned media (**Fig 4.2.1.b.2**). The CD80 and CD86 expression on monocytes, in THP1 cells were upregulated, whereas on MM6 cells, the expression was downregulated. Along with CD80 and CD86, MHC class II molecule, is involved in the antigen presentation. To understand how the MHC class II molecule expression drifts in the GB conditioned media, the HLA-DR marker was investigated on monocytes in the presence of GB conditioned media.

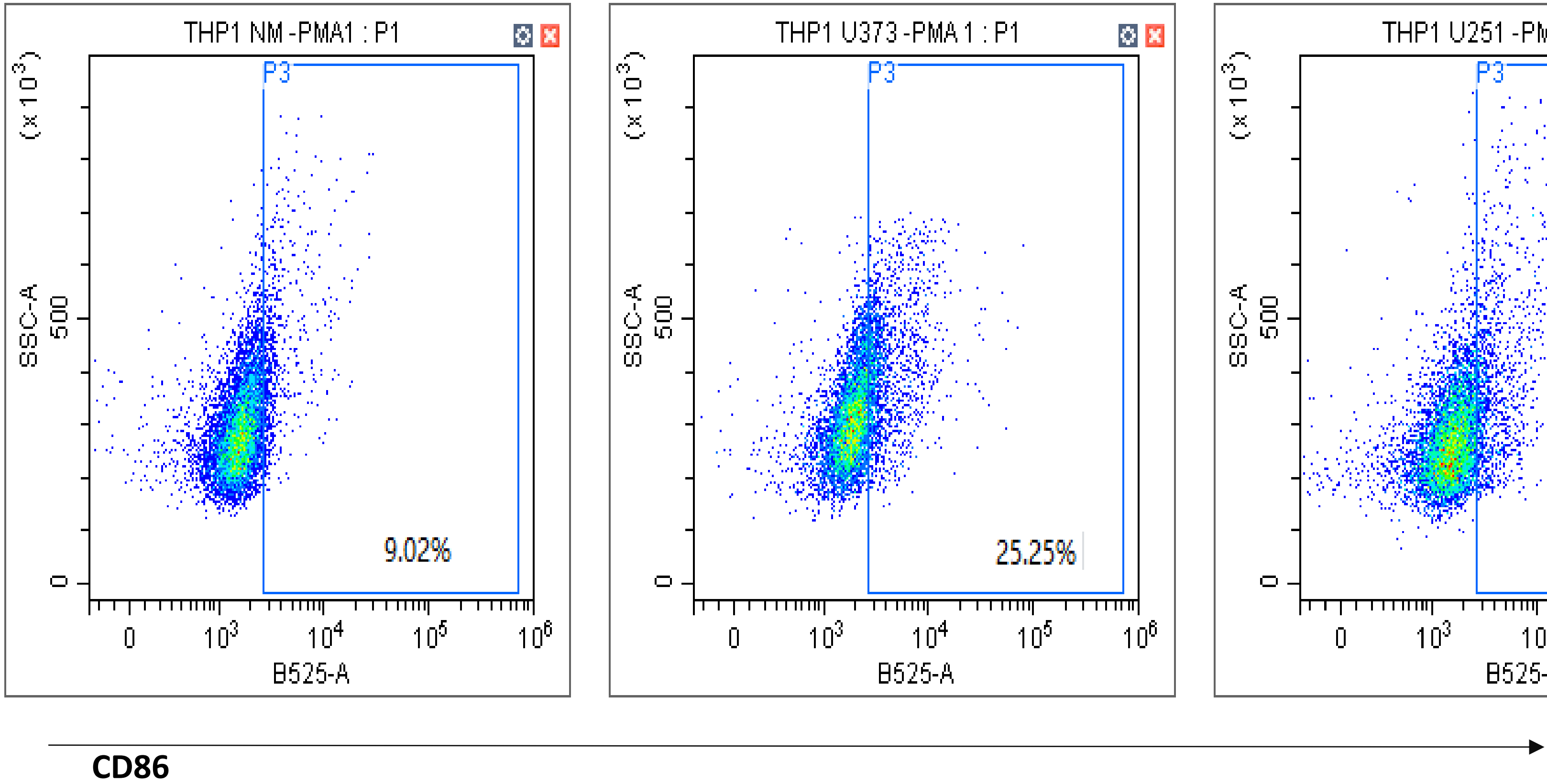


Fig 4.2.1. b.1 Representative dot plots for the expression of CD86 on monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the THP1 cells cultured in normal media, U373 and U251 conditioned media and stained with FITC conjugated anti-CD86. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

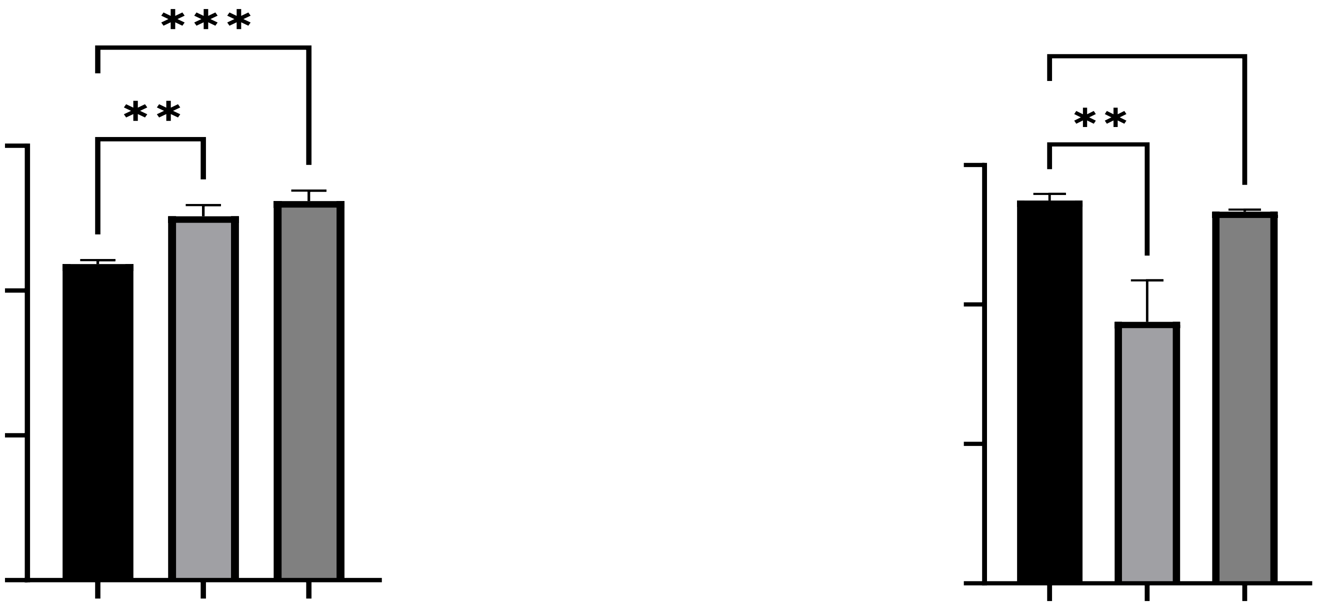
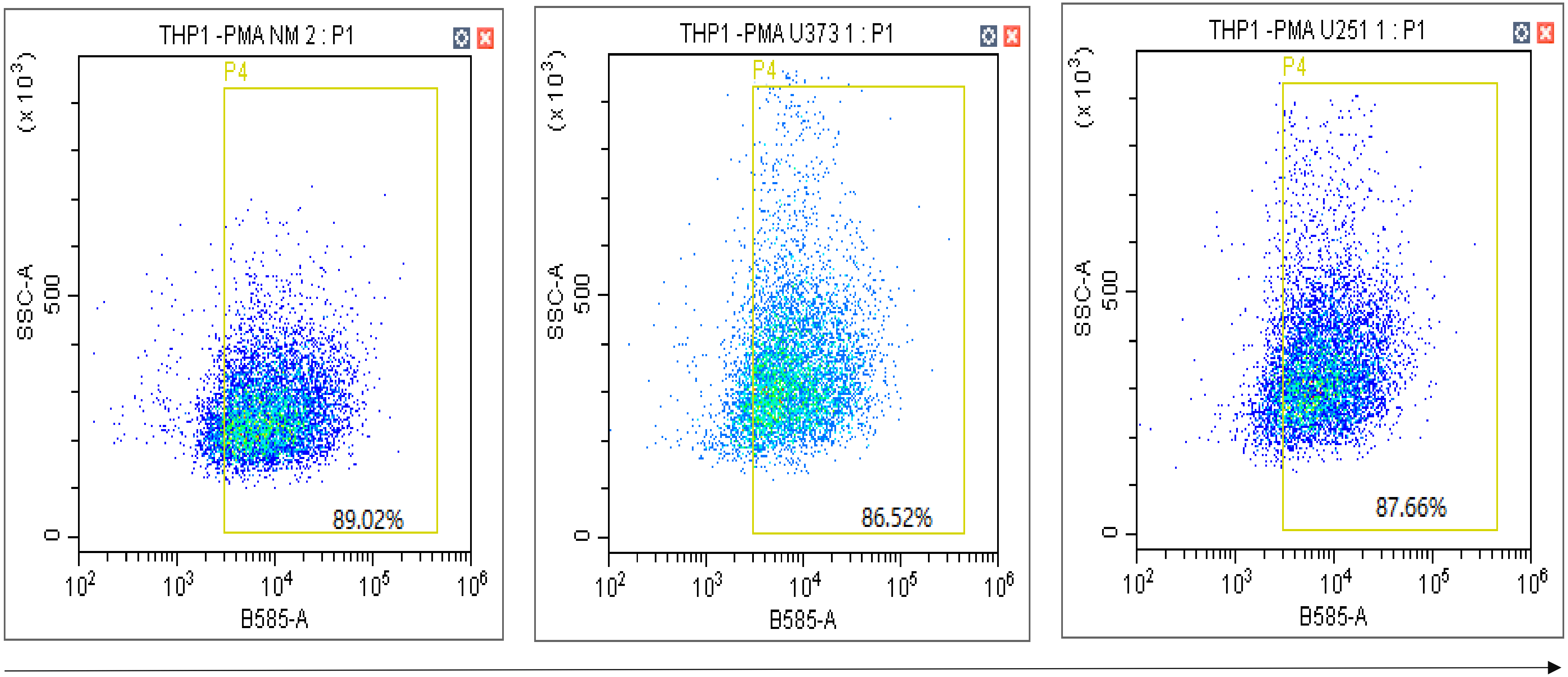


Fig 4.2.1. b.2 Bar graphs represent the MFI of CD86 on THP1 & MM6 cells with mean and SD with significance _** - p value <0.005, *- p value < 0.05 and ns nonsignificant_.n=3. The normal media is used as the control to analyse the change in the MFI of CD86.

The Major Histocompatibility Complex (MHC), also known as Human Leukocyte Antigen (HLA) in humans, are the molecules which aid in presenting antigen peptides for activation of CD4+ and CD8+ T cells, the MHC consists of two classes MHC class I and II. The MHC class I in humans are encoded by the genes *HLA-A, HLA-B* and *HLA-C* and the MHC class II are encoded by *HLA-DR, HLA-DQ* and *HLA-DP* (Neefjes et al., 2011). The MHC class II are generally present on antigen presentation cells and have the specificity for CD4+ T cell activity (Roche & Furuta, 2015). The loss of HLA-DR expression has been observed on various tumours including GB, leading to immune suppression (Mengos et al., 2019). The HLA-DR expression on monocytic cells, THP1 and MM6 cells in normal media, and conditioned media were analysed to investigate how the antigen presentation was affected by the GB secreted proteins.



HLA-DR

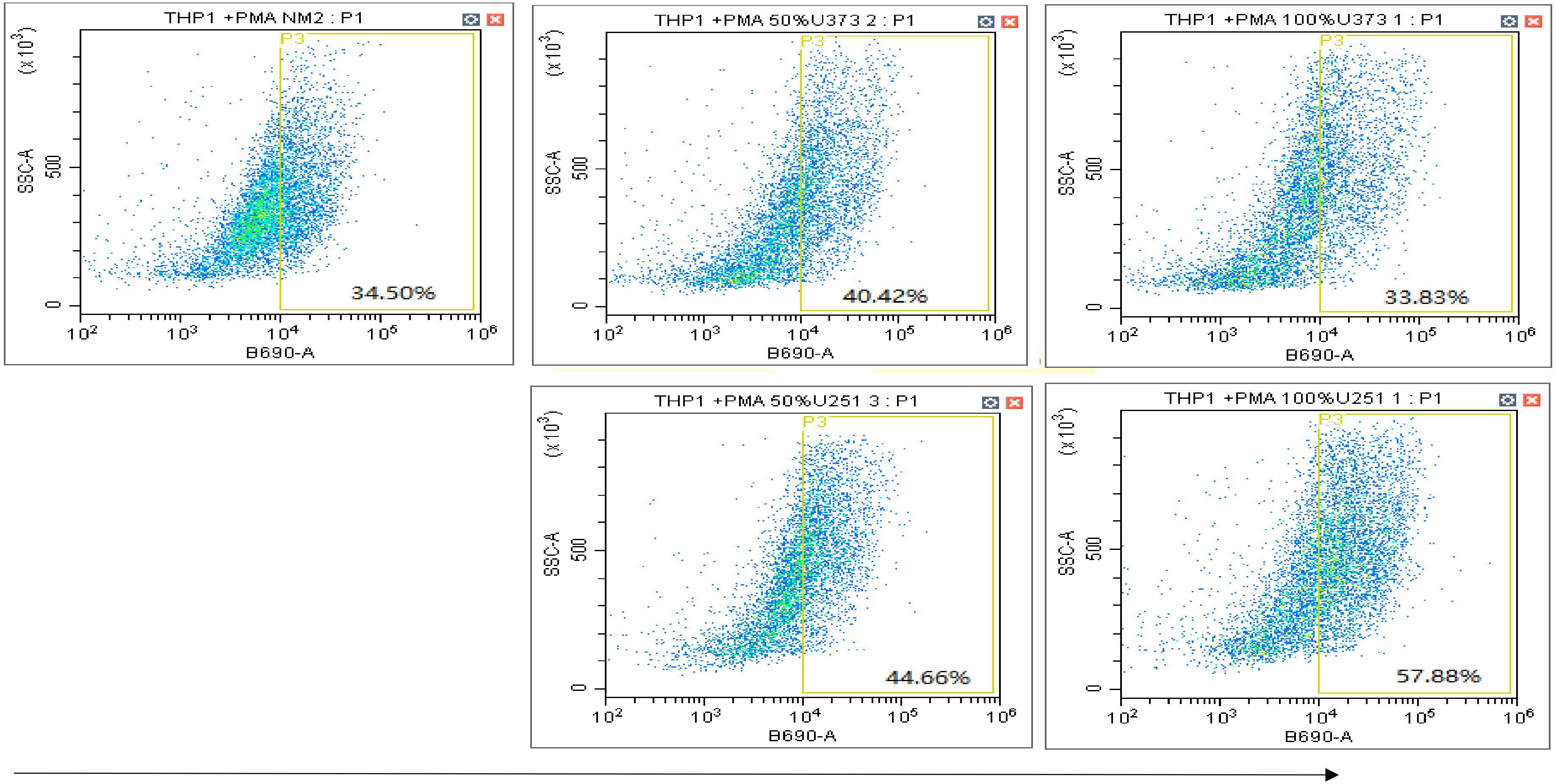
Fig 4.2.1.c.1 Representative dot plots for the expression of HLA DR on monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the THP1 cells cultured in normal media, U373 and U251 conditioned media and stained with PE conjugated anti-HLA DR. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

Fig 4.2.1.c.2 Bar graphs represent the MFI of HLA DR on THP1 & MM6 cells with mean and SD with significance of ***** - p value < 0.0001 ** - p value <0.005, and *- p value < 0.05,* n=3. The normal media is used as the control to analyse the change in the MFI of HLA-DR.

The dot plots from flow cytometry shows a reduction in cell population in the presence of GB conditioned media (**Fig 4.2.1.c.1)**. The MFI values of HLA-DR expression on THP1 cells grown in U373 conditioned media (MFI- 11017±478) showed a reduction compared to the expression on normal media grown cells (MFI-13260 ± 278). Similarly, in U251 conditioned media decreased in the HLA-DR expression (MFI-11902 ± 172) was observed (**Fig 4.2.1.c.2 first graph)**. The HLA-DR expression on MM6 cells showed a decrease in the HLA-DR expression on cells in U373 (MFI-22005 ± 2522) and U251 (MFI- 33963 ± 3637) conditioned media compared to cells in normal media (MFI- 45288 ± 585) (**Fig 4.2.1.c.2 second graph)**. In both THP1 and MM6 monocyte cells, the HLA-DR expression had considerably decreased in the conditioned media from both U373 and U251 cell lines (**Fig 4.2.1.c.2**). In the monocytic stage, the CD80 and CD86 expression were upregulated and downregulated respectively in the GB conditioned media, meanwhile, the HLA-DR expression was reduced in both monocytic cell lines grown in the conditioned media. The findings from these results show that the HLA-DR expression had affected by the GB secreted proteins and may contribute to the suppression of antigen presentation by the monocytes.

4.2.2 The Antigen Presentation markers in PMA stimulated monocytes

The monocytes recruited through blood had been differentiated into macrophages upon stimulation from the tumour showing M0 phenotype, where it would either polarize to M1 or M2 phenotype. In the present study, the expression of co-stimulatory molecules CD80/86 and HLA DR on M0 macrophages for antigen presentation to lymphocyte activation were analysed for the changes from GB secreted proteins. The co-stimulatory molecules CD80 which plays a crucial role in either stimulation or the inhibition of T lymphocytes through binding of CD80 to either CD28 or CD152, based on various stimulation factors in the immune response (Mir, 2015). CD80 on macrophages plays a role as either anti cancer or cancer promoting in the tumour microenvironment based on the stimulation for antigen presentation or to bind with immune regulator CTLA (Vackova et al., 2021). In this study, the drift in the CD80 expression on macrophages upon culturing in the GB conditioned media was analysed using flow cytometry with the PerCP/Cyanine 5.5 conjugated anti-CD80 on PMA stimulated THP1 and MM6 cells (**Fig 4.2.2.a)**. The cells were cultured in 100% of normal media, 50% and 100% of U373 and U251 conditioned media.



CD80

Fig 4.2.2.a.1 Representative dot plots for the expression of CD80 on PMA stimulated monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the PMA treated THP1 cells cultured in normal media, U373 and U251 conditioned media and stained with PerCP/Cyanine 5.5 conjugated anti-CD80. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

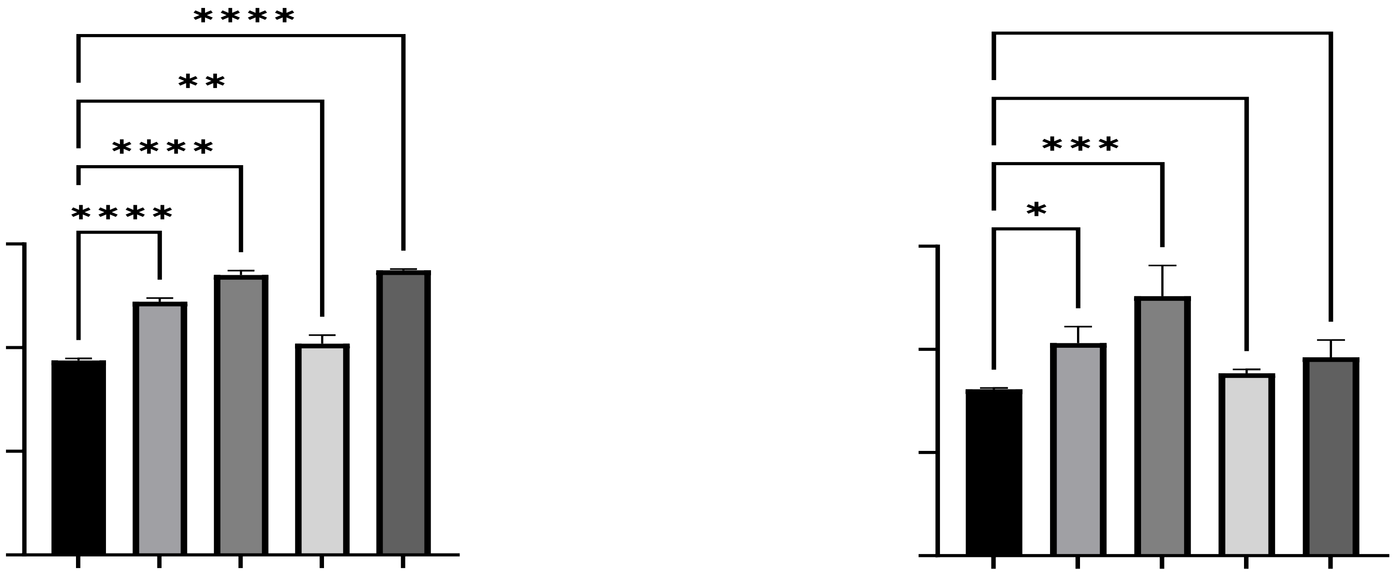


Fig 4.2.2.a.2 Bar graphs represent the MFI of CD80 on THP1 & MM6 cells with mean and SD with significance _**** - p value < 0.0001, ***- p value < 0.0005, ** - p value <0.005, *-p value < 0.05 and ns nonsignificant._n=3. The normal media is used as the control to analyse the change in the MFI of CD80.

The CD80 expression on THP1 cells had observed in each titration of GB conditioned media, comparing with the control, CD80 stained cells in normal medium. The dot plots show the variations in the cell population of anti CD80 antibody coated cells in the GB conditioned media **(Fig 4.2.2.a.1)**. CD80 expression was detected in THP1 cells and an increased expression on M0 macrophage stage was found in both the conditioned media from U373 (MFI for 50% - 24443 ± 351, 100%-27009 ± 437) and U251 (MFI for 50%-20399 ± 809, 100%- 27451 ± 137), when compared to the cells in the normal media (MFI-18770 ± 183) (**Fig 4.2.2.a.2 first graph**). Subsequently, Figure 4.2.2.a.2 graph represents the MFI of CD80 expression on MM6 cells in M0 phenotype where an increase in the expression of CD80 (MFI for normal media-16136±116, conditioned media U373 in 50%-20600±1603 and 100%-25165±2960, U251 in 50%-17681±393 and 100%-19237±1667), same as observed on THP1 cells, was observed. The results on CD80 expression on PMA treated THP1 and MM6 cells suggest that the GB conditioned media is upregulating the expression of CD80.

The CD86 expression on M0 macrophages was analysed in the presence of GB conditioned media. The CD86 is a co-stimulatory molecule rapidly expressed right after the activation of antigen presentation and thereby promotes immune response by T cell activation (de Vos et al., 2020). The CD86 expression on THP1 cells in normal media, 50% and 100% of U373 and U251 conditioned media (**Fig**

4.2.2 b.1) was investigated using flow cytometry. The results revealed an increase expression of CD86 in the conditioned media. The MFI of CD86 were increasing in both 50% (MFI-10257±489) and 100% (MFI-9150±220) of U373 and 50% (MFI-10513±301) and 100% (11042±377) of U251 conditioned media (**Fig 4.2.2 b.2 first graph**). However, in MM6 cells, the CD86 has increased only in 100% of U373 (MFI-13047±1643) and U251 (MFI-10485±866) conditioned media than the cells in the normal media (MFI-8716±433) (**Fig 4.2.2 b.2 second graph**). In both THP1 and MM6 cells, the CD86 expression was higher in the GB conditioned media (**Fig 4.2.2 b.2)**. The findings on both CD80 and CD86 suggests that the GB conditioned media may be influencing the expression of co-stimulatory molecules.

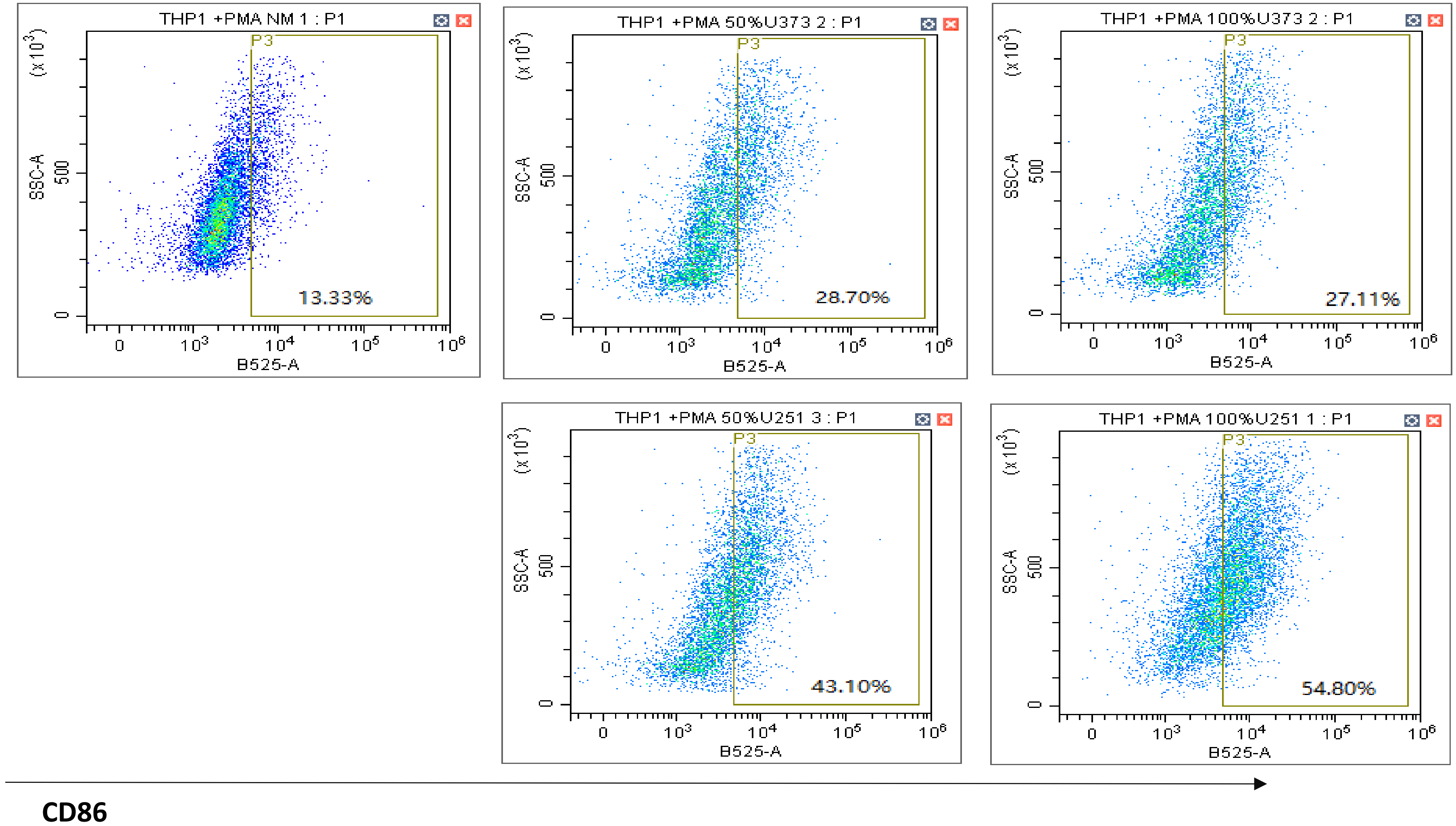


Fig 4.2.2.b.1 Representative dot plots for the expression of CD86 on PMA stimulated monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the THP1 cells treated with PMA, cultured in normal media, U373 and U251 conditioned media and stained with FITC coated anti CD86. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

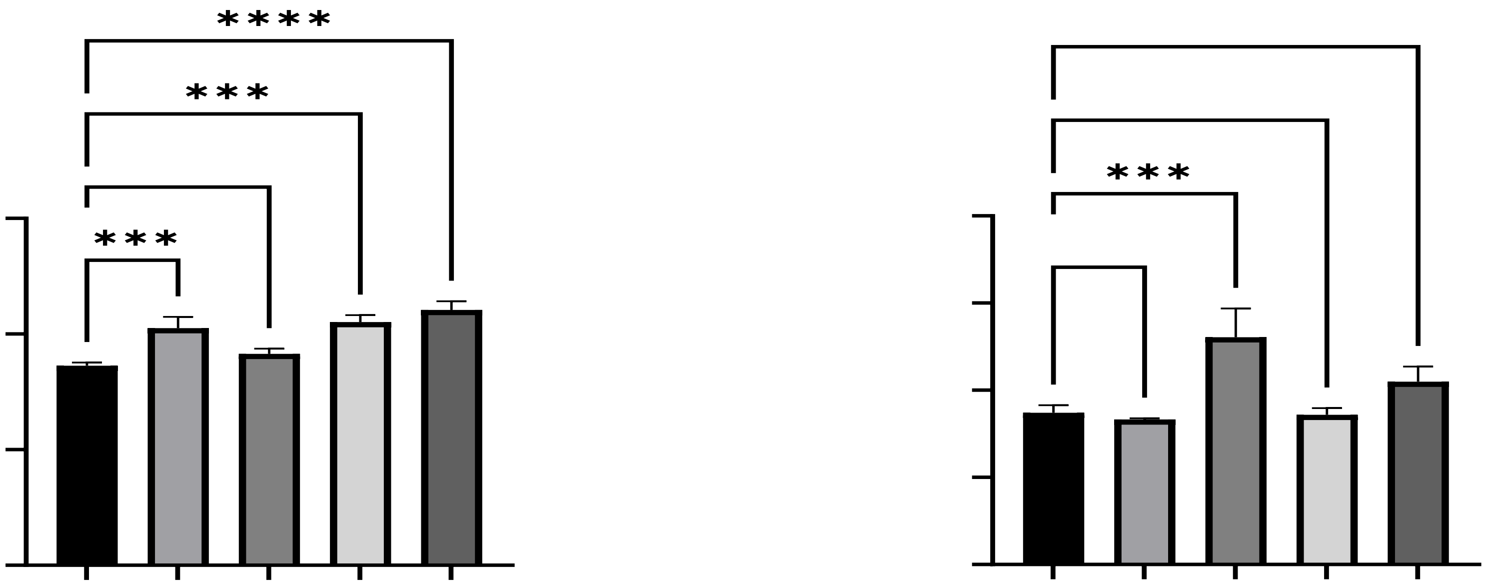


Fig 4.2.2.b.2 Bar graphs represent the MFI of CD86 on THP1 & MM6 cells with mean and SD with significance ***** - p value < 0.0001, ** - p value <0.005, *-p value < 0.05 and ns nonsignificant*, n=3. The normal media is used as the control to analyse the change in the MFI of CD86.

HLA DR, one of the class II MHC antigens is expressed on antigen presenting cells like macrophages for the immune response via CD4+ T helper cells as well as the CD8+ T cells (Li et al., 2020). The dot plot data has shown a reduction in cell population in the presence of GB conditioned media **(Fig 4.2.2.c.1**). The HLA DR expression on THP1 cells (MFI of normal media-34502 ± 331) is found to be reduced in 50% (MFI-21513 ± 722) and 100% (MFI-14626 ± 468) of U373 and 50% (MFI-27506 ± 1052) and 100% (MFI-19404 ± 962) U251 conditioned media (**Fig 4.2.2.c.2 first graph**). Similarly, the HLA DR expression has significantly downregulated upon the MM6 cells (MFI of normal media- 55067 ± 248) grown in the U373 (MFI for 50%-36383 ± 2184 and MFI for 100%-46444 ± 1251) and U251 conditioned (MFI for 50%-29572 ± 1025 and MFI for 100%-34929 ± 2499) **(Fig 4.2.2.c.2 second graph)**. The HLA DR expression on both THP1 and MM6 cells (**Fig 4.2.2.c.2)** in 50% and 100% of U373 and U251 conditioned media cultured cells were downregulated in the presence of GB conditioned media. These findings from M0 phenotype macrophages cultured in 50% and 100% of GB conditioned media showed an increase in the expression of co-stimulatory molecules CD80 and CD86 and a reduction in the HLA-DR expression on both THP1 and MM6 cells suggesting that the GB secreted proteins might be affecting the antigen presentation by the M0 macrophages.

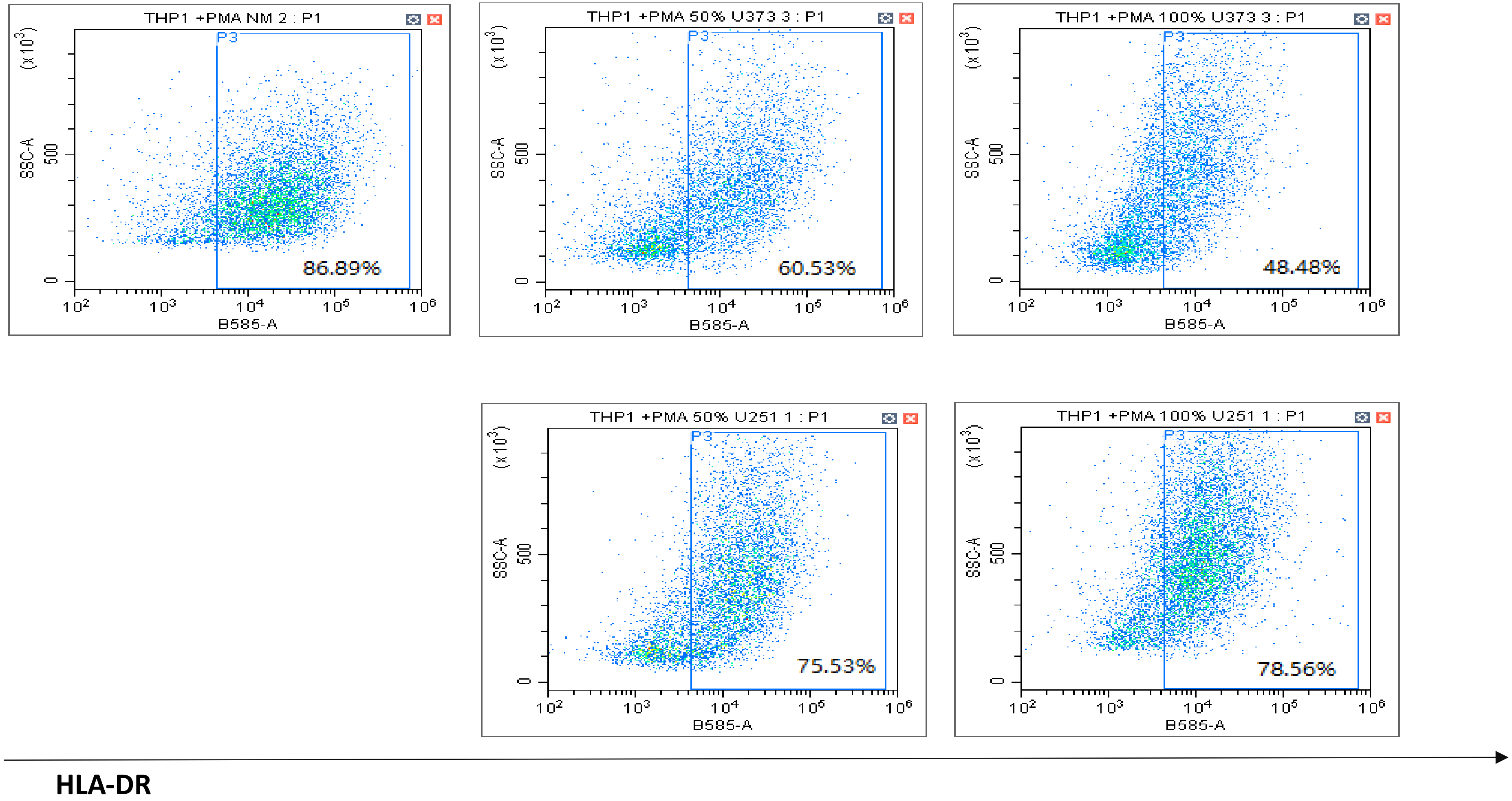
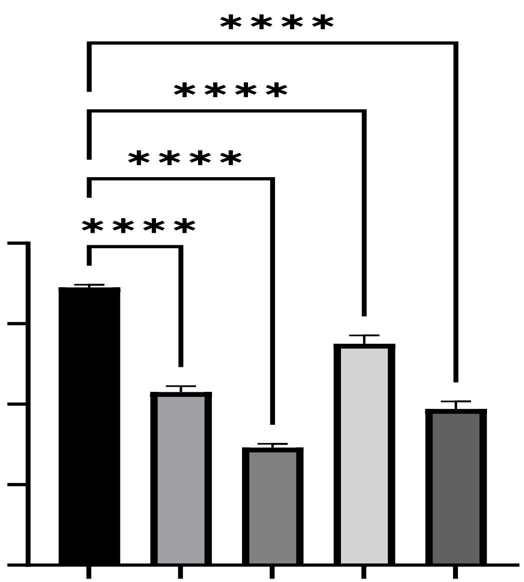


Fig 4.2.2.c.1 Representative dot plots for the expression of HLA DR on monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the THP1 cells cultured in normal media, U373 and U251 conditioned media and stained with PE conjugated anti HLA DR. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.



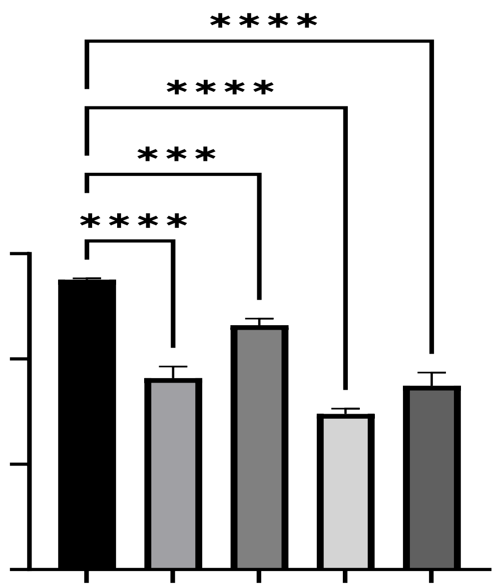


Fig 4.2.2.c. Bar graphs represent the MFI of HLA DR on THP1 & MM6 cells with mean and SD with significance ***** - p value < 0.0001, ***- p value < 0.0005, and ns nonsignificant*. n=3. The normal media is used as the control to analyse the change in the MFI of HLA-DR.

Similar to the monocytic stage, in M0 phase, the HLA-DR expression was reduced on both the cell lines in the GB conditioned media. To further validate, how the antigen presenting markers were observed in polarized stage, LPS was used to stimulate the PMA treated monocyte cells to M1 phenotype.

4.2.3 The Antigen Presentation markers in PMA and LPS stimulated monocytes

The macrophages in the immune response should be in either M1 or M2 polarized. The M1 macrophages are polarized from M0 stage in the presence of Lipopolysaccharide (LPS) (Yunna et al., 2020). The M1 macrophages have the CD80/86 as the surface markers, which could bind with CD28 for T cell activation and also it express MHC class II, HLA DR that bind with T cell receptor on T cell for activation leading to an immune response. In tumour microenvironment, the macrophage polarization is towards tumour promoting M2 phenotype (Boutilier and Elsawa, 2021). In GB, the tumour infiltrating macrophages are known as Tumour associated macrophags (TAMs) towards the tumour microenvironment and appears in M1 phenotype in the onset of tumour growth and later these macrophages are polarised into tumour growth supporting M2 macrophages (Ren et al., 2023). In the present study, the PMA stimulated monocyte cells were again stimulated with Lipopolysaccharide (LPS) which polarise to M1 stimulation and the cells were cultured in GB conditioned media from U373 and U251 cell lines.

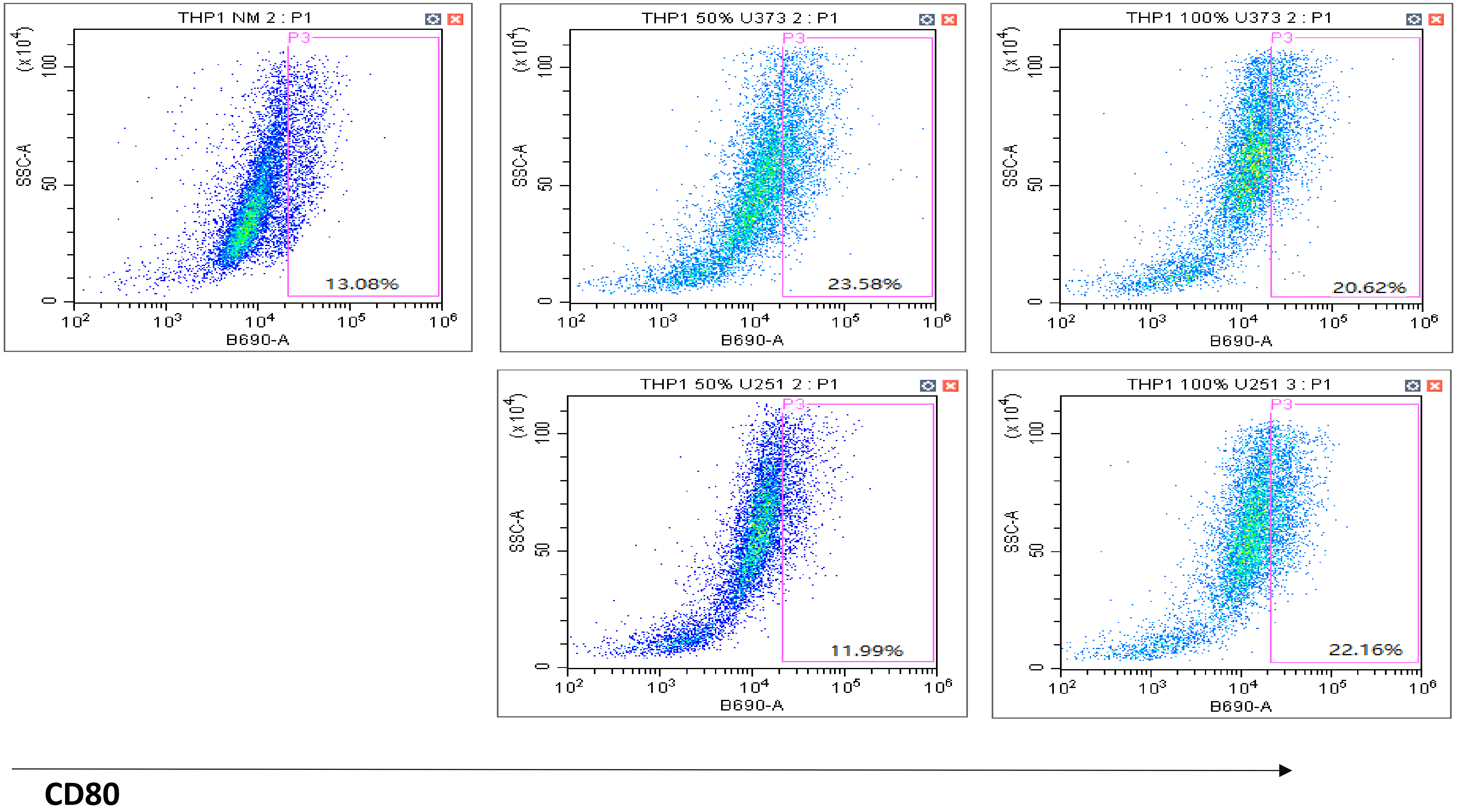


Fig 4.2.3.a.1. Representative dot plots for the expression of CD80 on PMA and LPS stimulated monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the THP1 cells stimulated with PMA and LPS treated, cultured in normal media, U373 and U251 conditioned media and stained with PerCP/Cyanine 5.5 conjugated anti CD80. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

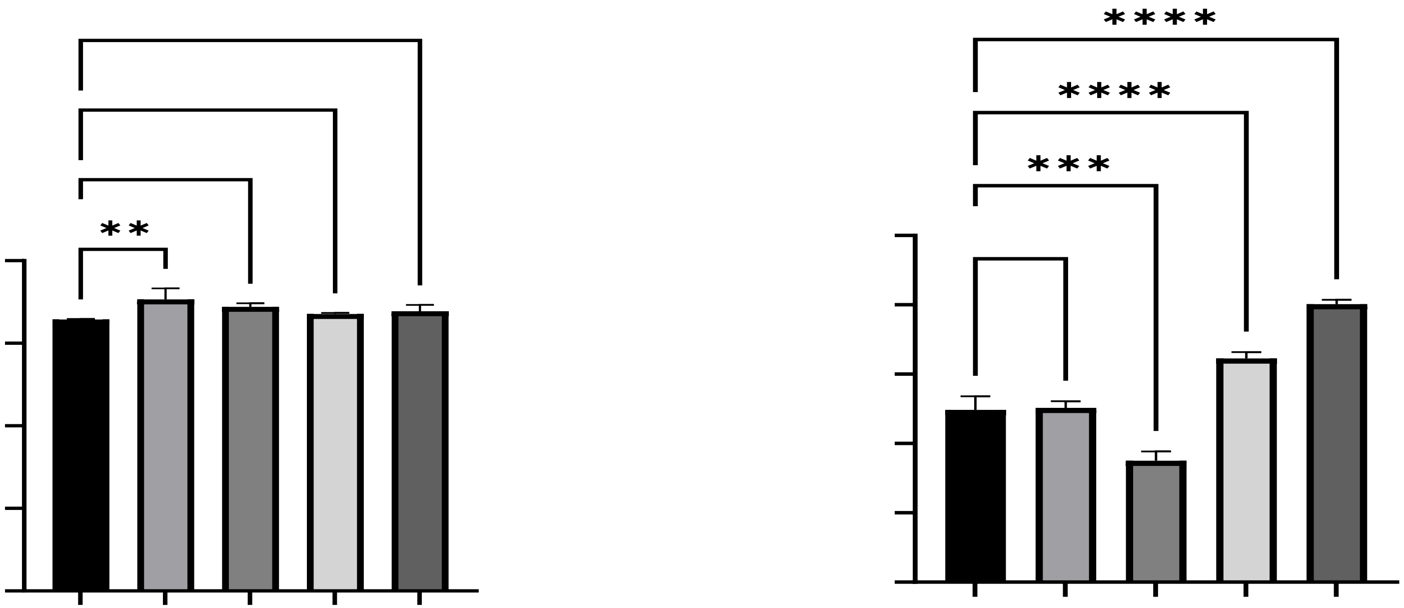


Fig 4.2.3.a. Bar graphs represent the MFI of CD80 on THP1 & MM6 cells with mean and SD with significance ***** - p value < 0.0001, ***- p value < 0.0005, and ns nonsignificant,* n=3. The normal media is used as the control to analyse the change in the MFI of CD80.

Upon PMA and LPS stimulation, the antigen presenting marker expression were analysed on THP1 and MM6 cells (**Fig 4.2.3.a.1)** following staining with CD80. The LPS stimulated THP1 cells had shown a slight increase in the CD80 expression, but it was small in 50% of U373 conditioned media (MFI- 35313 ± 1326). The expression on cells in 100% U373 condtioned media was small (MFI-34428 ± 406) than the normal media (MFI-32874 ± 91)(**Fig 4.2.3.a.2 first graph**). In U251 conditioned media, the MFI of CD80 was almost similar (MFI for 50%-33564 ± 114 and 100%-33873 ± 788)) to the control (MFI-32874 ± 91). The PMA and LPS stimulated MM6 cells were stained with CD80, and expression was unstable in both 50% (MFI-50313 ± 1888) and 100% (MFI-35046 ± 2626) of U373 conditioned media (MFI of normal media- 49683 ± 3957) whereas, in U251, there was massive upregulation of CD80 (MFI for 50%-64515 ± 1893 and 100%-80233 ± 1249) (**Fig 4.2.3.a.2 second graph**). The CD80 expression on both MM6 and THP1 cells in M1 phenotype were varying (**Fig 4.2.3.a.2**), as there were no stable upregulation or downregulation observed in the GB conditioned media. The CD86 expression was analysed along with CD80 on PMA and LPS stimulated monocytes which were treated with GB conditioned medium.

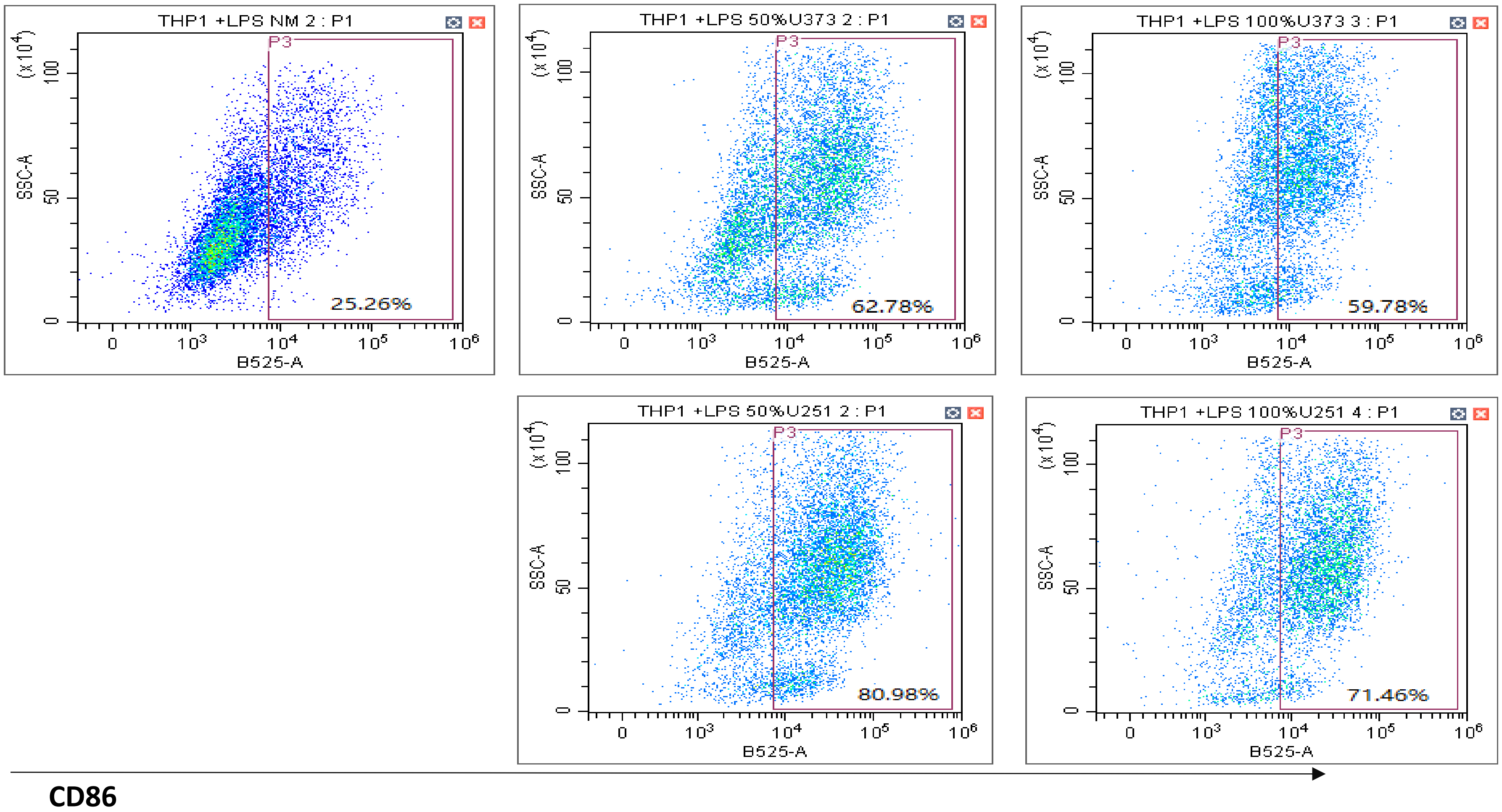


Fig 4.2.3.b.1 Representative dot plots for the expression of CD86 on PMA and LPS stimulated monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the PMA and LPS stimulated THP1 cells cultured in normal media, U373 and U251 conditioned media and stained with FITC conjugated anti CD86. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

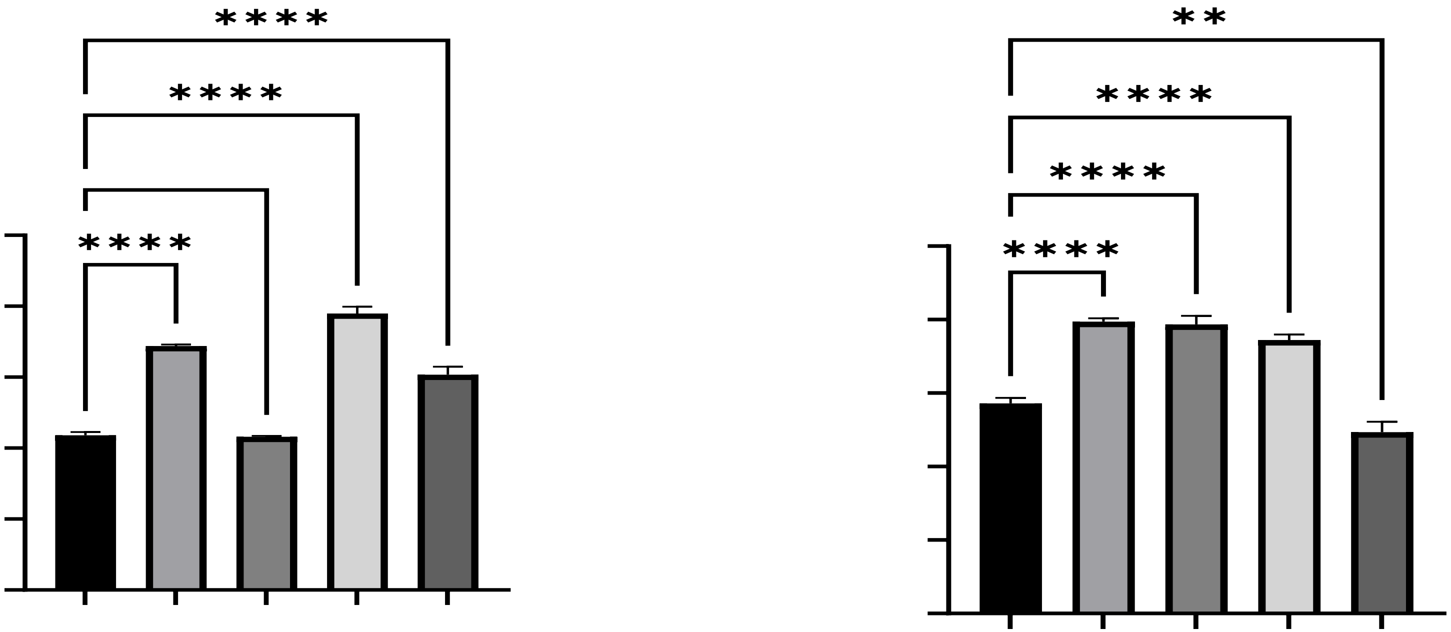


Fig 4.2.3.b.2 Bar graphs represent the MFI of CD86 on THP1 & MM6 cells with mean and SD with significance ***** - p value < 0.0001, and ***- p value < 0.0005*. n=3. The normal media is used as the control to analyse the change in the MFI of CD86.

The THP1 cells were stimulated with PMA and LPS grown in GB cell cultured conditioned media (**Fig 4.2.3.b.1**) was stained with anti-CD86 antibody and analysed its expression of the costimulatory molecule. Overall, the CD86 expression was increasing (MFI range between 21000 to 39000) in various percentages of both U373 and U251 conditioned media as compared to the control (MFI-21813 ± 440) (**Fig 4.2.3.b.2 first graph)**. The THP1 cells were stained with FITC conjugated CD86 and there was an increase in the CD86 expression in presence of 50 % of U373 (MFI for 50%-34402 ± 204) and both the 50% (MFI-38989 ±930) and 100% (MFI-30374 ± 1106) of U251 conditioned media. In MM6 cells, the CD86 expression was increased except in the 100% of U251 conditioned media (**Fig 4.2.3.b.2 first graph),** which showed a slight reduction than the control. In general, the CD86 expression had an upregulation on both THP1 and MM6 cells (**Fig 4.2.3.b.2)** in GB conditioned media. Furthermore, the HLA DR expression on M1 polarized macrophages treated with GB conditioned media was also observed.

The M1 phenotype macrophages express HLA DR for the antigen presentation making T cell and B cell activation and differentiation. In tumour microenvironment, the TAMs are mostly found with M2 phenotype which are having smaller expression of HLA DR and drives for the tumour progression (Li et al., 2020). The study from Fan et al., (2017), suggests that high expression of HLA DR in GB is associated with a lower survival rate in GB patients, suggesting that, this HLA DR plays a major role in immune modulation in GB (Fan et al., 2017). The HLA DR expression on macrophages and microglia in brain has supressed in the tumour microenvironment of GB (Lin et al., 2022). To determine how the GB cell cultured conditioned media affects HLA DR expression on M1 macrophages, THP1 and MM6 cells stimulated with PMA and LPS and stained with PE fluorescent conjugated anti-HLA DR antibody were analysed by flow cytometry. The dot plots on THP1 cells showed a decrease in cell population percentage (97% to 86%), when the cells were in GB conditioned media (**Fig 4.2.3.c.1**). In THP1 cells, there was a higher expression of HLA DR in normal media cells (MFI-31689 ± 969), but the conditioned media from U373 (MFI for 50%-19121 ± 735 and 100%-12552 ± 185) and U251 (MFI for 50%-14264 ± 515 and 100%-15445 ±390) has reduced HLA DR expression (**Fig 4.2.3.c.2 first graph**). Similarly, on MM6 cells showed the HLA DR expression was downregulated (MFI for normal media- 39076 ± 317) in both conditioned media from U373 (MFI for 50%-25461

± 1286 and 100%-18433 ± 238) and U251 (MFI for 50%-17668 ± 500 and 100%-15825 ± 1583) (**Fig 4.2.3.c.2 second graph**). The HLA-DR expression on both the cells THP1 and MM6 (**Fig 4.2.3.c.2**) were reduced in the GB conditioned media even in 50% and 100% titrations which was reducing from MFI 31000 to 12500 for THP1 cells and in MM6 reduced from 39000 to 15800 in MM6 respectively.

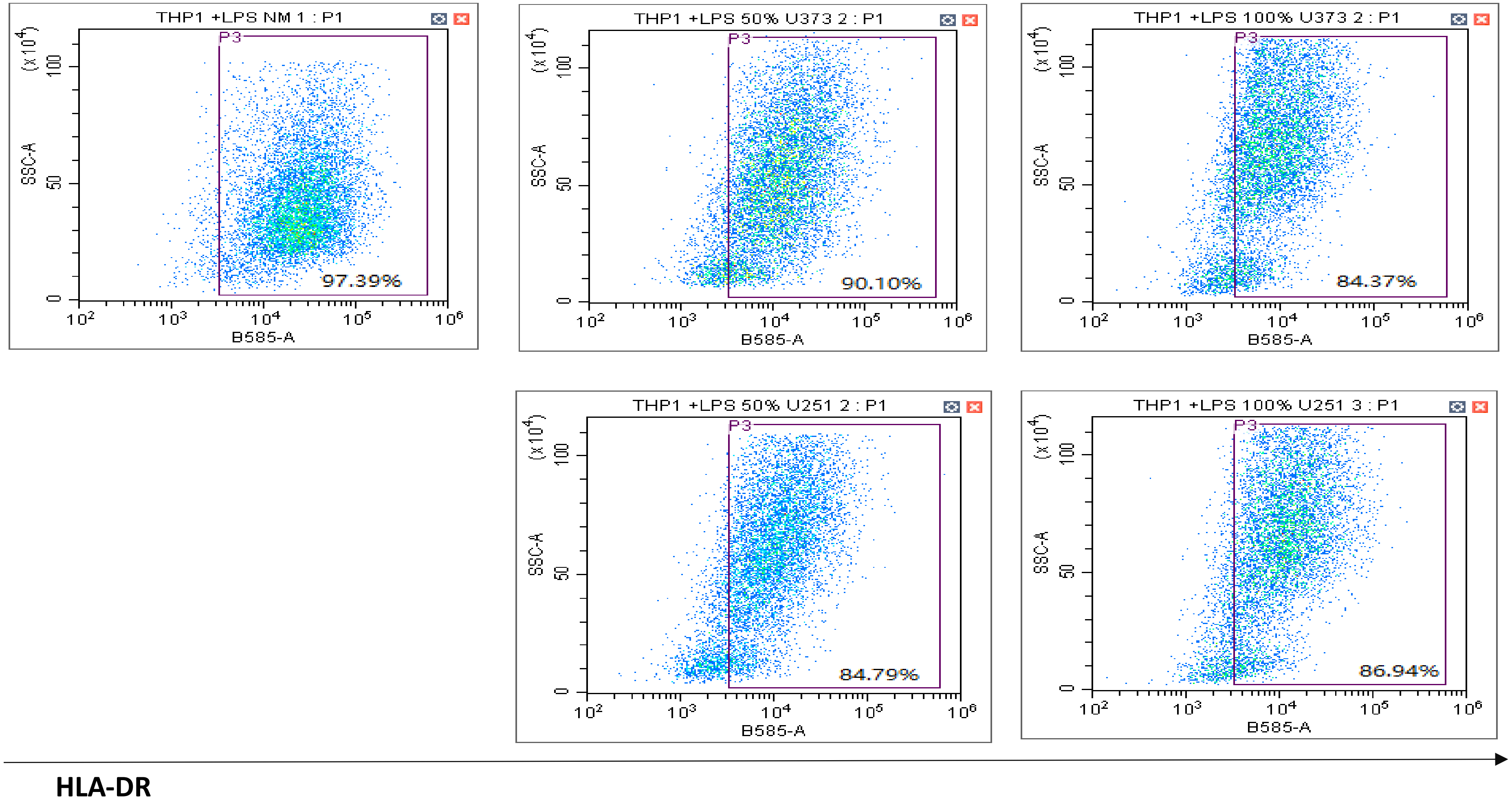


Fig 4.2.3.c.1 Representative dot plots for the expression of HLA DR of PMA and LPS stimulated monocyte cells THP1 & MM6 in GB conditioned media: The dot plots show the THP1 cells treated with PMA and LPS cultured in normal media, U373 and U251 conditioned media and stained with PE conjugated anti HLA DR. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

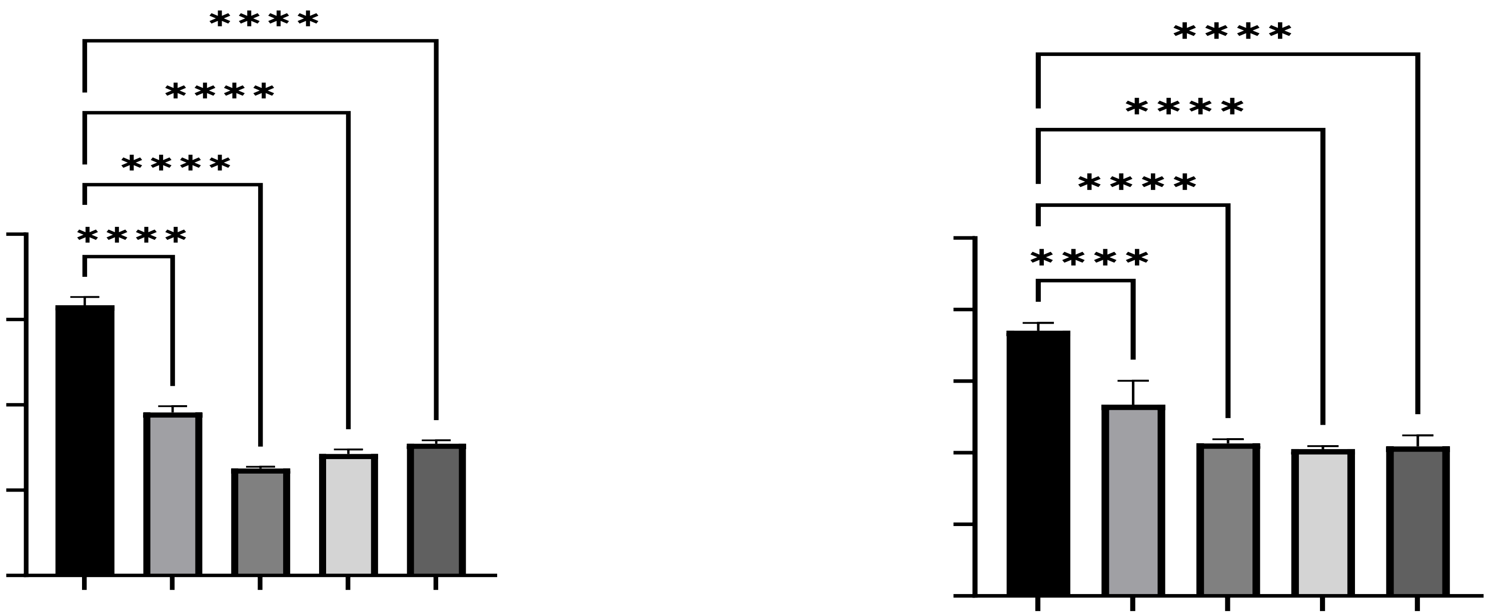


Fig 4.2.3.c.2 Bar graphs represent the MFI of HLA DR on THP1 & MM6 cells with mean and SD with significance ***** - p value < 0.0001, and ***- p value < 0.0005.* n=3. The normal media is used as the control to analyse the change in the MFI of HLA-DR.

The flow cytometry data suggests that the HLA DR expression had affected by the GB conditioned media in monocytic stage, PMA stimulated stage M0 phase and both PMA and LPS stimulated M1 phenotype. The HLA DR expression points out that the antigen presentation for the T cell activity might get inhibited from the monocytes and macrophages. The CD86 expression were increasing in PMA stimulated condition and in PMA and LPS stimulated stage in the presence of conditioned medium. This suggests that the proteins secreted from the GB cells present in the conditioned media might influenced the expression of CD86 and HLA-DR and the immune suppression might be through the macrophages and its antigen presentation for T cell activation.

Research from the immunology group at University of Wolverhampton focuses on the immune suppression by glioblastoma secreted proteins and particularly how it affects the macrophage activity and leads to T cell inhibition. Previous studies from this group on GB tumour samples using mass spectroscopy have shown expression of Galectin 1 protein in 58 out of the 59 samples analysed. Unpublished data from the same group also found accumulation of Galectin 1 around T cells in GB specimen using confocal microscopy accumulated around the brain blood vessel and suggesting a possible role in blocking the T cell movement from the blood vessel to the tumour and hindering the movement towards the tumour core by activating PD L1 and Galectin-9.

4.2 Galectin 1 presence in the GB conditioned media

Galectins are the carbohydrate binding proteins, which are conserved through eukaryotic ancestors. The galectin protein family are involved in the regulation of immune system, supressing the anti-tumour immunity favouring the tumour growth (Compagno et al., 2020). Galectin 1 is the protein discovered first among this lectin family which is encoded by the gene *LSGALS1*. This Galectin 1 is involved in immune regulation by maintaining the homeostasis in normal body whereas it is dysregulated in pathological conditions (Camby et al., 2006). Galectin 1 overexpression leads to immune suppression, tumour growth, invasion, and metastasis, and has been found in different types of cancer (Gheysen et al., 2021). The study from Van Woensel et al., (2017), suggests that the silencing of Galectin 1 in GB cells was shown to be associated with the switching of M2 to M1 macrophage phenotype in the GB microenvironment leading to the activation of CD4+ and CD8+ T cells, which could be the reversal of the antitumour activity of the immune system (Van Woensel et al.,2017).

The IFN-ϒ is one of the interferons produced by natural killer cells and T cells, for the clearance of pathogens and eliminating tumour from the body by apoptosis. This IFN-ϒ also causes the immune suppression in tumour by enabling the immune checkpoint inhibitor molecules (Jorgovanovic et al., 2020). Using an ELISA assay, the concentration of Galectin 1 in GB cultured conditioned media was analysed. Changes in Galectin 1 secretion was also investigated upon treatment of the GB cells and its effects with IFN-ϒ.

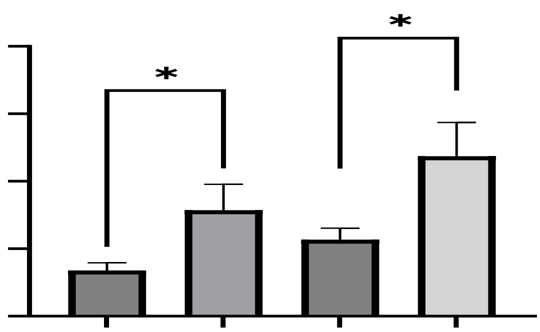


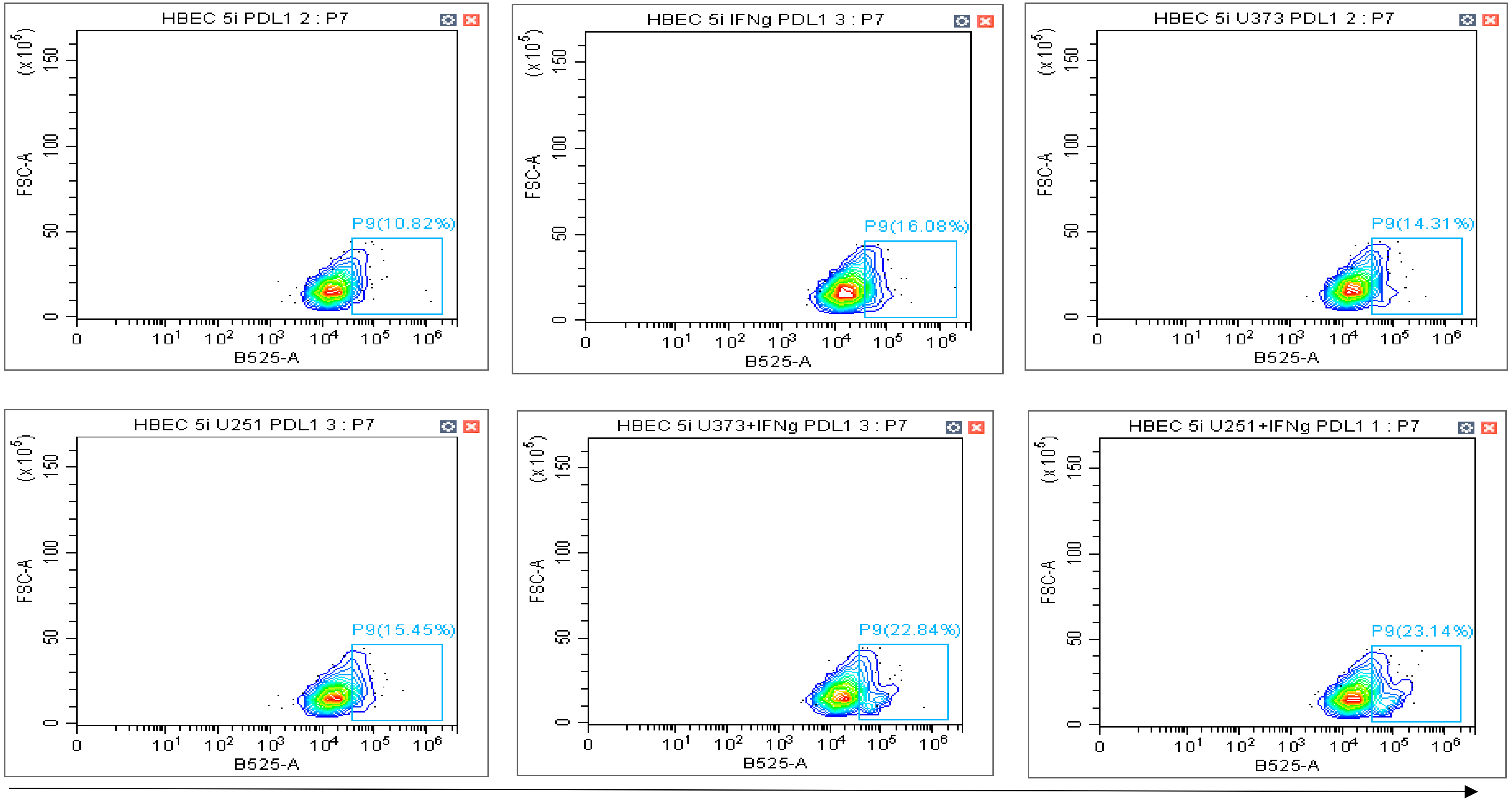
Fig 4.3, Inhibition of Galectin 1 by IFN-ϒ treated GB conditioned media: Galectin 1 secretion was reduced by treating IFN-ϒ with the GB cells. Quantification of Galectin 1 in U373 and U251 GB conditioned media with or without IFN-ϒ treatment by ELISA. The bar graph shows the Galectin 1 concentration with mean and SD with significance **- p value < 0.05,* n=3.

The experiment was performed using U373 and U251 GB cells following treatment with IFN-ϒ and the conditioned media were chosen. The Galectin 1 concentration in the U373 showed higher concentration (3.14ng/mL) than the U373 with IFN-ϒ treatment (1.4ng/mL). Similarly, the U251 had shown higher Galectin 1 concentration (4.7ng/mL) than the IFN-ϒ treated U251 conditioned media (2.3ng/mL) (**Fig 4.3**). This data demonstrates that IFN-ϒ addition decrease Galectin 1 secretion in the GB cells.

The previous studies from the unpublished data of the Immunology group had shown that the Galectin 1 was found near the T cells in brain blood vessels in the tumour. The PD L-1 and Galectin 9, the immune checkpoint ligands were active on the perivascular tissues and might get activated upon the binding of Galectin 1. This causes infiltration of the T cells into tumour core that may get inhibited at the blood vessel. To validate whether the conditioned media from GB and IFN-ϒ can regulate the expression of PDL-1 and Galectin-9 on brain blood vessels, flow cytometry was used in human brain endothelial cells.

4.3 PD-L 1 and Galectin-9 on brain endothelial cells

Programmed Death protein 1 or PD-1 and its ligand PDL-1 is one of major immune checkpoint molecules that facilitates the inhibition of the immune responses by controlling the T cell activity. The PDL-1 also known as CD279, plays a major role in the growth of different types of malignancies (Han Y., et al.,2020). In GB, the PDL-1 was found to be upregulated, especially in patients with low survival rates (Hao et al., 2020). The studies on PDL-1 expression on endothelial cells show that its expression varies in different inflammatory diseases (Veluswamy et al., 2020). The PDL-1 expression on brain blood vessels was analysed to find out whether it is influenced by the presence of IFN-ϒ, U373 and U251 conditioned media alone and the IFN-ϒ treated conditioned media from U373 and U251 cells. The brain endothelial cells HBEC 5i were used to analyse brain blood vessels and checked the expression of PDL-1 in the conditioned media and IFN-ϒ treated GB conditioned media. Results from flow cytometry analysis of HBEC 5i cells (**Fig 4.4.a.1**) demonstrated that the cell population find a better word largely towards the right in IFN-ϒ treated GB conditioned media. The PD-L 1 expression was observed on the cells grown in normal media and the cells treated with IFN-ϒ, the immune regulating interferon, showed almost similar expression of PDL-1 (MFI of PDL-1-55110 ± 1770 and IFN-ϒ-55956 ± 1375)). The endothelial cells grown in 50% of U373 (MFI-56436 ± 1720) and U251 (MFI-56560 ± 1059) conditioned media did not show much change in the PDL1 expression compared to the control. However, the U373 cells grown in IFN-ϒ, and those in conditioned media increased the PDL1 expression (MFI-65577 ± 1604). Along with U373, U251 with IFN-ϒ treatment showed higher expression (MFI-72969 ± 3181) of PDL-1(**Fig 4.4.a.2**). The expression of PDL-1 on IFN-ϒ treated GB conditioned media used shows the influence of IFN-ϒ on immune regulation in HBEC 5i cells.



PDL-1

Fig 4.4.a.1 Representative dot plots for the expression of PDL-1 on brain endothelial cells HBEC 5i in GB conditioned media: The contour plots show the HBEC 5i cells cultured in normal media, IFN-ϒ treated normal media, U373 and U251 conditioned media alone and IFN-ϒ treated conditioned media and stained with FITC conjugated anti-PDL-1 using flow cytometry. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

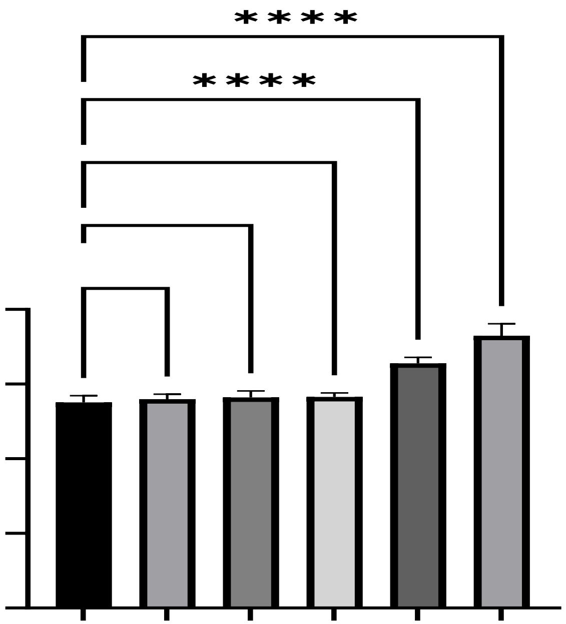


Fig 4.4.a.2 Bar graphs represent the MFI of PDL-1 with mean and SD with significance ***** - p value < 0.0001, and ***- p value < 0.0005, ** - p value <0.005, and ns nonsignificant*, n=3

The results from the flow cytometry data show that the IFN-ϒ plays a crucial role in upregulating the PDL-1 expression. The conditioned media from IFN-ϒ treated GB cells showed PDL-1 expression compared to the conditioned media without IFN-ϒ treatment. IFN-ϒ from T cells causes expression of PDL-1 that could lead to the immune checkpoint activation and inhibition of the immune response (Qian et al., 2018).

The Galectin 9 is one of the transmembrane transporters that plays a major role in regulating the innate and adaptive immune system and enhances the regulatory T cell activation leading to immune suppression. Galectin 9 is a ligand for the receptor T cell immunoglobulin and mucin domain containing 3 (TIM-3) and its interaction causes immune suppression (Lv et al., 2022). According to Yasinska et al. (2019), Galectin 9 silences the anti-tumour immunity by damaging T cells thereby supporting the tumour growth and the TIM3 reduces the activation of T cells by dysregulating through the Interleukin 2 production (Yasinska et al., 2019). In previous studies from unpublished data of Immunology group, activation of Galectin 9 was shown to block the migration of T cells to the tumour core at the blood vessels. In this study, the expression of Galectin 9 investigated on the brain endothelial cells, IFN-ϒ treated ones, U373 and U251 conditioned media grown cells and IFN-ϒ treated U373 and U251 conditioned media cultured cells using flow cytometry (**Fig 4.4.b.1**).

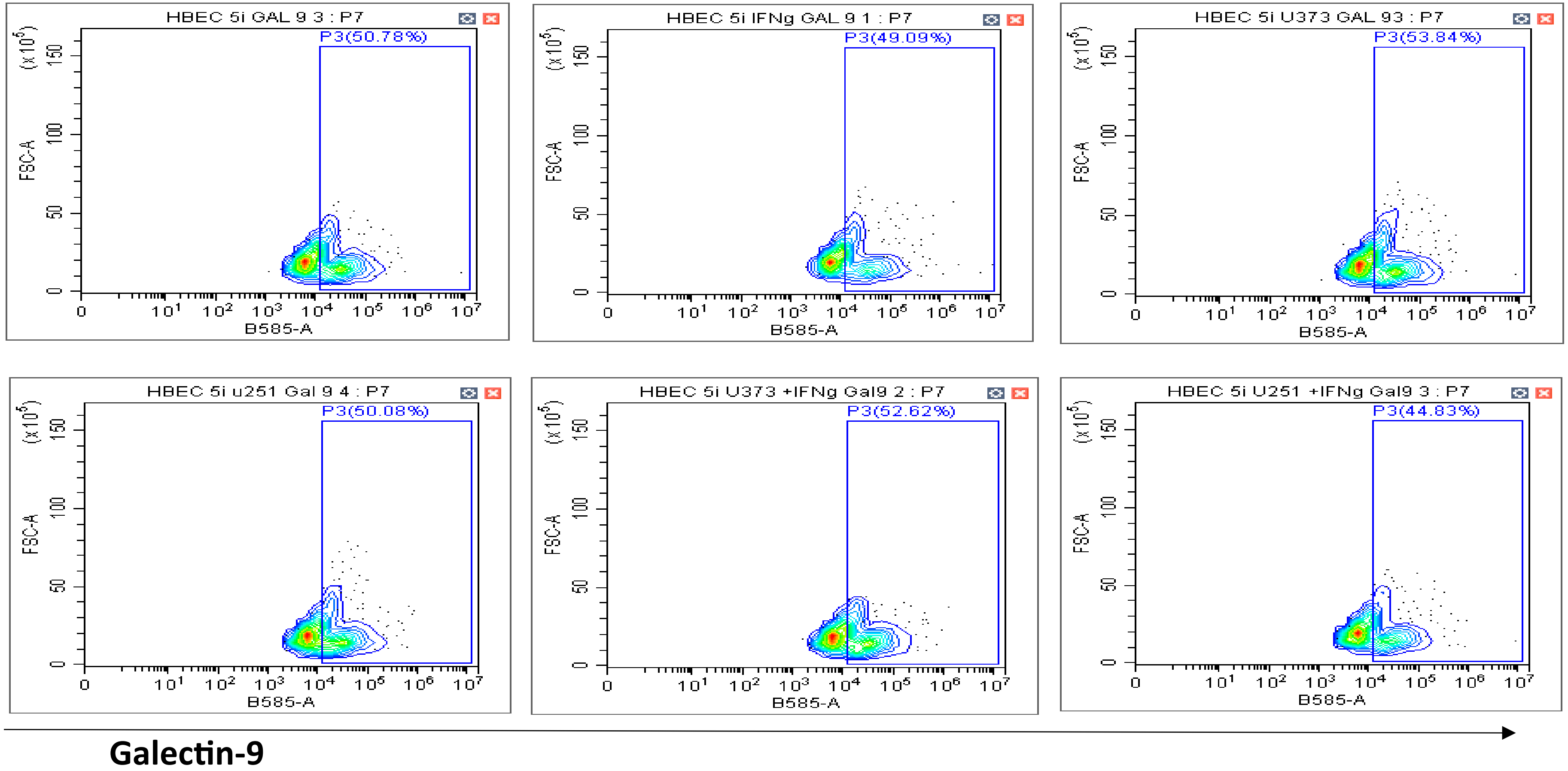


Fig 4.4.b.1, Representative dot plots for the expression of Galectin 9 on brain endothelial cells HBEC 5i in GB conditioned media: The contour plots show the HBEC 5i cells cultured in normal media, IFN-Ƴ treated normal media, U373 and U251 conditioned media alone and IFN-Ƴ treated U373 and U251 conditioned media and stained with PE conjugated anti-Galectin 9. The normal media is used as the control to observe the changes in cell population in the presence of GB conditioned media.

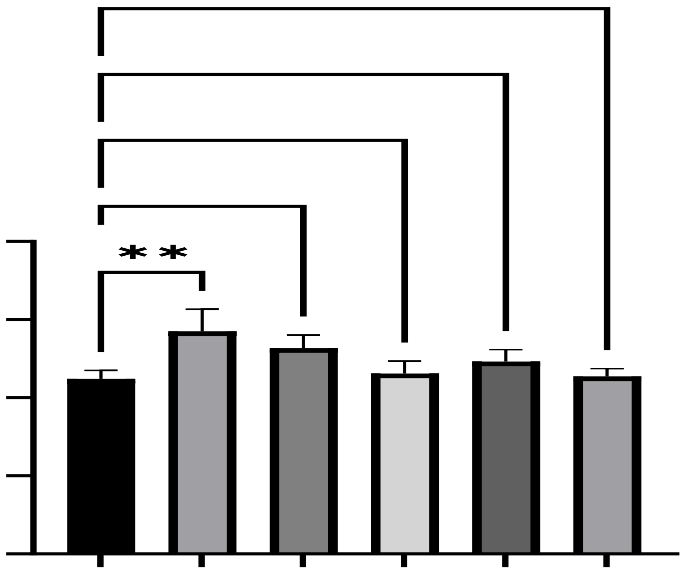


Fig 4.4.b, Bar graphs represent the MFI of Galectin 9 with mean and SD of three different experiments with significance ***** - p value < 0.0001, ** - p value <0.005, *-p value < 0.05 and ns nonsignificant.* n=3.

The PE conjugated Galectin 9 was expressed on HBEC 5i cells (MFI-44846 ± 2136) and there was rise in the expression of Galectin 9 in IFN-ϒ treated endothelial cells (MFI-56981 ± 5672). The Galectin 9 expression was also enhanced in U373 conditioned media grown HBEC 5i cells (MFI-52726 ± 3325). The Galectin 9 expression decreased (MFI-46146 ± 3211) in U251 conditioned media. There was no significant variation in the Galectin 9 expression on IFN-ϒ treated U373 (49230 ± 3065) and U251 (45414 ± 2037) conditioned media when compared to the positive control (**Fig 4.4.b.2**). The flow cytometry data on Galectin-9 expression on HBECs suggests that it would be upregulated in the presence of IFN-ϒ, and in the presence of conditioned media from U373.

The findings from the ELISA and flow cytometry data suggests that the IFN-ϒ plays a crucial role in the immune regulation. The Galectin-1 suppression by IFN-ϒ suggests that the IFN-ϒ from T cells could regulate the immune suppression which in turn can reverse the immune inhibition using some anti-Galectin-1 inhibitors. The upregulation of PDL-1 and Galectin-9 by IFN-ϒ leads to a finding that the T cell in the tumour microenvironment itself regulates and suppresses the immune response and causes immune inhibition. The expression of PDL-1 and Galectin-9 was further investigated on HBECs and the immune cells such as macrophages and T cells in the tumour, using the qualitative techniques.

5.Discussion

5.1 Phagocytosis and antigen presentation from macrophages

Glioblastoma is the primary tumour among the CNS tumours with an incidence rate of 3.2 per 100,000 people and mostly found in older patients with an average survival rate of 15 months (von Zuben de Valega Negrao et al., 2023, Kotecha et al., 2023). The treatment for GB includes surgical resection, chemotherapy, radiotherapy and mix of chemotherapy and radiotherapy and immunotherapy (von Zuben de Valega Negrao et al., 2023, Kotecha et al., 2023). Despite these treatment options, GB tumours still remain an incurable malignancy with a high recurrence rate (von Zuben de Valega Negrao et al., 2023, Kotecha et al., 2023).

The GB microenvironment is made up of cancerous and non-cancerous cells including normal brain cells, immune cells, GB stem cells, and blood vessel cells as well as various proteins and other biomolecules secreted by the tumour and other cells in microenvironment, all of which support the tumour growth and maintenance causing invasion and metastasis (Schiffer et al., 2018). The non cellular secretions from the tumours could contribute to immune modulations, EM transitions for invasion, migration and cell death inhibition, and angiogenesis through intercellular networking leading to tumour proliferation (da Cunha et al., 2019). The tumour microenvironment has been studied *in vitro* especially the secretions from GB tumours using the conditioned media or the culture supernatants that enabled researchers to investigate tumour's proliferation, metastasis, and immune suppressive abilities (Yavuz et al., 2021, Yavuz et al., 2022). In the present study, the conditioned media from two established GB cell lines U373 and U251 were analysed for total protein concentration of GB conditioned media following from 24, 48,72 and 96 hours (Day1 to 4) of the incubation. The conditioned media after 72 hours (day 3) showed highest protein concentration compared to days 1,2 and 4.

The BBB recruit pro-inflammatory secretions and immune cells which would be shifted to anti-inflammatory upon the stimulation and signalling from the GB makes the tumour more proliferative and aggressive (Segura-Collar et al., 2023). In GB, the microenvironment creates very complex immune suppressive through secretion of tumour supportive cytokines especially IL-10 and TGF-β which would be favouring tumour associated macrophages which causes T cell inactivation. Furthermore, the GB microenvironment stimulates myeloid derived suppressor cells and regulatory T cells and the PDL-1 which would lead to diminish the anti-tumour immune response (Jain et al., 2023).

The macrophages/microglia in GB expresses M1 or M2 phenotype, where M1 in the patients are considered as a sign of better survival rate, whereas the M2 favours for the poor survival (Gjorgjevski et al., 2019). The macrophages/microglia accounts for the most populated immune cells in the GB microenvironment, and these macrophages would be in tumour supportive phenotype with the expression of CD163 and CD204 markers in the tumour core and periphery (Ma et al., 2021). Previous studies have shown that, the conditioned media from GB cell lines influences the polarization of macrophages, mostly supports for M2 phenotype, helps in tumour invasion, immune suppression, and drug resistance (Zeren et al., 2023). To validate the macrophage studies, the conditioned medium from GB cell lines U373 and U251 was analysed and the medium displayed highest protein concentration (72 hours) was further analysed for the phagocytic activity of LPS in treated and non-treated macrophages. The antigen presentation marker which one expression was also investigated in the conditioned media.

The phagocytosis is the anti-tumour mechanisms from macrophages where it would be modified by the tumour microenvironment resulting in tumour growth favouring and reduction in the phagocytosis through expressing various regulatory molecules by the tumour and macrophage (Li et al., 2023). In the present study on phagocytic activity of macrophages, the macrophages in M0 and M1 phenotype were investigated, the M1 macrophages were obtained by stimulating with LPS (Nielsen & Schmid, 2017). The results from this study showed a reduction in phagocytosis was observed in M0 and M1 macrophages, or with and without LPS treatment, and the GB conditioned media. The phagocytic activity in MM6 and THP1 cells in M0 stage was also found to be reduced by the GB conditioned media. Indeed, there was a decrease in the percentage of phagocytosis from 9.8% to 1.45% (**fig 4.1.c.2** and **fig 4.1.d.2**). These findings suggest that the phagocytic activity of macrophages might be affected by the secretions from the GB tumour.

The macrophages and T cells have connected each other to elicit an immune response in physiological condition, where in GB, the TAMs interact with naïve T cells and causes activation of immune checkpoint molecules, secretion of immune suppressive cytokines and the differentiation to regulatory T cells which favours tumour growth (Tu et al., 2021). The monocytes are the innate immune cells in blood which would be recruited to the inflammation site and would be differentiated into macrophages or dendritic cells upon stimulation have the potential for the T cell activation through antigen presentation especially in tumours (Affandi et al., 2021). The monocytes express co-stimulatory molecules CD80 and CD86 and the antigen presentation marker HLA-DR and this would help in the processing of antigens for T cell activation and eliciting the immune response (Lee et al., 2017, Pinto et al., 2018). The monocytes cultured in GB conditioned media showed variations in the expression levels of CD80 and CD86 (**fig 4.2.1 a.2** and **fig4.2.1. b.2**). In THP1 cells, the CD80 and CD86 expression increased, but the MM6 cells showed decreased expression in the presence of GB conditioned media. However, the HLA-DR expression was reduced (**Fig 4.2.1.c.2**) in both cell lines suggesting that, the antigen presentation might get affected by the tumour microenvironment secretions on monocytes.

In the microenvironment of GB, the TAMs consist of brain resident microglia and the monocyte differentiated macrophages, which would be in the phenotypes M0, M1 or M2, and the expresses the MHC class II and CD80 and CD86 for the antigen presentation (Pires-Afonso et al., 2020). The co-stimulatory molecules CD80, CD86 and the MHC class II on the glioblastoma infiltrating macrophages are down regulated, which would be discouraged to present the antigen and inhibition of T cell activation (Hussain et al., 2006). In this study, the expression of CD80, CD86 and HLA-DR on the M0 and M1 macrophages in the presence of GB conditioned media was investigated. The CD80 expression of THP1 and MM6 cells which in M0 stage was increased in presence of 50% and 100% of conditioned media from both the U373 and U251 (**Fig 4.2.2.a.2**). Similarly, the CD86 expression was increased in THP1 cells but the MM6 cells only showed expression in 100% of both U373 and U251 conditioned media (**Fig 4.2.2.b.2**). In the M1 stage, where LPS were added, the CD80 expression (**Fig 4.2.3.a.2**) was fluctuating in the presence of conditioned media. However, the CD86 expression increased **(Fig 4.2.3.b.2**) in both THP1 and MM6 cells. The HLA-DR expression was decreased in both M0 and M1 stages in presence of conditioned media (**Fig 4.2.2.c.2** and **Fig 4.2.3.c.2**). The findings from the phagocytosis assay and the flow cytometry suggests that the conditioned media from GB might have some inhibitory effect on phagocytosis activity and the antigen presentation of macrophages. The findings from this study could speculate that the reduction in the percentage of phagocytosis and the downregulation in HLA-DR expression, might be responsible for inactivation of immune response in the GB cells. This inhibitory effect will be further studied using patient samples and in the presence of the target protein Galectin-1 and using co-culturing the macrophages and T cells.

5.2 Galectin-1 and Immune checkpoint molecules in GB conditioned media

Galectins are the soluble, glycan binding proteins having carbohydrate recognition domains, engaged in both intracellular and extracellular functions on cells, and would be activated in physiological and pathological conditions. (Liu et al., 2023). Galectin -1 has been found in different organs including brain, spleen, lymph nodes, lung, and heart and present in various cells such as epithelial cells, stromal cells, endothelial cells, and various immune cells (Elola et al., 2005, Camby et al., 2006, Hermenean et al., 2022). Galectin-1 are involved in a range of biological functions such as cell proliferation, apoptosis, immune modulations, wound healing, cell cycle arrest and engaged in diverse pathways for the regulation and maintenance of the tissue homeostasis (Elola et al., 2005, Camby et al., 2006, Hermenean et al., 2022). Galectin-1 has been studied in dual roles through its engagement in promoting various infections and diseases and favours in subsiding the pathogenicity (Mendez-Huergo et al., 2019, Li et al., 2020, Yu et al., 2023). It has been reported in different autoimmune disorders with a promoting and protective as well as its involvement in allergic reactions and in neurodegenerative disorders (Mendez-Huergo et al., 2019, Li et al., 2020, Yu et al., 2023). Galectin-1 is one among the prototype galectin family that is highly present in various cancers, and which would help in modulating immune system, and nearby tissues in the microenvironment causing tumour progression (Mariño et al., 2023). The study from Chen et al., (2019) has shown that the knockdown of *LGAL1* gene for Galectin-1 in GB has been down regulated the immune suppressive M2 macrophages, cytokines favouring tumour growth and the myeloid derived suppressor cells which would be reprogramming the tumour microenvironment (Chen et al., 2019).

According to Nambiar et al., (2019), the Galectin-1 from tumours have the immune suppressing ability through binding to the endothelial cells and affects T cell migration and activation through PDL-1 and Galectin-9 (Nambiar et al., 2019). The Galectin-1 knockout studies have shown that the IFN-ϒ from T cell increased in tumours suggesting the effect of Galectin-1 on T cells. However, the IFN-ϒ enhances the immune regulation through PDL-1 and Galectin-9 (Nambiar et al., 2019). Previous studies from Immunology group (University of Wolverhampton) suggested that Galectin-1 from the tumour accumulated around the T cells in the brain blood vessels, suggesting there might be a T cell inhibition by Galectin-1. The results from this study on Galectin-1 concentration using IFN-ϒ treated GB cell lines showed that the Galectin-1 concentration was reduced in both GB cell lines compared to the untreated cells (shown in **Figure 4.3**). The Galectin-1 knockout studies from Nambiar et al., and ELISA assay, the Galectin-1 and IFN-ϒ are co-related, where the IFN-ϒ from T cell activation could regulate the Galectin-1 secretion from GB, which might be potential therapeutic target for treatment. Future studies will therefore include knockout experiments of Galectin-1 gene in GB cells and culturing immune cells in Galectin-1 knockout GB conditioned media.

As the Galectin-1 binds to the blood vessels in brain, the research from Immunology group showed that the activation of immune checkpoint molecules, PDL-1 and Galectin-9 on the blood vessels, which was upregulated to control T cell migration to the tumour site leading to accumulation of T cells around the blood vessels. The PDL-1 expression on endothelial cells in GB has significant role in tumour progression and angiogenesis, and it impacts the pathological grade of GB (Xue et al., 2017). The T cells could be regulated at the BBB, the human brain endothelial cells, through the expression of PDL-1 especially in pathological conditions (Pittet et al., 2011). The study from Rodig et al., (2003), revealed that, the IFN-ϒ treated or activated endothelial cells expressed higher PDL-1, which could inhibit the T cell activation, that is the IFN-ϒ secreted by T cells regulate the immune responses through PDL-1 activation (Rodig et al., 2003). This study investigated GB secretion to the microenvironment, its involvement in the activation of PDL-1 on the blood vessels in the brain and the expression levels of PDL-1, especially after the T cell activation. The results revealed that an upregulation of PDL-1 on HBECs (MFI of PDL-1-55110 ± 1770) upon growing in IFN-ϒ treated GB conditioned media (MFI for U373-65577 ± 1604 and MFI for U251 -72969 ± 3181) (**Fig 4.4.a.2**). The PDL-1 was upregulated by IFN-ϒ treated GB conditioned media suggests that the immune regulation might be from the GB secretion and got upregulated from T cell activation, and thus anti-tumour responses get regulated.

Galectin-9 and Tim-3 interactions regulate immune responses by T cells and in context of GB, the molecules are upregulated and causing immune suppression making them highly malignant (Liu et al., 2016). The Galectin-9 are expressed on endothelial cells and are upregulated by IFN-ϒ, where it supports inflammation and immune regulation (Alam et al., 2011). In this study, the expression of

Galectin-9 on brain blood cells in the presence of GB conditioned media with or without IFN-ϒ was affected (**Fig 4.4.b.2**). The Galectin-9 was expressed on IFN-ϒ treated HBECs cells and on the U373 conditioned media treated cells. The IFN-ϒ treated GB cell line conditioned media did not show much expression of Galectin-9. The Galectin 9 upregulation on conditioned media from U373 cells suggests that the secretion from tumour might influences Galectin-9 expression but could not find change on U251 treated HBECs. The PDL-1 expression on HBECs in the presence of IFN-ϒ treated GB conditioned media was upregulated. The Galectin-9 was upregulated on HBECs in presence of IFN-ϒ and U373 conditioned media. The secretions from GB with IFN-ϒ had some influence on PDL-1 and Galetin-9 upregulation, which might lead to the inhibition of T cell movement and accumulation at the blood vessels.

IFN-ϒ, the cytokine having a major role in innate and adaptive immune responses against microbial infections and clearing tumours from the body and having proinflammatory activity. The IFN-ϒ are produced profoundly by the NK cells and both the helper and cytotoxic T cells (Alspach et al., 2018). IFN-ϒ regulates the lymphocyte mediated inflammation through PDL-1 expression which would be resolving the immune action or inflammation to reduce the damage to the tissues from the lymphocytes (de Kleijn et al., 2013). In GB, the IFN-ϒ from the activated T lymphocytes upregulated PDL-1 would leads to the inhibition of immune responses and helps the tumour to proliferate (Ricklefs et al., 2018). In addition to PDL-1, Galectin-9 is an immune checkpoint protein which induces T cell death through immune modulatory receptor TIM-3 and IFN-ϒ from T cells enhances the Galectin-9 expression (Merani et al., 2015) (Park et al., 2011). Galectin-9 regulates the immune response through promoting T cell apoptosis and activates regulatory T cells for the resolution of the immune response and any dysregulation causes autoimmune diseases and cancer (Mansour et al., 2022). The studies suggest that the PDL-1 and Galectin-9 are upregulated in the presence of IFN-ϒ, which would be from T cells and in the context of immune response, IFN-ϒ secretes and carries out the apoptosis of target cells and to resolve the response, the IFN-ϒ activates these PDL-1 and Galectin-9 and thereby the immune response gets regulated. As the abovesaid results suggests that IFN-ϒ have a role in the activation of immune checkpoint molecules, but how the IFN-ϒ is capable to activate the immune checkpoint molecules, and time point of its activation is unknown, we will be doing time-based assays using IFN-ϒ, conditioned media from GB and the GB cell lines and HBECs to validate and generate a pathway for the immune suppression in GB.

The current study adds that the secretions from GB were down regulating the phagocytosis and antigen presentation by macrophages. Furthermore, the IFN-ϒ from activated T cells might be downregulating the Galectin-1 secretions from

GB. Additionally, the PDL-1 and Galectin-9 on human brain endothelial cells are upregulated by IFN-ϒ and the GB conditioned media which suggests that the T cells get clustered at the blood vessels in brain, blocking the T cells movement through immune checkpoint molecules following the GB secretions and IFN-ϒ. To further validate the role of Galectin-1 in GB immune suppression, especially on macrophages and T cells, the co-culturing of macrophages and T cells in presence of GB conditioned media and human recombinant Galectin-1 and observe the markers which are activated for immune response and analyse whether the immune response is inhibited or not. Along with this, the migration of T cells through brain endothelial cells in presence of GB conditioned media and Galectin-1 will be investigated in order to offer insights into the role of PDL-1 and Galectin-9. To achieve this, confocal microscopy could be used to investigate the macrophage phenotype and find out whether it is accumulated or not at blood vessels in GB tissues. To make better understanding of the GB secretion, we will use the conditioned media from GB patient samples.

6. Conclusion

The tumour microenvironment of the glioblastoma is the major cause for immune suppression, proliferation, invasion, and metastasis. Macrophages are the most common immune cells present in the GB microenvironment. Macrophages have phagocytosis activity as well as the antigen presentation capability to activate T lymphocytes, but due to the stimulation from GB, macrophage would have polarized to tumour supportive phenotype. The present study suggested that the secretions from GB affect the phagocytosis activity and the antigen presentation by macrophages, leading to inactivation of T cells.

The mass spectrometry studies from Immunology group have identified Galectin-1 as the common protein highly expressed in different GB samples (58 out of 59 samples) and the Galectin-1 was found to bind to the endothelial cells causing the activation of PDL-1 and Galectin-9 to block the T cell migration. In the current study, the Galectin-1 from GB conditioned media was quantified using ELISA assay. The results revealed the expression of Galectin-1 is suppressed by the addition of IFN-ϒ in the established GBM cell lines U373 and U251. Along with this, the effect of GB conditioned media on PDL-1 expression on human brain endothelial cells was upregulated in the presence of IFN-ϒ treated GB cells compared to the untreated ones. The Galectin-9 on human brain endothelial cells showed upregulation with addition of IFN-ϒ and the conditioned media from GB cell line U373.

In this current study, conditioned media from U373 and U251 were used to validate the effect of GB secretions on phagocytosis activity and antigen presentation. Further studies need to be conducted to investigate the effects of Galectin-1 on macrophages and T cells by co-culturing cells to elucidate the effect on macrophage polarisation as well as the T cell activation and to assess the role of Galectin-1 with immune suppression. In addition to that, the migration of T cells through brain endothelial cells will be studied using conditioned media from GB and using human recombinant Galectin-1.

7. References

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8. Appendix

Ethical Committee Approval

Your project has been approved by the Life Sciences Ethics Committee (LSEC), University of Wolverhampton, please see details below:

Student: Joel Varghese

Supervisor: Kesley Attridge

Outcome date: 24/08/2022

Project Title: EXPRESSION AND TARGETING OF MACROPHAGES INHIBITORY PROTEINS IN GLIOBLASTOMA

Outcome: Approved

LSEC reference number: LSEC/202122/KA/145