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I, Leena Ahad, hereby submit this original work as part of the requirements for the degree of Master of Science in Cancer and Cell Biology.

It is entitled:

The Immunotherapeutic Approach to Glioblastoma Multiforme

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Title:

The Immunotherapeutic approach to Glioblastoma Multiforme

Leena Ahad

Submitted in partial fulfillment of the

requirements for the degree of Master of Science in

Cancer and Cell Biology

Committee Chair: Qing Lu

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ABSTRACT

Glioblastoma multiforme poses significant treatment challenges, attributed to its distinctive characteristics and immune evasion mechanisms, and carries a grim prognosis despite advancements in therapy. Therefore, rigorous efforts are necessary to uncover additional therapies or other unconventional strategies. Recently, immunotherapies have gained significant traction due to their success in certain malignancies. Immunotherapies use the host's immune system to enhance the ability to combat cancer. These therapies have been in development for over 100 years, and many of them are currently under investigation for treating Glioblastoma multiforme in preclinical and clinical studies. Immunotherapies include antibody therapies, including immune checkpoint blockade, adoptive T cell therapies, cytokine therapies, cancer vaccines, and oncolytic viruses. A deeper understanding of the origins of immunotherapy, immune system dynamics in response to cancer, immune evasion mechanisms of glioblastoma, and the various immunotherapeutic strategies may facilitate the development of novel and effective treatment strategies for glioblastoma. Here, we review the different immunotherapeutic approaches and propose possible combinatory strategies.

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I would also like to note that all the figures presented in this work are original and were generated using Biorender.com.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Figures:

Tables:

ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
αGITR	Agonistic GITR antibody
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cell cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
AE	Adverse event
APC	Antigen presenting cells
BBB	Blood-brain barrier
BCR	B cell receptor
BiCAR	Bispecific CAR
BiTes	Bi-specific T-cell engaging antibodies
Bregs	Regulatory B cells
CAF	Cancer-associated fibroblast
CAR-T	Chimeric antigen receptor (CAR) T-cell
CCL	Chemokine C-C motif ligand
CDC	Complement dependent cytotoxicity
CNS	Central nervous system
CSF	Colony stimulating factor

CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CT2A	Wild-type for p53 mouse glioma cell line
CXCL	Chemokine (C-X-C) ligand
DAMP	Damage-associated molecular pattern
DC	Dendritic Cells
Dex	Dexamethasone
dMMR	Mismatch repair deficiency
DNA	Deoxyribonucleic acid
ECG	External control group
ECM	Extra-cellular matrix
EDB	Extra-domain B
EGFR	Endothelial growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
eTCR	Engineered T cell receptor
FasL	Fas ligand
FcRs	Fc receptors
GBM	Glioblastoma multiforme
GEMMs	Genetically engineered mouse models
GITR	Glucocorticoid-induced tumor necrosis factor related protein

GL261	Glioma 261-murine glioma cell line
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCMV	Human cytomegalovirus
HER2	Human epidermal growth factor 2
HIF	Hypoxia-inducible factor
HSV	Herpes simplex virus
IC	Immune checkpoint
ICB	Immune checkpoint blockade
IDH	Isocitrate dehydrogenase
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukins
IL-13 Rα2	IL-13 α receptor 2
ILCs	Innate lymphoid cells
ITLs	Infiltrating T Lymphocytes
LAMP1	Lysosome-associated membrane protein 1
LTS	Long term survivor
M-MDSC	Monocytic MDSC
mAbs	Monoclonal antibodies
MAC	Membrane attack complex
MB-lectin pathway	Mannan-binding lectin pathway

MBL	Mannan-binding lectin
MCA	Methylcholanthrene
MDSC	Myeloid derived suppressor cells
MGMT	O6-Methylguanine-DNA Methyltransferase
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MOG	Myelin oligodendrocyte glycoprotein
MRD	Minimal residual disease
mRNA	Messenger Ribonucleic Acid
nGBM	Newly diagnosed GBM patient
NIVO	Nivolumab
NK cells	Natural Killer cells
NO	Nitric oxide
NSCLC	Non-small cell lung carcinoma
OS	Overall survival
OV	Oncolytic Virus
PAMPs	Pathogen-associated molecular patterns
PD-1	Programmed cell death - 1
PD-L1	programmed death-ligand 1
PDO	Patient derived organoids
PDOX	Patient derived organoid xenograft
PDX	Patient derived xenograft

PFS	Progression free survival
pGBM	Progressive GBM
PGE2	Prostaglandin E2
PMN-MDSC	Granulocytic/polymorphonuclear MDSC
PMNs	Poly morphonuclear Cells
PRRs	Pattern Recognition Receptors
PTM	Post-translational modification
rGBM	Recurrent GBM
ROS	Reactive oxygen species
RT	Radiation therapy
S100A8/A9	S100 calcium-binding protein A4
SCID	Severe Combined Immune Deficiency
SOC	Standard of care
STAT	Signal transducer and activator of transcription
synNotch	Synthetic Notch
TA	Tumor Antigens
TAA	Tumor associated antigens
TAMs	Tumor associated macrophage/microglia
Tan-CAR	Tandem CAR
TCR	T cell receptor
TCR-αβ	T cell receptor αβ
TGF-β	Transforming growth factor- β

TH	T-helper cells
TIBs	Tumor infiltrating B lymphocytes
TIGHT	T-cell immunoglobulin and ITIM domain
TILs	Tumor infiltrating lymphocytes
TIM3	T cell immunoglobulin and mucin domain 3
TLR	Toll-like receptor
TLS	Tertiary lymphoid structure
TMB	Tumor mutational burden
TME	Tumor microenvironment
TMZ	Temozolomide
TNF	Tumor Necrosis Factor
Tregs	Regulatory T cells
TTF	Tumor treating fields
VEGF	Vascular endothelial growth factor

INTRODUCTION

CNS and brain tumors are the fifth most common type of cancer, with Glioblastoma multiforme (GBM) ranking as the deadliest. GBMs are the most common and aggressive form of high-grade primary malignant brain tumors, with approximately 12,000 new diagnoses in the United States and an estimated 250,000 globally per year. Only around 7% of patients with GBM survive 5 years.

GBMs are grade IV gliomas originating from neural stem cells, oligodendrocyte or astrocyte precursors, or possibly from de-differentiated mature astrocytes. This disease primarily afflicts the elderly, with a median age of 64, yet it can also manifest in younger individuals. There is a negative correlation between age and survival rates. Certain factors, such as IDH mutations and MGMT-methylation, have also emerged as contributors to better therapeutic outcomes. However, treatment options are very bleak for these diagnoses as these tumors remain largely treatment-resistant with a high rate of recurrence.

The current standard of care (SOC) consists of surgery (if operable) followed by chemotherapy and radiation. Over the last decade, Tumor-treating fields (TTFields) and advancements in chemoradiation techniques, imaging technologies, and surgical mapping have improved outcomes. Despite these advancements, the median survival time remains approximately 15 months. Most therapies fail to significantly increase survival as GBMs are immunologically cold, non-inflamed, T cell exhausted with a blood-brain barrier (BBB), necrotic center, and are antigenically and cellularly heterogeneous. GBMs are also known to be highly migratory and diffuse to other regions of the brain. Recurrence may occur due to the challenges of eradicating diffuse invasive GBM cells within the surrounding regions. These unique features

of GBM present many therapeutic challenges and warrant further intensive investigation into additional treatment strategies for these tumors.

Immunotherapies have emerged as an appealing avenue due to their success in certain malignancies and are currently under investigation in preclinical and clinical trials for GBM. These therapies harness the body's natural defenses to combat diseases. Substances made in the body or a laboratory boost immune response to disease. Immunotherapies can be classified into two major subtypes: the activating therapies, which elicit or amplify the immune response, and the inactivating therapies, which suppress components of the immune response. Based on the type of substance and their mode of action, they can be further classified into antibody therapies, immune checkpoint blockade, T cell therapies, cancer vaccines, cytokine therapy, and oncolytic viruses.

Here, we summarize the roots of immunotherapy development, components of the immune system, and mechanisms of immune suppression and evasion by GBM. We further discuss a variety of immunotherapies and their efficacies in treating GBM, emphasizing therapies that have been or are currently under assessment in clinical trials. Finally, we summarize the challenges in developing new therapies for GBM and potential future directions.

CHAPTER 1

HISTORY OF IMMUNOTHERAPIES

Immunotherapy is a treatment for cancer in which it harnesses the body's immune system to combat diseases. Substances made in the body or in a laboratory are used to boost the immune response to disease. The beginning of the development of immunotherapies can be dated back to the 1800s, a time when not much was known about the immune system or its responses to disease. What started as a handful of case reports led to the development of a new line of treatments and a new field of study.

Coley's Toxins

The inception of immunotherapy development can be traced back to the late 1800s. Dr. William Coley, now known as the "Father of Immunotherapy," was the Chief of the Bone Sarcoma Unit at Memorial Hospital in New York. The loss of a patient prompted him to delve into the search for additional or novel treatment options for his patients. He combed the available literature at his hospital and uncovered multiple reports of partial or complete tumor regression in patients who had survived an erysipelas attack – an infection of the skin caused by the streptococcus bacteria. He concluded that if accidental infections could cure sarcomas, then artificial infections should yield similar results. This led him to conduct the first systematic study of immunotherapy to treat malignant tumors.

In 1891, he began injecting his patients with live and attenuated Streptococcus pyogenes and Serratia marcescens. He demonstrated inducing a true erysipelas reaction (fever) and showed improvement or full recovery of tumors [1]. Coley obtained variable results in which some

tumors would diminish completely, some would regress and maintain size, and others would recur. He also found that the curative effect was much greater in sarcoma patients than in carcinoma patients in the ratio of 3:1. At the time, it was presumed the infection was causing the immune system to target the cancer cells. Additionally, during his investigations, he reported that treating inoperable malignant tumors with repeated inoculations of erysipelas was practical and did not pose a great risk. However, this treatment method resulted in a few fatalities in patients who were too physically frail to withstand the infection while combating cancer [2].

During his research, he also uncovered Fehleisen's and Bruns' work in Germany in the 1880s. Fehleisen's experiments on dogs and, later on, a few humans demonstrated it was possible to induce erysipelas by inoculating subjects with pure cultures of streptococcus erysipelatis [3] . Bruns reported observations of accidental erysipelas or disease caused by inoculation in 14 cases of malignant disease, showing promising results and demonstrating greater effectiveness in sarcoma patients [4]. Both of these reports supported his hypothesis.

These observations lead to the his life-long study of immunotherapy and development of Coley's toxins, which were a form of immunotherapy that utilized bacterial products to stimulate the immune system [5]. However, due to the risks associated with this method and the unknown mechanism of action, and the variability of his results, oncologists in the early 20th century adapted surgery and radiotherapy as the standard treatment.

Ehrlich’s dyes/ Paul Ehrlich

A few years later, in the early 1900s, Paul Ehrlich demonstrated it was possible to visualize the individual components of the cell by staining using various dyes [6-9]. With his work, he also developed the entire field of histopathology. It was believed that there were

different physical or chemical components of the dye that bound to different components of the cell, suggesting that there were specific combinations in which the dye and a part of the cell bound to each other. This initiated the "lock and key" theory, which led to the development of the "side chain theory of immunity." This theory proposed that the integral parts of circulating cells could also link/interact with each other through "receptors" on the cell surface [10]. This further lead scientists to hypothesize that if a cell survived being bound to a toxin via a specific receptor and if other cells had the same receptor, that they would also survive the viral attack and the same side chains would in turn help prevent infection in the future. Sequentially, this lead to the identification of "antibodies" - circulating receptors in the body that recognize specific proteins known as “antigens”, further supporting the “lock and key” (antibody + antigen) theory [11, 12]. Ehrlich had termed these antigens as a "Magic Bullet" – encompassing the idea that if antigens on neoplastic cells could be identified, those specific cells could be targeted [13]. He synthesized a series of compounds with the capability to specifically targeting pathogenic cells without harming the hosts cells. One famous example is Arsphenamine (Salvarsan), the first synthetic drug to target syphilis. His work eventually led to the discovery and development of penicillin.

Immunosurveillance

Ehrlich also proposed that the hosts immune system could inhibit the progression of neoplastic cells into tumors [14]. Although, this hypothesis could not be proven at the time due to limited knowledge and experimental tools, it laid the foundation for the theory of “Immunosurveillance” which was later explored in the 1950s.

In 1905, Clowes and Baeslack demonstrated that mice had the capability to develop resistance to re-inoculation of tumor cells, if they had previously spontaneously regressed the same cancer [15]. This is indicative that the initial priming/exposure of the cancer cells ultimately lead to their regression later. Further suggesting that the mechanism of immune response to cancer is synonymous to vaccines for other diseases. In the 1930's this lead to the concept of cancer vaccines and scientists at the time developed a method of active immunization against tumors which consisted of inoculating animals intradermally with a very small volume of ground tumor cell suspension [16, 17].

Gross, in 1943, reported the first clear demonstration of tumor cells specific capability to stimulate an immune response using intradermal immunization of C3H mice [18]. He induced tumors in C3H mice using methylcholanthrene (MCA) and transplanted the tumor cells into a different cohort of the same mice. The transplanted mice were able to spontaneously regress the tumors and developed immunity consequently supporting the use of cancer vaccines.

In 1953, Foley demonstrated that mice only had the ability to spontaneously regress and develop immunity against tumors if the previous exposure of tumors cells were of induced tumor lineage [19]. However, the mice could not regress or develop immunity against tumors of a spontaneous tumor lineage. This suggested that induced tumors could stimulate an immune response, but spontaneous tumors could remain undetected by the immune system.

These findings lead to Lewis Thomas's proposal, in 1959, of the presence of an "immunological surveillance mechanism" against oncogenic cells [20]. He suggested that the immune system was capable of identifying neoplastic cells due to their expression of specific neoantigens on the cancerous cells and had the ability to eliminate them. Sir Frank Macfarlane Burnet is also credited with contributing to the theory of immune surveillance in cancer. He

proposed the immune system defined the concept of "self" and that the neo-antigens on the tumor cells could induce an immunological response [21, 22]. Together these theories supported Foley's findings.

A Step Backward

To test the theory of immune surveillance, in the 1970's, Stutman injected MCA into athymic nude mice and control mice. At the time it was believed that athymic nude mice, lacking the thymus gland, were completely immunologically incompetent [23, 24]. Stutman's experiments revealed nude mice did not form more MCA-induced or spontaneous tumors than in control mice [25, 26]. The tumors developed at the same rate and frequency in both cohorts. This directly opposed the theory of immune surveillance against cancer and the concept was considered dead by 1978 [27].

Resurgence of Cancer Immunosurveillance

In the 1980s, it was revealed that nude mice are not completely immunodeficient, they are immunocompromised. Despite possessing fewer T cells and B cells than their wild-type counterparts, nude mice have also been shown to have detectable populations of functional T cell receptor αβ-(TCR-αβ)-bearing lymphocytes [28, 29] and natural killer (NK) cells, which are not thymus dependent [30]. These discoveries prompted Stutman's experiments to be repeated on nude BALB/c and control mice [31]. Both cohorts were injected with varied doses of MCA and monitored for tumor development. The nude mice developed more tumors than the controls supporting the role of the immune system in cancer control and the theory of immune surveillance. Similar experiments were conducted on C.B-17 severe combined immune

deficiency (SCID) mice and their controls which also demonstrated increased tumor proliferation and volume in immunodeficient mice compared to controls [32]. Furthermore, the MCA induced tumors grown in both cohorts were transplanted into syngeneic immunocompetent hosts. The non-SCID cohort gave rise to more aggressive tumors, suggesting the immune system of the immunocompetent host eliminated the highly immunogenic cancer cells, leaving the non-immunogenic tumor cells to grow. This proposed a level of immunoselection in the control mice. RAG2 mice were also found to be more susceptible to tumors induced by MCA compared to WT mice [33].

Further studies supporting the role of the immune system, along with the discovery of other immune components such as interferon-γ (IFN-y), protecting the host from chemically induced and transplanted tumors [34, 35] and perforin deficient mice being more susceptible to MCA-induced tumors [27, 36-44], renewed interest in the theory of cancer immunosurveillance. Collectively, these events propelled the research into cancer immunotherapies (Fig 1).

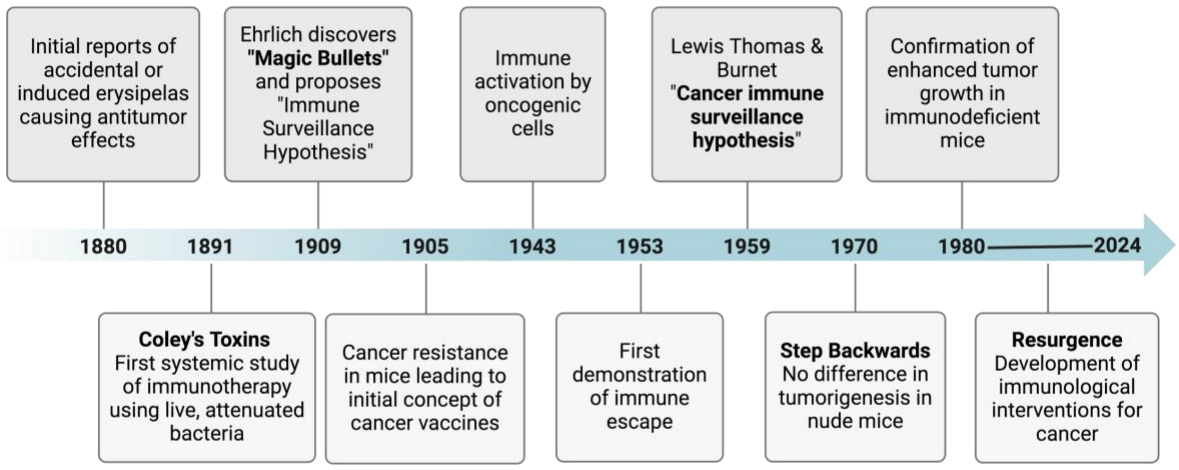

Figure 1: Evolution of Cancer Immunotherapy: A Historical Timeline

This diagram highlights the significant events that lead to the development of immunotherapies for cancer today.

CHAPTER 2

OVERVIEW OF THE IMMUNE SYSTEM IN RESPONSE TO CANCER

Immune surveillance plays a vital role in protecting the body against pathogens and abnormal cells [45]. However, despite its rigorous efforts, cancer cells have developed various mechanisms to evade immune detection and attack, leading to tumor growth and progression. Therefore, the immune system can also promote cancer progression via immunoselection of resistant variants, suppressing anti-tumor immunity and chronic inflammation. Today the dual host-protective and tumor-promoting nature of the immune system is referred to as cancer immunoediting [27, 46]. This widely accepted theory proposes the 3 E's of immunoediting – elimination, equilibrium and escape.

Elimination

The elimination phase embodies the theory of immune surveillance in which the immune system identifies and eliminates transformed cells before they become clinically apparent. The immune system consists of 2 arms - the innate immune response induced by germline-encoded pattern recognition receptors (PRRs) and the adaptive immune response induced by antigen-specific receptors on somatic cells [47-49]. The innate response provides a rapid generic response whereas the adaptive system provides a slow and specific response and are crucial for immune memory. Together they collaborate to try to protect the host from pathogens, neoplasms, viruses, and cancer.

Innate Immune system

The innate immune system is a sophisticated sentinel that serves as the initial defense against non self (i.e. Infections) or damaged self (i.e. tissue injury). The cells of the innate immune system constantly survey the body to detect and eliminate abnormal cells. The components of the innate immune system include soluble recognition molecules such as natural antibodies, pentraxins and the complement system; immune cells that originate from the myeloid lineage, these include dendritic cells (DC), macrophages, mast cells, monocytes, polymorphonuclear cells (PMNs – neutrophils, eosinophils, basophils); and innate lymphoid cells (ILCs), such as natural killer (NK) cells [50, 51]. Together, they play a vital role in the immediate defense against infections or other threats to the body by initiating inflammatory responses and programmed cell death to promote clearance and limit infections. Epithelial cells can also be considered as unofficial members of the immune system. They play a vital role as physical barriers, cytokine/chemokine producers, and can detect and process signals indicating potential danger.

The PRRs present on the innate cells play a crucial role in identifying distinct molecular patterns from microorganisms, termed pathogen-associated molecular patterns (PAMPs) and endogenous compounds released by injured and dying cells referred to as damage-associated molecular patterns (DAMPs) [52]. The structures identified by the PRRs are frequently vital for the survival of microbes [53]. The PRRs are encoded in the germline, therefore they have a limited capacity to recognize molecular patterns. They can be located in the nucleus, the cytosol and on the cell membrane. Activated PRRs on innate immune cells can induce three major responses: phagocytosis, maturation of APCs and certain PRRs, such as Toll-like receptors (TLRs), have the capacity to initiate the assembly of an inflammasome, a multiprotein complex.

This event results in the processing and subsequent release of proinflammatory chemokines and other cytokines, along with cell death manifested as pyroptosis. Cytokines are a family of small glycoproteins that communicate with both the innate and adaptive immune systems. There are many classes of cytokines, namely interleukins (IL) , interferons (INF), chemokines, lymphokines, colony stimulating factors (CSF) and tumor necrosis factors (TNF) [54]. Cytokines may be classified as pro-inflammatory or anti-inflammatory depending on their role [55].

Once abnormal cells are identified, cytokines are released to attract innate immune cells and instruct them to become activated, multiply or eliminate the threat. Neutrophils are a type of PMN that are generally the first to respond and remove the cell via phagocytosis. Phagocytes contain various enzymes in their granules that eliminate and digest the engulfed cells. Macrophages and DC are phagocytes that are also specialized antigen presenting cells (APCs) capable of engulfing pathogens and abnormal tissue, processing to obtain proteins from the foreign entities and expressing them as antigens on their cell surface. The stimulation of TLRs induces maturation of DCs, leading to increased antigen presentation and costimulatory molecules [56]. Macrophages also release chemokines to attract additional immune cells to the site. NK cells are classified as cytotoxic lymphocytes and only targets defective host cells such as tumor cells or virally infected cells. They are capable of releasing perforins and granzymes to eliminate their targets [57]. Perforins are glycoproteins that polymerize and form channels in target cell membranes leading to cytolysis [58]. Granzymes are a family of serine proteases that eliminate their target cells by inducing apoptosis [59]. Perforins and granzymes can synergize to efficiently induce cell death in target cells. This leads to the release of soluble mediators, chemokines, cytokines and growth factors to recruit other immune cells to the site of

inflammation. Furthermore, the innate cells are capable of activating the adaptive immune response via various mechanisms [51].

Myeloid-derived suppressor cells (MDSCs) are the negative regulators of the innate immune system [60]. They can be classified into granulocytic/polymorphonuclear MDSCs (PMN-MDSCs) or monocytic MDSCs (M-MDSCs) based on their cell lineages. Both MDSCs can mediate lymphocyte immune responses from both innate and adaptive immune systems by upregulating expression of signal transducer and activator of transcription 3 (STAT3), S100A8/A9, arginase 1 and inducing ER stress. Furthermore, M-MDSCs use immunosuppressive cytokines such as IL-10 and transforming growth factor- β (TGF-β), nitric oxide (NO), and increased expression of immune regulatory proteins such as programmed death-ligand 1 (PD-L1) whereas, PMN-MDSCs use peroxynitrite, oxygen reactive species (ROS), arginase 1 and prostaglandin E2 (PGE2) to regulate different parts of immune response to protect from chronic inflammation and autoimmune diseases [61].

Adaptive immune system

Although the innate immune response can combat certain threats independently, others necessitate a more organized and targeted immune approach. The adaptive immune response, while taking a slightly longer time to develop compared to the innate immune response, is typically more robust and specific. APCs and cellular debris play a vital role in the activation of the adaptive immune response. Immature DCs are present in the peripheral inflammatory tissues, where the pathogens or neoplasms are present, and engulf antigens via a process known as "antigen capture." These antigens are then cleaved into peptides. This initiates the maturation process of the DCs in the lymphoid system and stimulates upregulation of major histocompatibility complex (MHC) and co-stimulatory molecules. The MHC-I glycoproteins

express endogenous proteins and are present on all nucleated cells and MHC-II glycoproteins are only present on APCs and express exogenous proteins as “antigens”. Additionally, APCs are capable of cross-presentation of antigens in which exogenous antigens are presented on MHC-I molecules [62-64]. The primary function of DCs are to capture and process antigenic material and present MHC-antigen complex on their surface to “prime” cells of the adaptive immune system. In the lymph nodes, the mature DCs prime the lymphocytes, comprising of B cells and T cells. The lymphocytes with high affinity to self-antigens undergo negative selection during development thereby building tolerance to self-antigens. However, interactions with foreign antigens will initiate lymphocyte activation and clonal selection [65-69].

T lymphocytes modulate tumor progression via cell-mediated mechanisms. Priming of naive T cells in the lymph nodes initiates maturation of the T cells into $CD4^+$ T helper cells (THCs) or $CD8^+$ cytotoxic T cells. Maturation of the $CD4^+$ T cells begin once the tumor antigens presented by the MHC-II molecules bind to the T cell receptors (TCRs) on the naïve $CD4^+$ T cells [70]. The costimulatory CD80/CD86 (B7) receptors on the DC then bind to the CD28 ligand on the $CD4^+$ T cells and further promote maturation of the $CD4^+$ T cell [71]. The release of IL-2 via autocrine signaling is also essential for initiating and activating the effector-T-cell response, this leads to subsequent clonal selection and expansion as well as differentiation of effector and memory $CD4^+$ T cells [72]. $CD8^+$ cytotoxic T cells are also activated through DCs however, they are activated via cross-priming in which the DCs cross-present exogenous antigens on MHC-I molecules [73, 74]. These cytotoxic T lymphocytes (CTLs) are then further activated through a similar co-stimulation response, further leading to their clonal selection and expansion [75]. Moreover, DCs produce chemokines CXCL9 and CXCL10 to attract additional

infiltrating T lymphocytes (ITLs) to the tumor tissue, facilitating communication between cancer and immune cells [76].

The mature T cells then travel through the lymphoid system to their target destination and respond to the pro-inflammatory cytokines that are released from immune cells or dead tumor cells. Upon binding to their target tumor cells, the lymphocyte's interaction with co-stimulatory or co-inhibitory molecules expressed on the target cells becomes pivotal in determining the fate of the cancer cells [77]. In the presence of abundant co-stimulatory molecules, the T cells continue to execute the immune response; however, the presence of predominantly co-inhibitory molecules will render the T cells inert leading to T cell anergy or death. These molecules are also present on APCs and can determine T cell fate [70].

Once stimulated, the infiltrating CTLs launch the immune assault on the cancer cells by releasing perforins, granzymes, IFN- γ and TNF-α [78]. The antitumor function of IFN-γ includes creating a proinflammatory microenvironment and enhancing tumor immunogenicity by upregulating the expression of MHC class I and II on APCs [79-81] and MHC-I on tumor cells [33, 82]. Additionally, IFN-y stimulates the expression of chemokine receptors on T cells and posttranslational modification (PTM). TNF- α plays a role in the formation and effective operation of the immune system by facilitating signaling pathways that regulate both cell survival and cell death [83].

There are many subsets of THC, each with their own functions [84]. THCs release pro-inflammatory cytokines to enhance the immune response by stimulating the macrophages to destroy engulfed neoplasms, NK cells to release granzymes and perforin and further initiate mature CTLs and B cells [85-87]. THCs aid in the priming of CTLs when both $CD4^+$ THCs and $CD8^+$ T cells bind to their respective antigens on the same DC, the THCs stimulate the DC to

increase antigen presentation and to express specific co-stimulatory signals and cytokines to the CTL that promote clonal expansion and differentiation into memory or effector T cells [88-91]. The THCs can also trigger gene expression programs in CTLs to enhance their function through diverse molecular mechanisms thus allowing the CTLs to overcome challenges that typically impede antitumor immunity [92, 93]. These enhancements are largely due to CD27 costimulation and include but are not limited to downregulating coinhibitory receptors, upregulating chemokine receptors and increasing metalloprotease activity thereby increasing CTLs motility, migration and infiltration.

Additionally, some THCs can possess cytotoxic capabilities. Cancer cells are identified through the MHC-I bound tumor antigens (TA) on the surface of the cells by the TCRs on the THCs [94]. This process is dependent on the presence of costimulatory molecules. The tumor cells can then be eliminated directly through cytolytic mechanisms or indirectly by modulating the tumor micro environment (TME) [95, 96].

Regulatory T cells (Tregs) are a specialized subset of T cells involved in modulating T cells in immune tolerance and preventing autoimmune diseases. They execute their regulatory functions through various mechanisms: by releasing anti-inflammatory cytokines such as TGF-β, IL-10, and IL-35 [97]; by cytolysis of APCs and T cells by perforin and granzyme excretion; by metabolic regulation and modulating available IL-2, which is crucial for THC proliferation and survival [98, 99]; by DC modulation by upregulating co-inhibitory molecules such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1) [100, 101]; and by promoting trogocytosis to selectively remove the antigen-MHCII complexes from APCs [102].

B cells are another subset of lymphocytes involved in the adaptive immune response and are responsible for the humoral immune response. The B cells play a pivotal regulatory role in the immune system. They function by generating antibodies [103], serving as APCs [104], providing support to other mononuclear cells, and directly contributing to inflammatory pathways. All B cells have B cell receptors (BCRs) on their surface that undergo negative selection during development to bind to non-self-antigens thereby defining the specificity of the cell. They are also capable of expressing both MHC-I and MHC-II [105].

B Lymphocytes predominantly engage in antigen presentation through BCRs. Interaction with an antigen specific to the BCR triggers B cell activation and proliferation, along with the internalization of the antigen [106]. This internalization process results in the antigen's processing and subsequent presentation. In polyclonal B cell activation, the binding of numerous BCRs to repetitive epitopes on the surface of the antigen is followed by TLRs engaging with PAMPs or interacting with components from the complement system [107, 108]. Lipidic antigens and polysaccharides will typically trigger this type of T-independent activation. Once activated, the B cells will undergo clonal proliferation and the eventual differentiation of daughter cells into short-lived plasma cells. This type of activation lacks the capacity to produce memory B cells.

Linked recognition is another form of B cell activation that is dependent on T cells. It requires internalization and processing of cognate antigen by the B cells, followed by expression of the antigen through MHC-II to be recognized by the THC with TCRs corresponding to the same antigen. The B cells can then be activated by the interaction between the CD40 ligand present on the surface of TH lymphocytes and the CD40 molecule and the release of several cytokines which will facilitate the development of B lymphocytes into plasma cells that secrete

antibodies and differentiation into memory B cells [109]. Plasma cells are produced in large quantities but have a short lifespan, whereas memory cells are generated in smaller numbers but exhibit a longer lifespan. Additionally, B cells can efficiently express low abundance antigens, costimulatory molecules (CD80, CD83, CD86, CD40 and more)[110], cytokines (CCL22 and CCL17)[111, 112] and cross present antigens to further stimulate both CD8 and CD4 T lymphocytes [110, 113-116]. Furthermore, the antigen presentation capacity of B cells can be significantly enhanced under certain conditions to match that of DCs [71, 114, 117, 118].

B cells also generate various other immunostimulatory cytokines such as IL-6, IFN-y, TNF, C-C motif chemokine ligand 3 (CCL3), IL-2 and CSF-2 to attract and stimulate other immune cells [119]. The cytokines released by the THCs following B cell antigen presentation, influence antibody isotope switching, also known as class switching, which takes place when naïve B cells transition from producing IgM and IgD to different isotypes such as IgA, IgG and IgE [120]. In both mice and humans, cytokines such as IL-4 [121], IL-5 [122], TGF-β [123], and IFN-γ [124] are known inducers of isotype switching after activation. The antibodies produced will have the same specificity as the BCRs and will bind to their corresponding antigens. Binding of the antibody to its antigen activates the classical pathway, one of three pathways, of the complement system [125]. This leads to opsonization, where antibodies create a coating layer around the target cell which attracts phagocytes to target and eliminate that cell. This is known as complement-dependent cytotoxicity (CDC). Additionally, innate immune cells express Fc receptors (FcRs) [126] complementary to the antibodies released by the B cells, leading to further cell death via antibody dependent cellular phagocytosis (ADCP) by myeloid cells or antibody-dependent cell cytotoxicity (ADCC) by myeloid cells and NK cells [127]. Antibodies can also mediate intracellular neutralization by inducing proteasomal degradation of intracellular

proteins [128, 129], signaling interference by modulating signaling pathways [130, 131] and transcytosis [131].

The complement system, a crucial component in the innate and adaptive immune response, consists of over 30 proteins present in both the plasma and on cell surfaces [132]. Initiation of the classical pathway occurs when C1q, the first protein in the complement cascade, binds directly to the pathogen surface. This pathway can also be activated through the interaction of C1q with antibody-antigen complexes. The initiation of the mannan-binding lectin pathway (MB-lectin pathway) occurs through the binding of mannan-binding lectin (MBL), a serum protein, to carbohydrates containing mannose on bacteria or viruses. In the alternative pathway, activation takes place when a complement component spontaneously becomes active and binds to the pathogen's surface.

Triggering the complement system initiates highly effective proteolytic cascades that culminate in both the opsonization and lysis of the pathogen [133]. The selective lysis of the pathogenic surface occurs by assembling membrane-penetrating pores, referred to as the membrane attack complex (MAC) [134]. Simultaneously, this process generates an inflammatory response by producing potent proinflammatory mediators (anaphylatoxins) stimulating chemotaxis and activation of innate immune cells/phagocytes [135].

A subset of B cells that suppress inflammation by producing cytokines such as IL-10 [136], or IL-35 [137], have been observed in both human and murine cancers and are referred to as B regulatory cells (Bregs) [138]. These Bregs have demonstrated the ability to convert conventional $CD4^+$ THCs into Tregs and have been linked to diminished survival rates for cancer in humans [139-141]. Additionally, Bregs can produce TGF- β, facilitating the induction of Tregs [142-144].

The prime targets for the lymphocytes are neoantigens which arise from mutations that are often unique to the host, and the occurrence of non-synonymous mutations vary across different types of cancers [145]. T cells and B cells are both able to retain antigenic information and become memory cells. These memory cells will not be activated at the initial exposure but will become activated during any secondary exposures and will provide a more rapid response. This is the foundation of immune memory and the basis for vaccine treatments.

Additionally, the presence of tumor infiltrating T lymphocytes (TILs) or B lymphocytes (TIBs) have been linked with favorable treatment outcomes and better prognosis in various types of cancers [146-148]. Intratumoral B cells are often located within organized structures known as tertiary lymphoid structures (TLS) which resemble the structures/arrangements found in lymphoid organs. Within the TLS, B cells and T cells are interspersed and occasionally organize into separate compartments similar to the arrangement observed in lymph nodes [127, 149]. In certain malignancies, B cells within TLS further organize into germinal centers, actively producing antibodies capable of identifying TAs. A higher abundance of TLS, or the expression of TLS-related genes have been correlated with improved patient survival and TIBs residing in TLS exhibit elevated MHC-I and MHC-II expression levels [150-152]. Moreover, TIBs in non-small cell lung cancer (NSCLC) tumors could actively participate in the presentation to and activation of $CD4^+$ T cells in the presence of human TA and some patients could activate TILs without exogenous antigens [153]. Co-culturing TIBs from NSCLC patients with $CD4^+$ T cells resulted in $CD4^+$ T cells adopting a Th1 phenotype, while B cells displaying exhaustion markers led to the generation of $CD4^+$ T cells with a regulatory T cell phenotype. Th1 $CD4^+$ T cells are responsible for increasing inflammation through the release of cytokines such as IFN-γ, TNF-α and IL-2 [154]. In other cancers TIBs have shown higher expression/increased expression of

IFN-y and chemokines such as CCL3, CCL4 and CCL5, to attract NK cells, macrophages and T cells, consequently exhibiting increased T cell infiltration [155, 156]. Consequently, the presence of TILs, TIBs and TLS have been associated with better responses to immunotherapy treatments for various cancers [150, 152, 157, 158]. Furthermore, some immunotherapies rely on the presence of TILs.

The degree of lymphocyte infiltration contributes to the classification of tumors as immunologically “hot” or “cold” tumors [159]. Hot tumors are defined by a high immunoscore, determined by high T cell and CTL infiltration, checkpoint activation, genomic instability and pre-existing antitumor immune responses. Cold tumors are defined by a low immunoscore, low mutational burden, poor antigen presentation and minimized T cell priming. A third classification of “altered tumors” have been proposed with intermediate immunoscores [160]. The altered tumors can further be subclassified into immunosuppressive and immune-excluded tumors. Altered-immunosuppressed tumors are defined by poor T cell infiltration and presence of immunosuppressive cells, inhibitory mediators and immune checkpoints. Altered-excluded tumors have been defined as accumulation of T cells at tumor borders (invasive margin) but with no/minimal infiltration, epigenetic regulation and reprogramming of the TME, activation of oncogenic pathways, aberrant tumor stroma and/or vasculature and hypoxia. Hot tumors are associated with better responses to immunotherapies [161, 162] whereas altered and cold tumors require combined therapeutic approaches to obtain desired outcomes and many are ongoing pre-clinical and clinical trials [163, 164].

The cells or active molecules involved in the innate and adaptive immune systems must work together, to induce an effective immune response, to fight infectious diseases or cancer (Fig

2). When both systems respond appropriately during the initial stages of cancer, it allows for the elimination of cancer cell thus leading to restoration of the tissue to its normal state.

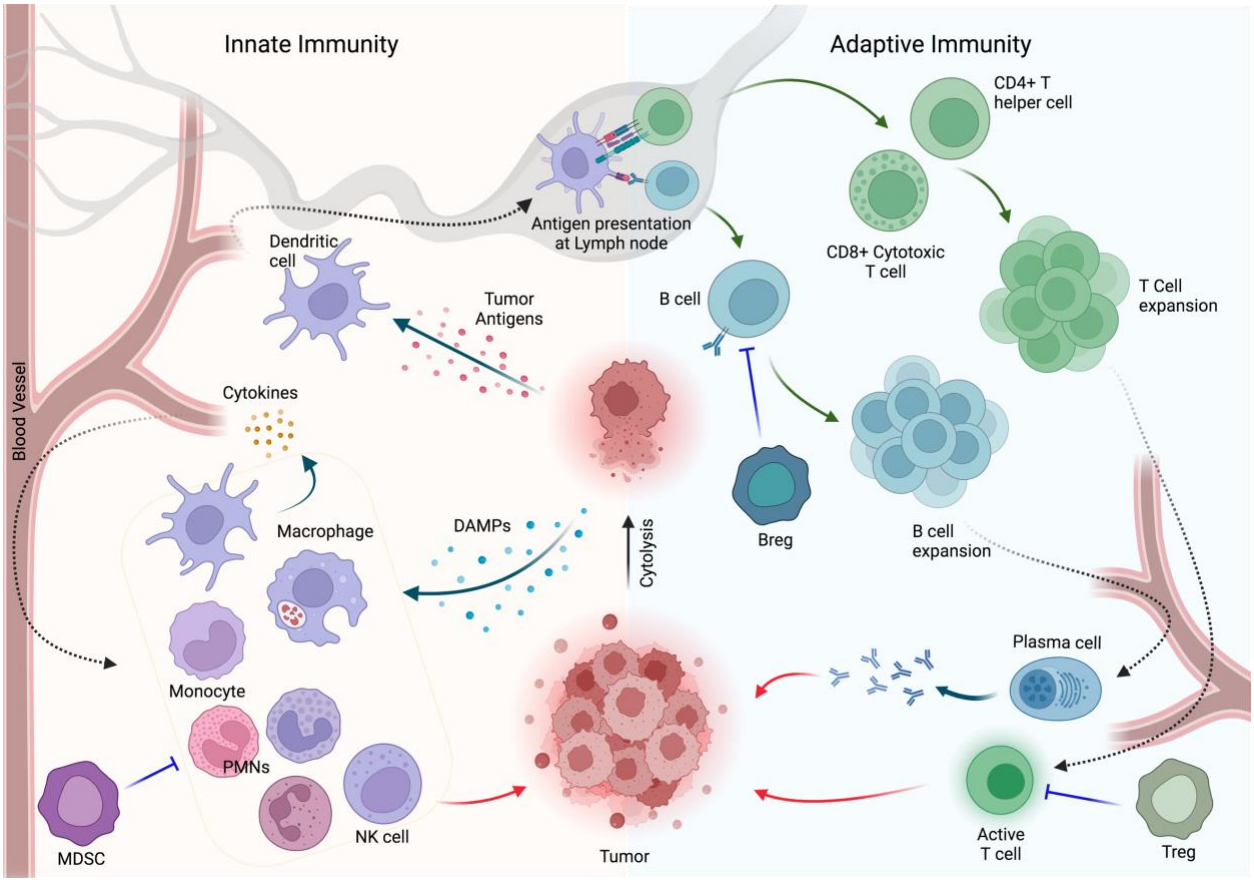

Figure 2: Collaboration of innate and adaptive immune cells to eliminate cancer

This figure provides an overview of the collaborative functions of the innate and adaptive immune responses. The innate immune cells are the NK cells, Monocytes, Macrophages, DCs and PMNs. These cells are activated upon the release of DAMPs by damaged/dead cancer cells. This leads to subsequent targeting and elimination of tumor cells leading to more activation of additional innate immune cells. Moreover, the innate cells release cytokines to attract additional innate and adaptive immune cells. MDSCs negatively regulate the cells of the innate immune system. Cytolysis of tumor cells also release antigens which are engulfed by DC, processed and

presented on DC cell surface. The DC then travel to the lymph nodes where antigen presentation to T cells and B cells occur. Antigen presentation activates $CD4^+$ T cells, $CD8^+$ T cells and B cells which leads to their clonal selection and expansion. The activated T cells travel through the blood vessel to eliminate the tumor. The B cells differentiate into plasma cells that produce antibodies to eliminate the tumor. Tregs and Bregs can negatively regulate T cells and B cells respectively.

Equilibrium

In this phase, the residual occult tumor cells that survived the elimination phase remain in a state of tumor dormancy and are maintained at equilibrium by the immune systems. This theory is supported by the occurrence of minimal residual disease (MRD) in hematologic and solid cancers [165]. In MRDs, a limited number of malignant cells persist in the body, remaining below the threshold of cytogenic or morphological detection. The occurrence of organ transplant recipients developing tumors after transplantation, even when absent in donors in an overt form, implies the possibility of their dormant presence in donors at the same site or at a distant site [166, 167]. The immunosuppression in the recipient serves as the stimulus required for immune escape and the development of a fully manifested cancer [168].

Mice administered with small doses of MCA developed small, stable masses at the site of injection that only progressed into fully developed cancer when specific components of the immune system were deactivated. This suggests the immune response had previously been suppressing the growth of these tumors [42].

Studies have indicated two mechanisms of equilibrium. In one, the individual tumor cells may remain inactive, experiencing neither cell division nor apoptosis [169]. Here the immune

system may ultimately eradicate all tumor cells, ultimately leading to elimination. In the second scenario, the proliferation is counterbalanced by apoptosis, preventing an increase in size [170]. However, prolonged habitancy in the second state allows for immune exhaustion and the tumor the opportunity to further mutate and become unrecognizable to the immune system. This is referred to as immunologic selection and leads to tumor growth. The ongoing dynamic interplay between the host's immune response and the invasive processes of the tumor is widely accepted as a pivotal factor in shaping tumor progression [171-174].

Escape

Cells that further mutate and are no longer detected or eliminated by the host's immune system grow, induce and immune suppressive microenvironment and develop into clinically apparent tumors and could lead to metastatic tumors. This is consistent with the theory of immunoediting in which the tumor cells with highly immunogenic neoantigens are "edited out," allowing the less immunogenic cells to continue growing undetected [175, 176]. Furthermore, it has been shown that tumors originating in individuals with an intact immune system are typically less immunogenic compared to those that are from an immunodeficient microenvironment [33].

Cancer cells have also developed various strategies of immune escape, including impairments in antigen presentation, heightened expression of negative regulatory pathways and the recruitment of immunosuppressive cells to create an immunosuppressive TME to evade immune surveillance [177]. These tactics lead to compromised effector function in immune cells and the inhibition of anti-tumor immune responses. The mechanisms vary between cancers, but some common methods include reduced MHC and antigen presentation, impairing DC maturation, and an immunosuppressive TME. Although many tumor cells with immunogenic

antigens can be removed by the immune system in the elimination phase, the antigens that persist may be enough to facilitate tumor regression via targeted immunotherapies.

CHAPTER 3

MECHANISMS OF GBM IMMUNE ESCAPE/SUPPRESSION

The brain is thought to be an immune privileged organ with neuroprotective mechanisms to minimize excessive inflammation. While these mechanisms protect the brain, they can also add to creating a more tolerogenic environment for tumor growth. The brain is protected by the blood brain barrier (BBB) making certain immune molecules and drug delivery complicated. While naïve T cells are absent from the brain, activated T cells and antibodies have access to the central nervous system (CNS) [178]. Therefore, the CNS is not immunologically isolated. Additionally, the brain has a specialized glial lymphatic system to remove waste products and macromolecules and traffics the metabolites and APCs to the deep cervical and lumbar lymph nodes [179]. While various types of macrophages exist within the CNS, Microglia are the most prominent residents.

GBM are typically immune cold, with a low mutational burden and a low neoangiogenic burden leading to heterogenicity within the tumors. There are many factors that aid in GBM immune escape (Fig 3). The TME is composed of tumor cells, stromal cells, endothelial cells, pericytes, TAMs and microglia. Microglia are the resident macrophages in brain. Additionally, immunosuppressive cytokines in the TME, such as IL6, IL10, TGF-B and PG-E, inhibit the innate and adaptive immune responses by inactivating NK cells, suppressing T cell activation and function and activating Tregs to further inhibit immune cell functions.

Decreased antigen presentation

GBM can downregulate, remove or transform MHC molecules and antigen expression through mutations and epigenetic or transcription inhibition of gene expression in GBM cells and microglia [180]. Microglia present antigens via MHC-I and MHC-II molecules and are the primary APCs in the CNS [181]. However, IL-10 and TGF-B in the TME reduce microglial MHC expression. Low MHC levels are also seen in GBM stem cells thereby enabling immune escape from T cells and leading to tumor initiation, progression and therapy resistance [182].

However, NK cells cannot be evaded in this manner as they induce immune responses to abnormal cells by evaluating the degree of MHC-I expression. Therefore, GBMs have adapted by expressing NKG2D ligands to escape lysis by NK cells [183].

Impaired DC maturation

As previously discussed, DC maturation is stimulated by DC interactions with TA and DAMPs. Although DCs are not usually present in heathy CNS and represent less than 1% of the immune cells in the CSF, DC accumulation has been observed in neuropathological conditions and aged brains [184]. Oncogenic cells can inhibit the maturation of DC through the release of tumor-derived factors such as TGF-B [185], IL-10 [186], vascular endothelial growth factor (VEGF) [187], prostaglandin E2 (PG-E2) [188]and indoleamine 2,3-dioxygenase (IDO) [189]. Additionally, immunosuppressive cells in the TME, such as MDSC and Tregs, can express inhibitory factors to suppress DC maturation, which in turn reduce the expression of MHC and costimulatory factors, thus downregulating inflammatory cytokines, such as IL-12, leading to inhibited T cell activation/proliferation and IFN-y [190, 191].

Suppression of T cell activity, migration and infiltration

Typically, GBMs are poorly infiltrated with T cells, thus supporting the immune cold phenotype. The T cells that are found in the tumors are often dysfunctional due to anergy or exhaustion [192]. Anergy is a result of partially activated T cells due to coinhibitory signals or the lack of costimulatory signals leading to inhibited functionality. Exhaustion is defined by upregulated inhibitory receptors and is a result of prolonged activation and chronic antigen stimulation. Both states can eventually lead to T cell death. GBMs can modulate T cells by decreasing costimulatory molecules or increasing coinhibitory molecules, leading to T cell anergy/exhaustion. Programmed death ligand – 1 (PD-L1) is a coinhibitory immune checkpoint (IC) protein that is often upregulated in cancers and are a marker of T cell exhaustion. This has been confirmed to be prevalent in many human cancers, including GBM [193].

VEGF is a cytokine that is frequently upregulated in GBMs. This leads to the formation of leaky and abnormal blood vessels and consequently poor perfusion thus, hindering the infiltration of T-cells and therapeutic agents [194]. Simultaneously, it compromises T-cell effector functions and may even induce T-cell apoptosis within the TME [195]. Furthermore, VEGF can affect T cell activation by inhibiting DC maturation. Additionally, IL-10, PG-E2 and VEGF increase death mediator Fas ligand (FasL) expression in EC and induces apoptosis of CTLs [196]. In some instances, even if CTLs are able to migrate through the BBB and into the tumor, they may not be able to infiltrate due to additional physical barriers. Cancer-associated fibroblasts (CAFs) and other immunosuppressive immune cells, in the periphery of the tumor, generate extracellular matrix (ECM) proteins that restrain T cells or produce chemokines such as CXCL12, that impede T cell infiltration [197].

Converted Immunosuppressive cells

The TME is also known to take advantage of the innate and adaptive immune responses and convert the immune cells into pro-tumorigenic phenotypes. High MDSC and Treg frequencies in tumors are typically associated with poor patients' prognosis [198]. Additionally, microglia and other macrophages in the brain can be transformed to support tumorigenesis.

The microglia and macrophages present in the TME are referred to as tumor associated macrophages/microglia (TAMs)[199]. Macrophages were originally identified as phagocytic cells that could eliminate cancer cells. The M1 phenotype is triggered by pro-inflammatory cytokines, such as TNF and INF, and bacterial components like lipopolysaccharides. These M1 macrophages produce angiostatic factors such as IL-12 and CXCL10 that contribute to anti-tumor immunity. However, M2 macrophages are induced by immunoregulatory cytokines, such as TGF-B, IL-4 and IL-10, and stimulate the release of tissue-remodeling and pro-angiogenic factors like matrix metalloproteinases (MMPs) and VEGF, which are associated with tumor promotion. TAMs often do not exhibit clear M1 or M2 phenotypes, making the binary classification of these cells challenging due to their inherent complexity [200-205]. The immunosuppressive GBM TME is capable of converting M1-like microglia/macrophages into M2-like phenotypes and elevated levels of these M2-like TAMs have been associated with reduced patient survival and therapy resistance in GBM [206]. TAMs, specifically microglia, are also the most abundant cells in the GBM TME and secrete anti-inflammatory cytokines and attract MDSCs and Tregs.

MDSCs are recruited by the TME to reduce T cell response and induce Tregs. MDSCs decrease the cytotoxic activity of NK cells and CTLs by reducing the expression of cytotoxic factors, granzyme and perforin. Tregs promote tumorigenesis by inhibiting CTLs and inducing T

cell tolerance [207, 208]. Additionally, Tregs in the TME decrease lymphocyte counts [209] by increasing the expression of ICs, such as CTLA-4 and PD-1. These coinhibitory molecules inhibit and can induce apoptosis of T cells.

Tumor-associated cell in the TME can also downregulate antigen expression thereby protecting them from elimination by the immune system [198] and induce apoptosis of CTLs [199]. Immunosuppressive enzymes, such as IDO and Arginase 1, further contribute to the immunosuppressive TME [210, 211]. IDO induces a tryptophan metabolite, kynurenine, which is known to suppress T cell function while stimulating MDSCs and Tregs. Arginase 1 cooperates with IDO to inhibit the function of DCs. Additional metabolites and inflammatory mechanisms have also been known to affect immune response to cancer cells [212].

Hypoxia

The TME contributes to tissue hypoxia due to increased tumor oxygen consumption and irregular blood vessels which leads to the increased expression of hypoxic growth factors, such as hypoxia-inducible factor-1α (HIF1α) [213]. Hypoxia further recruits TAMs, MDSC and Tregs and stimulates VEGF, angiogenesis and the immunosuppressive STAT3 pathway [214]. Additionally, the hypoxic necrotic core also leads to the formation of a pseudo palisade layer consisting of highly migratory cells. Studies have shown this layer is saturated with TAMs that can clear the necrotic area [215]. This could promote tumorigenesis in the newly cleared area, thus presenting an additional immune evasion mechanism.

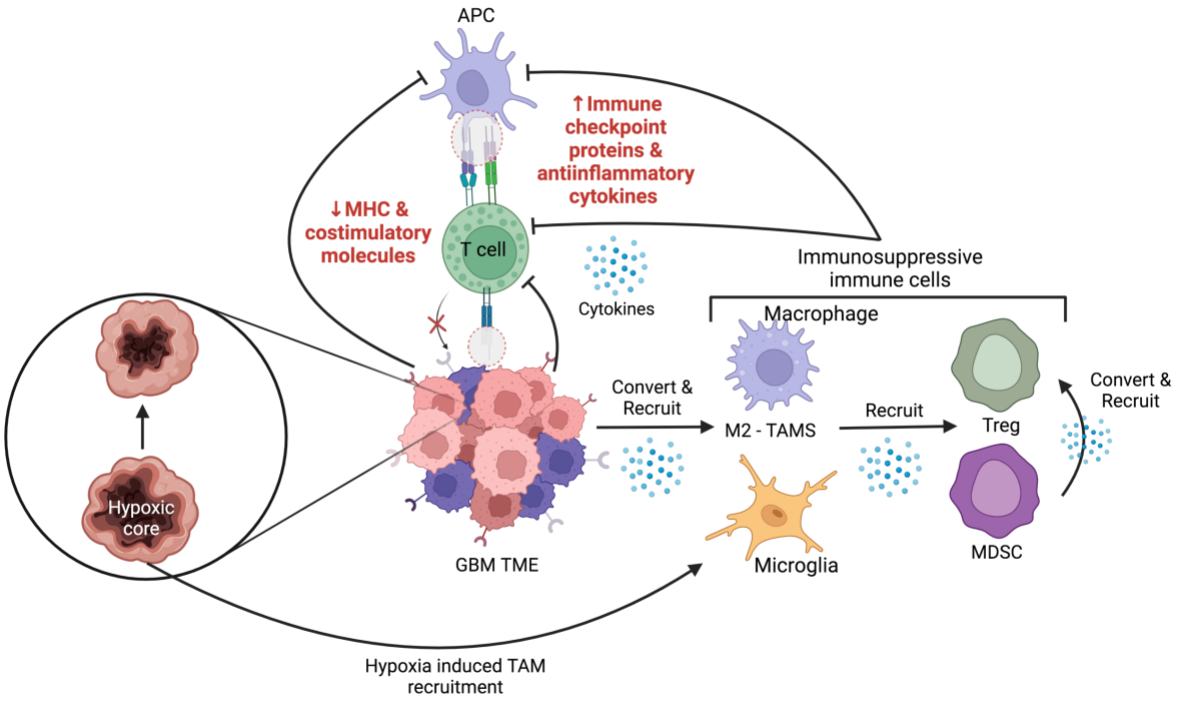

Figure 3: The GBM TME Immunosuppressive mechanisms supporting tumorigenesis

The GBM TME consists of a variety of cells with immunosuppressive mechanisms. The GBM cells decrease antigen and costimulatory molecule expressions, increase coinhibitory molecules and cytokines to escape elimination by T cells and disrupt the function of APCs. Cells in the GBM also release cytokines to recruit and convert TAMs into M2 phenotypes. The TAMs then release additional cytokines to recruit Tregs and MDSCs. MDSCs can further convert and recruit Tregs. These immunosuppressive cells can disrupt T cells and APCs by increasing immune checkpoint proteins and anti-inflammatory cytokines. The hypoxic core also recruits TAMs to clear necrotic tissue and allow for tumorigenesis.

Therapeutic immune suppression

Current standard of care (SOC) therapies can also enhance the immunosuppressive TME. Several reports indicate chemotherapy and radiation therapy have been reported to cause lymphopenia and decreased overall survival (OS) in GBM patients [216, 217].

Dexamethasone (Dex) is a corticosteroid that is routinely utilized in GBM treatment protocols to relieve edema in patients by modifying the permeability of blood vessels. However, studies have reported Dex also supplements the immunosuppressive TME by impairing NK cell and T cell function by disrupting DC maturation, upregulating PD-1 and CTLA-4 and suppressing inflammatory cytokines [218]. Additionally, Dex reduces the efficacy of chemotherapy and TTFields resulting in lower patient OS [219].

Besides potentially leaving residual oncogenic cells, surgical tumor excisions pose a risk of releasing tumor cells into the bloodstream [220]. This could result in elimination by the immune system. However, if the immune system is suppressed due to treatment using Dex, this could lead to immune evasion [221]. Additionally, immune suppression induced by chemoradiation could exacerbate the likelihood of recurrence and metastasis.

For many of the mechanisms GBM tumors utilizes to evade/suppresses immunity, there is a vulnerability that can be exploited using immunotherapies. The goal of immunotherapy for cancer treatment is to harness the hosts immune system to eliminate tumors. The potential cumulative immunosuppressive impact of chemoradiation and Dex contribute to the formidable challenge of treating GBMs. This emphasizes the need for in-depth exploration of alternative therapeutic avenues or novel treatment strategies to effectively target this form of cancer.

CHAPTER 4

IMMUNOTHERAPIES FOR GLIOBLASTOMA MULTIFORME

Immunotherapies have had considerable success in some cancer types and are used as the first line of therapy. There are many types of immunotherapies such as antibody therapy, checkpoint blockade, T cell therapy, cancer vaccines, cytokine therapy and oncolytic viruses. Existing therapies can enhance the immune response, trigger new responses or add immune components designed to specifically target cancerous cells. They can also be administered on their own or in combination with other strategies such as chemotherapy, radiation, or surgery. However, their implementation in the treatment of GBM has posed significant challenges. Here, we review the different types of immunotherapies that have been or are currently being evaluated in clinical trials for GBM.

Antibody therapy

Monoclonal antibodies (mAbs) can specifically bind to their target molecules and activate or suppress their molecular function, promote innate immune responses, such as ADCC, CDC and ADCP, or induce proapoptotic or neutralizing effects [222]. Antibodies can also form antibody-drug conjugate (ADCs) and bi-specific T-cell engaging antibodies (BiTEs) which facilitate T cell binding to cancer cells. Furthermore, mAbs can be designed to be tumor specific, targeting upregulated receptors such as EGFR, co-stimulatory/inhibitory molecules, immune checkpoints, anti-cytokine or anti-angiogenic.

GBMs are attractive targets for anti-angiogenic therapies due to their highly vascularized nature [223]. Increased expression of VEGF family proteins are correlated with poor patient

prognosis [224, 225]. Transcription of VEGF can be regulated by hormones, hypoxia, acidosis, oncogenes, tumor suppressor genes and various signaling molecules [226-230]. VEGF can also be expressed by cells in the TME and ECM [226, 231]. The only FDA approved immunotherapy for GBM, Bevacizumab, disrupts tumor blood vessel growth by binding to VEGF-A, and inhibiting the interaction with its receptor, VEGFR, thereby attempting to normalize the TME (Fig 4.A) [232]. Bevacizumab, alone and in combination, has been shown to inhibit tumor angiogenesis and growth. This mAb when used in combination with SOC treatments improved patient performance and prolonged progression free survival (PFS) but did not have much effect on overall survival (OS). Additionally, newly diagnosed GBM (nGBM) and first, recurrent GBMs (rGBMs) have a tendency to develop resistance after initial response to treatment [233]. The loss of available VEGF could stimulate other compensatory tumorigenic pathways. However, it is still considered a valuable last-line treatment and has shown PFS and OS in patients who have failed other therapies [234].

EGFR amplification is found in ~50% of all GBMs with EGFRvIII being the most common mutation within these tumors [235]. This mutation leads to a constitutively activated EGFR resulting in an increase of downstream pro-survival signaling pathways such as STAT3 and PI3K/AKT signaling [236]. Nimotuzumab is a mAb that targets EGFR without stimulating its intrinsic activity (Fig 4.A) and has been shown to increase radiation and chemotherapy sensitivity in U87MG and LNZ308 mouse models [237, 238]. These preclinical results eventually lead to a phase III clinical trial evaluating the efficacy of Nimotuzumab in GBM (NCT00753246) [239]. However, this study only showed a slight effect on prolongation of OS or PFS in Nimotuzumab + Temozolomide (TMZ) + radiation therapy (RT) vs TMZ + RT alone. Of note, this study did not select patients based on molecular markers thereby potentially affecting

the outcome of this trial. However, upon analysis of PFS and OS in EFGR amplification and EGFRvIII mutation, there was a slight but non-significant benefit observed in the amplification cohort with the experimental arm vs without amplification but not in EGFR mutated. This suggests this combination may not benefit those with EGFR amplifications/mutations. These results may be due to a variety of other reasons including the upregulation of supplementary compensatory pathways involving other receptors [240]. Additionally, these results could be attributed to the heterogeneity of the tumors.

Various other mAb therapies for GBM targeting CD27, CD47, PD-1/PD-L1, PDGFR, CD105, VEGF, EGFR, CTLA4 and IL-6, are currently being evaluated in preclinical and clinical trials (Table 1). Radio-labeled mAbs have also been developed to directly eliminate tumor cells or deliver cytotoxic substances are also currently being evaluated in clinical trials. Despite mAbs emerging as the first-line standard-of-care in some cancers, such as HER2+ breast cancer [241], they have not yet proven to be as effective in GBM.

Drug	Target	Target Classification	Reference
Bevacizumab	VEGF-A	Cytokine	[232]
Nimotuzumab	EGFR	Upregulated receptor	[238]
Pembrolizumab	PD-1	Immune checkpoint	[242]
Olaratumab	PDGFR	Upregulated receptor	(NCT00895180)
Ramucirumab	VEGFR-2	Upregulated receptor	(NCT00895180)
Tanibirumab	VEGFR-2	Upregulated receptor	(NCT03033524)
Nivolumab	PD-1	Immune checkpoint	[243]
Retifanlimab	PD-1	Immune checkpoint	(NCT06160206)
Atezolizumab	PD-L1	Immune checkpoint	(NCT03174197) (NCT05039281)
Avelumab	PD-L1	Immune checkpoint	(NCT03291314) (NCT03750071)
Durvalumab	PD-L1	Immune checkpoint	(NCT02794883)
Ipilimumab	CTLA-4	Immune checkpoint	[244]
Tremelimumab	CTLA-4	Immune checkpoint	(NCT02794883)
Relatlimab	LAG-3	Immune checkpoint	(NCT02658981)
Urelumab	CD137	Immune checkpoint	(NCT02658981)
Varlilumab	CD27	Immune checkpoint	(NCT03688178)

Table 1: List of mAbs evaluated in clinical trials for GBM.

Immune checkpoint blockade

Immune checkpoint blockade (ICB) are a class of mAb that inhibit or mimic ligand binding of co-stimulatory and co-inhibitory immune checkpoints (IC), such as CTLA4 and PD1/PD-L1, to reduce the immunosuppressive effect on CTLs (Fig 4.B) [214, 244]. PD-1 is expressed on T cells and it's interaction with PD-L1 promotes self-tolerance by suppressing effector T cell activity, allowing GBM to escape immune responses. PD-L1 is expressed in 88-100% of GBM and also expressed on TAMs and microglia within the TME, therefore is an attractive target [245-249]. ICIs targeting PD-1, PD-L1 and CTLA4 have already been approved for various cancers and are currently being investigated for GBM.

Pembrolizumab, targeting PD-1, has been FDA approved for a subset of patients with advanced nervous systems or brain tumors with DNA mismatch repair deficiency (dMMR) or high tumor mutational burden (TMB-H) and is currently being evaluated in GBM. Multiple pre-clinical anti-PD1 studies on murine GBM models exhibited promising anti-tumor response, especially in the GL261 model [250]. In the mouse models, anti-PD1 was evaluated as monotherapy or in combination to SOC therapies and lead to increased overall survival. These results lead to a single-arm phase II trial (NCT02337686) with operable rGBM evaluated administering Pembrolizumab before and after surgery however, this study did not observe any clinical benefits over SOC therapy [242]. Single RNA-seq of the resected tumors revealed low levels of T cells and high levels of immunosuppressive macrophages suggesting monotherapy with anti PD-1 may not be sufficient to induce effector immunologic responses in GBM. However, the immunosuppressive SOC treatments administered in relation to surgical swelling could have impacted the results of this trial. Consequently, a study evaluated Pembrolizumab as a neoadjuvant therapy in combination to adjuvant Pembrolizumab + surgery in patients with

operable rGBM [251]. This study revealed significant PFS and OS in the neoadjuvant + adjuvant + surgery arm. Further supporting potential PD-1 therapy in combination to SOC. Therefore, a phase IV clinical trial (NCT05235737) is currently ongoing to compare pembrolizumab neoadjuvant + adjuvant + SOC (chemo-radiotherapy) with adjuvant + SOC and SOC alone, to determine the benefits when combined to other SOC, potentially for unresectable GBM tumors.

Another anti-PD-1 therapy, Nivolumab (NIVO), is also undergoing clinical trials. In a phase III trial (NCT02017717), NIVO was evaluated against Bevacizumab in patients with rGBM who had previously undergone RT and TMZ [252]. NIVO did not demonstrate a significant improvement in OS compared to Bevacizumab however, NIVO did show a longer duration of response and this warrants further investigation. The observed outcomes may stem from Bevacizumab's normalization of the immunosuppressive TME through the reduction of abnormal blood vessels, thereby facilitating the appropriate migration of immune cells to the tumor site. NIVO, on the other hand, might have triggered a more sustained response owing to its targeting of the adaptive immune system, which typically exhibits a slower response.

Preclinical GL261 murine models have exhibited enhanced anti-PD1 efficacy in combination with RT [243]. This prompted investigations to determine if anti-PD1 could also have enhanced efficacy with other SOC treatments. Since unmethylated MGMT is known to cause TMZ resistance in GBM [253], a phase III trial (CheckMate 498) in GBM with unmethylated MGMT promoters was conducted. NIVO + RT was compared to TMZ + RT to determine if anti-PD1 could replace TMZ in patients predicted to have TMZ resistance. TMZ + RT demonstrated superior OS [243]. This suggests the use of NIVO may not be a suitable replacement for chemotherapy in these patients. One possible reason anti-PD1 failed to show better outcomes may be due to inadequate T cell responses due to the immunosuppressive TME.

Additionally, the RT could have also contributed to the immunosuppression. Another phase II/III clinical trial (NCI-2020-03404) is currently ongoing to determine if modulating multiple ICs using NIVO + Ipilimumab (anti-CTLA4) + RT will have a better PFR and OS compared to TMZ + RT for these TMZ resistant tumors.

One of the limitations of ICB is that there may be insufficient T cells within the TME to produce anti-tumor effects. Additionally, the targets may be downregulated or there may be a disruption in converting exhausted T cells to cytotoxic effector function. Furthermore, the immunosuppression by the TME, especially the TAMs, may overpower the effects of these therapies.

Converting Tregs to THC is another attractive therapeutic approach to antibody therapy in GBM [254]. There is an increased presence of Tregs in the GBM TME compared to healthy brain tissue and the phenotype of these infiltrating Tregs are different to those found in peripheral organs. Additionally, since the TCRs of converted Tregs recognize self-antigens expressed by the GBM cells, converting Tregs to effector cells have the potential to generate large quantities of effectors cells, already present in the TME, to target the cancer cells. Glucocorticoid-induced tumor necrosis factor related protein (GITR), an immune checkpoint protein, constitutively expressed on Tregs, has emerged as an attractive ICB target. Activation of GITR in Tregs leads to instability and depletion leading to a decrease in Treg's suppressive influence whereas in CTLs and $CD4^+$ effector cells, it increase their function and proliferation (Fig 4.B). In preclinical GBM models (GL261, CT2A, and 005GSC), agonistic GITR antibodies (αGITR) demonstrated Treg conversion to CD4 effector cells and an increase in antitumor activity when combined with PD-1 inhibitors [254]. The results from a small phase I study (NCT03707457) in rGBM comparing αGITR + Nivolumab (anti-PD-1) to other combination

therapies will provide valuable insight into the proof of concept in GBM patients. This combination targets two immune checkpoints therefore, their synergistic effects may reverse Tregs to THC and increase CTL effects. Further investigations are required to determine αGITR's ability to shift the immunosuppressive nature of GBM to become more tumoricidal.

To date, all completed mAb phase III clinical trials have failed. However, responses to ICBs have been reported in metastatic brain lesions originating from primary melanomas, lung tumors, or renal cell tumors [255-257]. The effectiveness of ICB in these tumors is believed to be due to their high TMB, leading to an abundance of neoantigens [258]. While recent reports indicate the presence of neoantigens and spatially restricted T cell clone expansion in glioblastoma patients [259], a higher TMB did not show a correlation with improved ICB response in primary brain tumors [260, 261]. In fact, low TMB has been associated with increased inflammation, better ICB response, and extended survival in both primary and recurrent tumors [262]. The limited ICB and SOC treatment efficacy in GBM can be attributed to the sparse infiltration of effector lymphoid cells and a myeloid-dominated immunosuppressive TME [263, 264]. Furthermore, studies have shown Tregs can contribute to radio-immunotherapy resistance and T cell activation in GBM [265].

Additional immune checkpoints, such as T cell immunoglobulin and mucin domain 3 (TIM3), T-cell immunoglobulin and ITIM domain (TIGIT) and CD96, are expressed on lymphocytes and are being explored in preclinical models due to their association with GBM [266]. Furthermore, ICBs are linked to diverse immune-mediated toxicities [267]. To overcome these side effects, immunoregulatory medications such as cytokine inhibitors could be used however, this could directly impact the effectiveness of the ICB. In GBM, the potential for transient increases in tumor volume/edema as a result of increased inflammatory infiltrates in the

confined space of the cranial vault, may lead to elevated intracranial pressure, requiring prompt medical or surgical intervention [268]. In addition to the poor outcomes in clinical trials, these adverse events could negatively impact the application of ICB as a SOC treatment option for GBM.

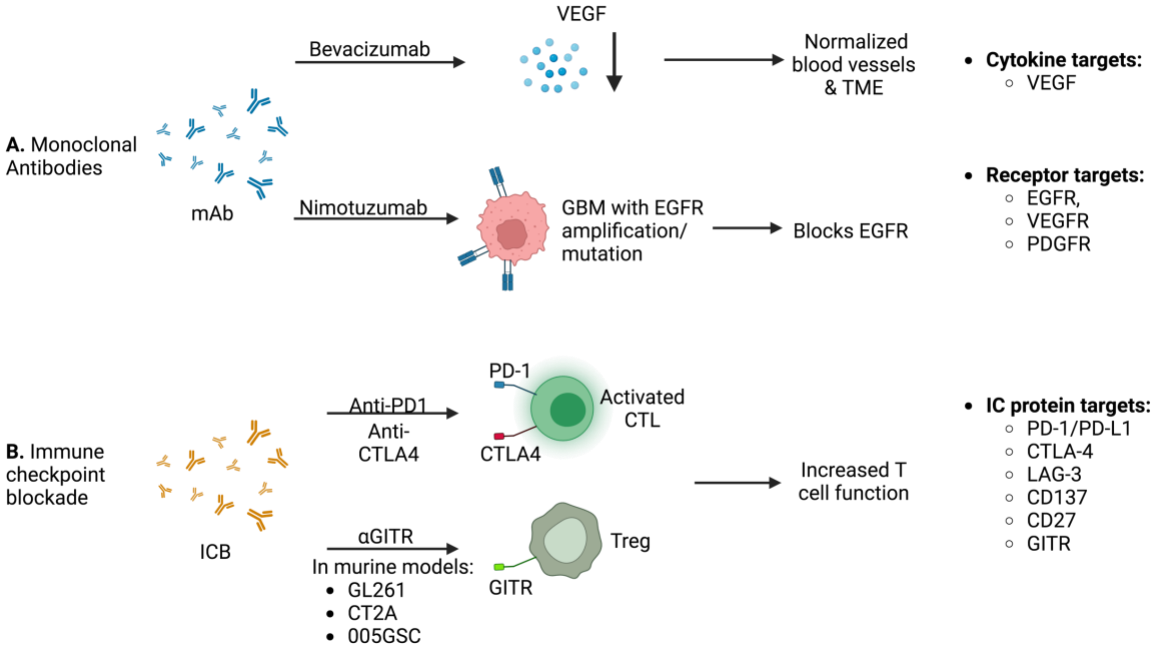

Figure 4: Monoclonal antibody targets in GBM

This figure depicts the various GBM targets for mAbs. A) Targets of mAb include cytokines and tumor associated antigens such as receptors. mAb mode of function and effect vary based on targets. B) Immune checkpoint blockades are a type of mAb that can target co-stimulatory or co-inhibitory molecules on T cells to increase T cell function.

Adoptive T cell therapies

The basis of T cell therapies involve removing T cells from the host, growing them ex vivo and returning to the host to fight cancer. There are various types of adoptive T cell therapies such as TIL therapy, TCR engineered T cell therapy (eTCR) and chimeric antigen receptor (CAR)-T therapy.

TIL Therapy

Typically, TIL therapy involves extracting TILs from resected tumor samples, harvesting with growth factors and returning large quantities of the TILs to the host (Fig 5.A)[269]. This method has shown promising results in cancer regression and remission in patients however, it requires anti-tumor TILs to preexist within the tumor biopsy which can be a challenge in GBM [259].

A pilot study was conducted in 1999 (NCT00002572) to evaluate the safety and efficacy of cytotoxic TIL therapy in combination with IL-2, post-surgery, in recurrent grade III or IV gliomas [270]. IL-2 enhances the expansion and activity of T cells [271]. Of the patients diagnosed with GBM, 2 exhibited partial regression [270]. However, this response could not definitively be attributed to the TIL therapy alone as the subjects were also offered chemotherapy post TIL therapy completion. The study did establish the safety of this method as the only complications were low grade fevers and asymptomatic brain edema. Currently, there is an ongoing study (NCT05333588) to evaluate the efficacy of TIL therapy following lymphocyte depletion by chemotherapy in GBM patients. Another early phase I trial (NCT03347097) evaluated PD1 antibody secreting TILs (PD1-TIL) compared to TIL therapy in 18 rGBM patients. Preliminary reports indicate infusions in both arms were tolerated well with no high grade adverse events (AEs) and OS was extended in the PD1-TIL arm [272].

There are many limitations for the use of TIL therapy in GBM. Some include the limited ability to resect tumors based on location, few effector TILs, heterogeneity of the tumor and the time-consuming preparation of the T cells [273]. A possible future direction for TIL therapy for patients with resectable tumors, could involve combining TIL therapy with αGITR. The influence of αGITR on T cells has been reviewed above. This method would ensure Treg conversion to $CD4^+$ effector cells and could enhance their ability to specifically target the oncogenic cells. This theory warrants further investigation.

TCR engineered T cells

TCR engineered T cells (eTCR) are another form of T cell therapy. This method involves the manipulation of TCRs on T cells to target specific MHC bound TAs, thereby capitalizing on the natural mechanisms of T cells. Although this method has shown in clinical trials to regress tumors in some cancers [274], the limitations of this therapy outweigh the benefits. These limitations include difficulties in manipulating the TCR DNA and TCR recognition of target related epitopes, specificity to MHC bound TA and off-target toxicity in healthy cells. There are currently no eTCR clinical trials for GBM.

CAR T Therapy

Another type of T cell therapy that has received increased interest in recent years is chimeric antigen receptor (CAR) – T cell therapy. Currently there are six FDA approved CAR-T therapies for the treatment of certain hematologic malignancies. The receptors on CAR-Ts are designed with the specificity of mAbs and bind to TA or tumor associated antigens (TAA) in the absence of MHC and co-stimulatory molecules (Fig 5.B) [275]. Additionally, CAR-T can recognize TAAs as proteins, glycoproteins and carbohydrates whereas eTCR can only recognize short peptide sequences [276]. These features allow for enhanced TA and TAA recognition and

overcomes the immunological barrier presented by elevated coinhibitory signals and antigen/MHC downregulation in GBM. Most CAR-T studies in GBM explored targeting EGFRvIII, IL-13 α receptor 2 (IL-13 Rα2) and human epidermal growth factor 2 (HER2).

Preclinical studies conducted in vitro and in mouse models (U87MG and U87-EGFRvIII) demonstrated CAR-T targeting EGFRvIII (CART-EGFRvIII) inhibited the growth of GBM [277, 278]. This led to a pilot study (NCT02209376) evaluating a single dose of CART-EGFRvIII in rGBM patients. The study reported CART-EGFRvIII expansion in the blood, traffic to the brain and on target effects on oncogenic cells [279]. However, they also reported a decrease in antigen presentation, increase in Tregs and upregulation of immunosuppressive signals such as IDO1 and PD-L1, which are all mechanisms of GBM immune evasion. These findings initiated additional trials to evaluate CART-EGFRvIII in combination with Pembrolizumab (anti PD-1) (NCT03726515). Likely to determine if this combination could prevent further T cell inhibition and increase CAR-T efficacy. The results of this trial will provide valuable data on the safety and efficacy of combining these immunotherapies for EGFRvIII+ GBM patients.

HER2 is a receptor that is expressed in approximately 80% of GBM but is also expressed in host cells. Therefore, targeting this antigen could lead to autoimmunity. Preclinical GBM U373 and patient-derived xenograft models in SCID mice exhibited HER2-CAR T anti-tumor activity through median survival increasing from 15 days to 90 days in control vs treatment cohort respectively [280]. An early trial did not produce encouraging results with one subject experiencing acute toxicity from a cytokine storm leading to fatal outcomes [281]. This could be due to the CAR-T targeting the hosts cells expressing HER2. To overcome this challenge, a subsequent phase I (NCT01109095) study evaluating CD28-costimulated HER2-CAR T

demonstrated better results particularly in one patient who exhibited partial response to therapy [282]. CD28 is the costimulatory molecule that competes with CTLA4 for the same ligand, CD80/86, present on APCs and tumor cells. Thereby, increasing CAR-T specificity for target tumor cells.

Other CAR-T trials have targeted IL-13Rα2 as it is expressed in up to 50% of GBM and have low expression in normal brain tissue. A U87 orthotopic xenograft model using SCID mice reported complete regression and no recurrence post intra-tumoral injection of IL13Rα2-CAR T cells [283]. Of note, the U87 glioma cells were modified to secrete IL-2 which plays a key role in T cell survival and expansion, this could have contributed to their successful outcomes. Therefore, suggesting IL-2 can also enhance CAR-T cytotoxic capabilities. In one study, CAR-T targeting IL-13Rα2 in a single patient with rGBM resulted in regression of all spinal and cranial tumors with increased immune response following a regimen of intracavity and intraventricular infusions [284]. Despite the recurrence of tumors at new locations in this patient, additional trials investigating this therapeutic option were initiated. An ongoing phase I trial (NCT04003649) is evaluating combining IL13Rα2-CAR T cells with anti PD-1 and CTLA4 to possibly enhance the T cell function.

Additional trials using CAR-T as a monotherapy or in combination are being evaluated in GBM patients [285]. These include targeting B7-H3, PD-L1, CD70, CD44, CD133, NKG2D and MMP2. Although some trails have demonstrated the benefits of CAR-T cell therapy, there are many limitations and risks involved. CAR-Ts have been shown to induce potent immune response which can lead to cytokine storms and ultimately death [286]. Strategies to manage potential cytokine storms related to CAR-T therapy may provide enhanced clinical outcomes, however, it could also affect the efficacy of CAR-T. One way to overcome this is to develop

CAR-T with multiple target antigens (Fig 5.B). Tandem CAR (Tan-CAR) and Bispecific CAR (BiCAR) are bivalent CAR-T that co-express two CARs to target multiple antigens. In murine U373 GBM models, BiCAR T cells overcame antigen escape, enhanced anti-tumor efficacy compared to unispecific CARs and improved overall survival [287]. This led to BiCAR-T cells currently undergoing phase I trials [288]. However, tumor and interpatient heterogeneity could still impact the effectiveness of this method. Therefore, a trivalent CAR-T cells, known as UCAR-T cells, have been developed to be specific to IL-13Rα2, HER2 and EphA2 as almost 100% of GBM patients have aberrant expression of these antigens [289]. The use of UCAR-T cells have been validated in orthotopic U373 murine models and patient-derived xenograft (PDX) mouse models. The U373 cell line was confirmed to express all 3 antigens. The study demonstrated nearly 100% tumor clearance in nearly all 15 patient derived GBM models. Additionally, CAR-T cell specificity to GBM cells can be further enhanced using synthetic Notch (synNotch) CAR circuits (Fig 5.B) [290]. The synNotch receptor can be programmed to be primed by specific antigens such as EGFRvIII or myelin oligodendrocyte glycoprotein (MOG), a CNS tissue specific antigen. The T cell can be programmed to only express antitumor CARs that target homogenously expressed TAA, once synNotch has been primed. This method ensures that the cytotoxic activity of the CAR-T cells is limited to the desired cells by requiring both synNotch receptors and CARs to bind to their target antigens. The synNotch receptors have successfully been engineered into BiCAR T cells targeting EphA2 and IL13Rα2 to form EGFRvIII synNotch–α-EphA2/IL13Rα2 CAR T cells and have been validated in GBM U87 murine and PDX mouse models. The U87 murine models exhibited selective suppression of intracranial tumors and did not affect tumors in the flank, therefore exhibiting regional selectivity. A phase I trial (NCT06186401) has been initiated to evaluate EGFRvIII synNotch–α-

EphA2/IL13Rα2 CAR T cells in GBM patients. These CAR-T cells lose CAR expression as they migrate away from the snyNotch priming environment thereby decrease the risk of cytokine storms and off-target effects. In order for synNotch CAR-T to be effective in other GBMs, similar complementary antigen combinations will need to be identified.

Despite the limited success of CAR-T therapy in preclinical and clinical models, there are many challenges to this mode of immunotherapy [288]. CAR-T success may be hindered by limited access of the immune cells to the brain due to the BBB or vascular anomalies and by the immunosuppressive TME. Other obstacles include antigen identification and escape after CAR-T therapy. CAR-T therapies can be potential therapeutic options for GBM patients but due to these limitations, it needs further evaluation as a standalone therapy or in combination.

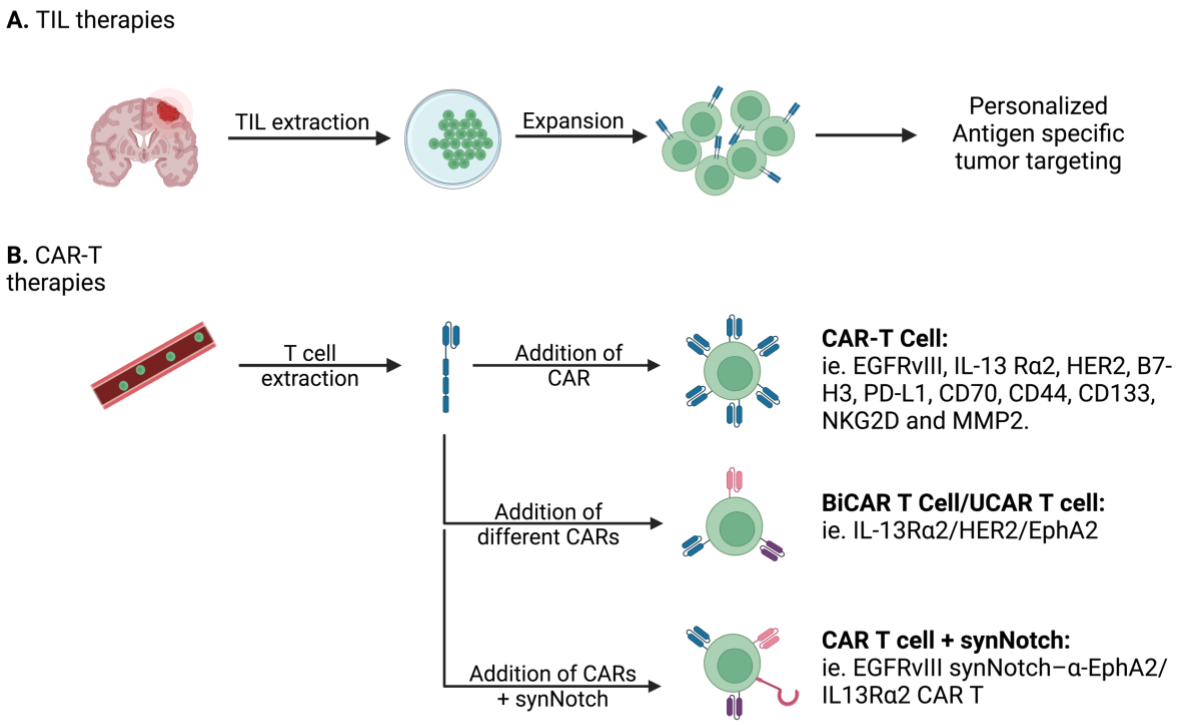

Figure 5: Types of Adoptive T cell therapies for GBM

This figure provides an overview of the types of T cell therapies being evaluated for GBM. A) Schematic of how TIL therapy is produced. TILs are extracted from the tumor, expanded and returned to the host B) T cells extracted from the host can be modified by adding one or multiple CARs and synNotchs.

Cytokine Therapy

As previously reviewed, cytokines released by T cells, endothelial cells, DC, macrophages and cancer cells play an important role in regulating the immune response. They can be classified into pro- or anti- inflammatory cytokines. To date, only IL-2 and IFN-α have been FDA approved for the treatment of certain malignancies.

The SOC regimen for GBM often induces severe and prolonged lymphopenia in the majority of GBM patients [291, 292]. IL-7 therapy has been shown improve the immune response and repair lymphopenia caused by SOC treatments in GL261 and CTA GBM murine models (Fig 6.A) [291, 293]. A recent study evaluated the use of IL-7 therapy alone and in combination to mAb or chemotherapy in 18 rGBM patients (NCT04289155). They reported recovery from lymphopenia induced by chemotherapy in both primary and secondary GBM patients [294]. Additionally, increased lymphocyte counts were maintained regardless of concurrent therapies, including Dex. 2 patients showed partial responses and one had stable disease for more than 2 years. However, due to the small sample size, lager studies will need to be conducted to validate these results and determine if there are any survival benefits. A larger current trial is currently ongoing (NCT03687957) to evaluate IL-7 therapy tolerability and lymphocyte counts in patients treated with chemoradiation.

MDSCs in GBM play a major role in the immunosuppressive TME by inhibiting CTL activation and proliferation via increased arginase-1 expression consequently leading to increased secretion of TGF-B and IL-10. TAMs also add to the immunosuppressive TME by secreting decreased levels of proinflammatory cytokines and inhibiting T cell function. Various immunoregulatory cytokines are or have been investigated in clinical trials for GBM including IFN-α, IL-12, CXCR4, TGF-β, TNF-α and GM-CSF but most have not shown significant

clinical benefits [295]. Many of these cytokines have been targeted using CAR-T and small molecule inhibitors. This section will focus on the direct use cytokines in GBM management, of which IFN-α, TNF-α and IL-12 have been investigated (Table 2).

IFN-α has been shown to inhibit immunosuppression-related gene expression and tumor angiogenesis in addition to increasing T cell activity and reducing T cell and macrophage exhaustion [296]. Additionally, IFN-α has been shown to decrease MGMT and increase TMZ sensitivity in GBM U251 and SKMG-4 xenograft models [297]. These preclinical results eventually led to a multicenter, randomized phase III clinical trial (NCT01765088), evaluating the SOC (radiation + TMZ) with or without IFN-α in 199 newly diagnosed patients with high-grade gliomas. The median OS for patients in the temozolomide + IFN-α group was 26.7 months compared to 18.8 months in the SOC group [298]. This study also reported an increase in OS in unmethylated MGMT patients in the combined arm, 24.7 months vs 17.4 months in SOC. However, the combination group experienced a higher incidence of seizures and influenza-like symptoms possibly due to the enhanced activation of the immune responses. While the immune regulatory properties of cytokines position them as promising candidates for cancer immunotherapy, their clinical implementation is hindered by undesirable side effects and a short serum half-life [299]. To extend the half-life of IFN-α, a pegylated version was created and evaluated in a phase II trial in combination to TMZ [300]. This study reported similar results to the previously reviewed trial. On the other hand, a phase III with 275 newly diagnosed high grade glioma patients failed to see any OS benefits when combined with Carmustine, another chemotherapy drug [301]. TMZ is a methylating agent and carmustine acts as an alkylating agent. Therefore, the beneficial combinatory effects of IFN-a may be dependent on the type of

chemotherapeutic agent used. Thus, emphasizing the need for drug-interactions to be carefully evaluated.

To minimize undesirable effects, some cytokines can be fused with mAbs (Fig 6.B). L19-TNF is a fusion of the L19 mAb and TNF-α. L19 targets the extra-domain B (EDB) in fibronectin (a marker for tumor vasculature) which has been shown to be expressed in GBM but not normal blood vessels [302, 303]. TNF-α is an inflammatory cytokine that facilitates the maturation of dendritic cells, subsequently promoting T cell stimulation and stimulates a range of pathways within a cell that ultimately lead to apoptosis or necrosis [304]. L19-TNF enables the targeted delivery to the tumor cells thereby potentially minimizing off target effect. When combined with alkylating agents (a class of chemotherapeutics), L19-TNF has been reported to induced complete responses in GBM mouse models [303]. This study also reported eradicated tumors and long-term survival in 80% of the mice. Furthermore, the surviving mice demonstrated increased protective immunity and resistance to tumor rechallenge. The authors demonstrated that the combined treatment altered the TME by downregulating the tumor-suppressive factors and promoting DC maturation and T cell infiltration. However, the effectiveness of the treatment was dependent on the presences of functional T cells. Based on these findings, a phase I/II trial was initiated for rGBM patients evaluating L19-TNF in combination with Lomustine (alkylating agent) (NCT04573192). Reports of the phase I trial indicate the treatment was well tolerated in all six patients and the median PFS was 43.3 weeks compared to the reported 4-12 weeks for Lomustine monotherapy. Recruitment for a larger, randomized trial is currently underway. Additionally, L19-TNF is also being evaluated in a phase I/II trial in combination with chemoradiotherapy in nGBM patients (NCT04443010).

IL-12 is another cytokine that has been recently explored in GBM. This cytokine can activate NK cells and induce IFN-y. It is also associated with improved CAR-T cell efficacy, heightened infiltration of $CD4^+$ T cells, and a reduced frequency of Tregs in the TME [305]. A preclinical study showed that IL-12 can reprogram the TME and support T cell mediated antitumor immunity but is not sufficient as a monotherapy to eradicate tumors in mouse models [305]. However, when combined with CAR-T, a single dose delivered intratumorally, was sufficient to elicit a complete response. IL-12 has also been fused with mAb and evaluated in clinical trials for other malignancies however, there have been no trials in GBM patients.

Undergoing these treatments may result in severe side effects such as capillary leak syndrome and cytokine release syndrome, which have been implicated in fatalities among certain patients. In numerous cases, the cytokine concentration triggers diverse effects, contributing to undesired off-target toxicities. The challenge of cytokine pleiotropy, signifying their capacity to influence various cell types in the immune system and peripheral tissues, further complicates clinical translation due to potential off-target effects [306]. Therefore, these therapies need to be heavily investigated to improve tumor-directed delivery or in combination with other therapies.

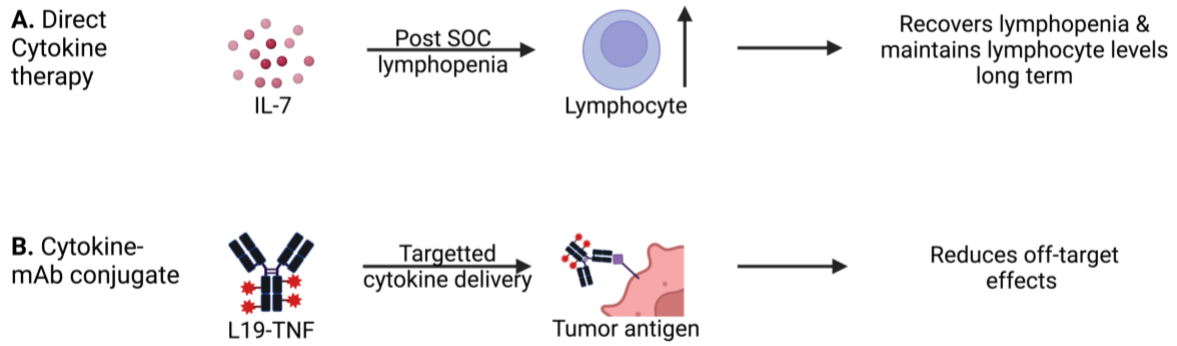

Figure 6: Modes of Cytokine delivery

Cytokines can be delivered in many ways. A) Direct use of cytokines B) Targeted delivery by forming conjugates such as Cytokine-mAb conjugates to reduce off-target effects.

Cytokine	**Function**	**Reference**
IFN-a	Inhibit immunosuppression-related gene expression & tumor angiogenesis. Reduce T cell and macrophage exhaustion. Increase T cell activity. Decrease MGMT expression.	[298]
IL-12	Induce NK cells & IFN-y. Associated with improved CAR-T cell efficacy, heightened infiltration of $CD4^+$ T cells, and a reduced frequency of Tregs in the TME	[305]
TNF-a	Maturation of dendritic cells. Stimulates T cells. Stimulates a pathway that lead to apoptosis or necrosis.	(NCT04443010) (NCT04573192)
IL-7	Reverts SOC-mediated lymphopenia	[294]

Table 2: List of promising Cytokines undergoing evaluation for GBM.

Cancer Vaccines

Therapeutic cancer vaccines are another form of immunotherapy that have gained a lot of traction in recent years. They are designed to program the immune system to elicit immune responses against neoantigens or TAA. Ideally, these antigens should be expressed in all cancer cells and necessary for their survival [307]. There are various types of cancer vaccines including whole cell-based vaccines, nucleic acid-based vaccines, peptide-based vaccines and virus-based vaccines. To date, two cancer vaccines have been approved by the FDA for bladder cancer and prostate cancer.

Whole cell-based vaccines

Whole cell-based vaccines can be classified into tumor cell vaccines and DC vaccines. A recent preclinical study showed vaccination with irradiated GBM cells transfected to produce GIFT-7, a fusokine of IL7 (critical for T cell response) and GM-CSF (influences DCs), resulted in 100% tumor clearance and 50% of long term survivor (LTS) in older GL261 and CT2A mouse models [308]. Furthermore, all of the LTS mice rejected tumor rechallenge. This study also demonstrated GBM cells from human tumor samples could be manipulated in the same way to produce the fusokine. This warrants further preclinical in vitro and in vivo testing before translation into phase I clinical trials.

Dendritic cell vaccines have been heavily investigated in recent years, especially since they were FDA approved for prostate cancer. DC vaccines are produced ex vivo by cultivating patient derived hematopoietic progenitor cells or monocytes and treating with a combination of cytokines to induce DC maturation (Fig 7). Subsequently, these matured DCs are loaded with the chosen tumor antigen. Additionally, antigens can be directly delivered to DCs in vivo by fusing

the antigen with mAbs targeting DC specific receptors. Both methods have shown therapeutic benefits in preclinical and clinical models in a range of cancers [309]. Preclinical GBM models exhibited increased activated T cell infiltration and indicated tumor regrowth prevention [310]. These results led to many global clinical trials evaluating DC vaccines as a monotherapy or in combination for GBM that have been completed or are currently ongoing.

A phase III trial (NCT00045968) was conducted using DCVax-L in combination with TMZ or TMZ with placebo [311]. This trial had a cross-over trial design with 90% all patients in both cohorts had received DCVax-L as it was offered to subjects who relapsed after initial response. OS for nGBM patients was 19.3 months compared to 16.5 external control group (ECG) from another trial. In the placebo cohort of patients who received DCVax-L after initial recurrence, OS was 13.2 months vs 7.8 month in ECG. Furthermore, this trial highlighted the potential benefits of DCVax-L as an adjuvant therapy with tumor-treating fields (TTF) following recurrence. The survival of the DCVax-L + TTF patients ranged from 22.6 to more than 72.7 compared to 8.9 – 29.2 months in only TTF. All of these patients had previously been treated with DCVax-L + TMZ. This trial also reported better OS in MGMT methylated patients thereby suggesting a synergistic effect of combining DCVax-L with TMZ. These results warrant further investigation into the use of DC vaccines as a monotherapy and in combination to other available therapies.

A different DC vaccine, DOC1021, was recently granted FDA fast track designation as the preliminary results from a phase I trial (NCT04552886) were encouraging with increased patient survival and no AEs reported.

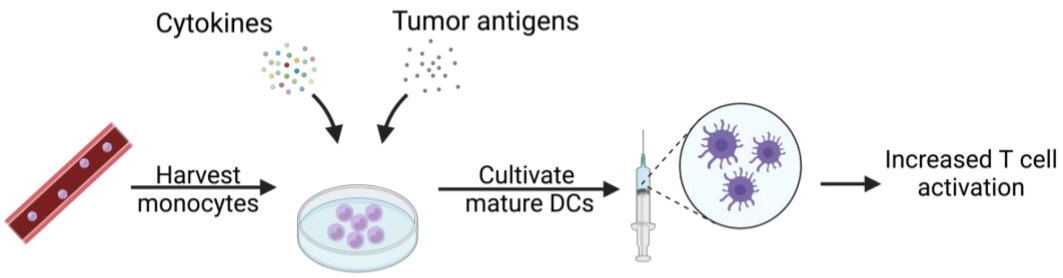

Figure 7: Schematic of DC Vaccination production

DC vaccines require hematopoietic stem cells from the host. The cells are then treated with cytokines and tumor antigens to prime and stimulate maturation. The mature DCs are then given to the patient to increase T cell activation.

Nucleic acid vaccines

Nucleic acid vaccines transport genetic information containing tumor antigens to the host, prompting the synthesis of antigen proteins to elicit immune responses against cancer cells. Initially, DNA vaccines were preferred due to their enhanced stability and prolonged presence in the body compared to mRNA [312]. The DNA needs to enter the cell nucleus for transcription, resulting in a relatively diminished immune response compared to mRNA vaccines, which can directly translate and express antigens in the cytoplasm. However, once within the nucleus, DNA vaccines can generate multiple mRNA copies, increasing antigen production. On the other hand, DNA vaccines entail a potential risk of insertion mutations, while mRNA vaccines carry no such risk of insertion or integration into the genome.

An ongoing phase I study (NCT05698199) is evaluating the use of ITI-1001 in in combination to SOC in nGBM patients. ITI01001 is a DNA vaccine containing plasmids that code for three human cytomegalovirus (HCMV) proteins that are fused to lysosome-associated membrane protein 1 (LAMP1) [313]. HCMV sequences and gene expression in GBM was initially controversial but a symposium in 2011 determined there was sufficient evidence from several institutions to indicate HCMV presence in most, if not all, GBMs [314]. Prior studies have also shown antigen fusion with LAMP1 increases antigen expression by MHC-1 and MHC-II, thereby stimulating CD4 and CD8 T cells [313]. In orthotopic CT2A murine GBM mouse models, ITI1001 was administered intradermally as a preventative vaccine and first dose was administered 24hrs prior to tumor cell implantation followed by three additional doses at fixed intervals. 5 out of 9 mice in the treatment group did not develop tumors [313]. The mice in the treatment group that had tumors also exhibited higher CD4 T cell infiltration and increased CD8 T cells. The results of this study imply ITI1001 may have preventative benefits in GBM settings however, further rigorous testing will need to be completed to determine its validity and potential benefits after tumor formation.

A phase I trial (NCT02718443) evaluated the effect of VXM01 in patients with progressive GBM (pGBM). VXM01 is a bacterial plasmid encoding VEGFR-2 [315]. Preclinical studies using murine analogs of VXM01 have shown anti-angiogenic and anti-tumor activities and preliminary data from this study reported 58% of patients showed detectable T cell response against VEGFR-2 and a correlation of prolonged survival in patients with decreased Intratumoral PD-L1 expression. This initiated another phase I trial (NCT03750071) in pGBM patients with VXM01 in combination with Avelumab (anti-PD-L1). This study reported preliminary data suggesting a few patients obtained PR and SD for more than 12 months [316]. Due to the small

number of patients in these trials and lack of data, larger trials are needed to determine the therapeutic benefit of this DNA vaccine as a monotherapy or in combination with anti-PD1/PD-L1.

Other GBM cancer vaccines are being developed to target the mRNA of the ADAMTSL4, COL6A1, CTSL, CYTH4, EGFLAM, LILRB2, LSP1, MPZL2 and SAA2 genes [317]. These genes modulate the GBM TME and have been associated with enhanced immune cell infiltration and unfavorable survival outcomes. ADAMTSL4 is linked to immune cell infiltration and has been proposed to be an independent biomarker for GBM. COL1A1 in tumor stem cells is crucial for anti-VEGF therapy. CTSL plays a key role in the radiation induced EMT transition of GBM stem cells as well as invasion and metastasis. CYTH4 is associated with MHC molecules and cytokines. EGFLAM is connected to GBM proliferation and metastasis. LILRB2 serves as a prognostic marker for GBM. LSP1 is associated with RT and CT response and is also involved in immunosuppressive cell infiltration and enhances PD1 expression. SAA2 promotes inflammatory diseases mediated by TH17 cells.

The advancement of nucleic acid vaccines offers several advantages: they are not restricted by MHC specificity, are cost-effective, can target both tumor-specific and tumor-associated antigens, and are capable of eliciting a diverse T cell response. Although the concept of these vaccines may seem like attractive options for GBM, not many have reached clinical trials and only a few have reported their results.

Peptide Vaccines

Peptide vaccines utilize identified TAA or neoantigens to elicit an immune response. While neoantigens are specific to the tumor, TAA can be expressed on non-malignant and

malignant cells with a higher expression in the latter. Therefore, personalized neoantigen vaccines have emerges as an attractive therapeutic option.

Rindopepimut, an EGFRvIII-targeting peptide vaccine, showed promising preclinical results against intracerebral tumors [318]. These results led to the eventual phase III randomized, double-blind clinical trial (NCT01480479) involving nGBM patients with confirmed EGFRvIII expression [319]. The participants had undergone maximal resection and standard-of-care radiation therapy with concomitant temozolomide. The trial randomly assigned patients to receive monthly intradermal vaccine injections or a control, alongside adjuvant oral temozolomide. With a total enrollment of 745 patients, 371 underwent vaccine treatment, and 374 received the control. The study was terminated due to futility as there were no difference in median OS between the two groups. Notably, around 57–59% of tumors in both treatment and control arms exhibited a loss of EGFRvIII expression. However, this loss was not linked to vaccine treatment or anti-EGFRvIII antibody titers, indicating a change associated with GBM progression rather than a response to the vaccine treatment. These results could stem from the immunosuppressive effects of radiation and chemotherapy affecting vaccine function, thereby producing similar results in both cohorts.

SurVaxM is another peptide vaccine targeting Survivin that is currently undergoing multiple clinical trials in GBM patients. Survivin is highly expressed in GBM cells and has been related to chemotherapy resistance and recurrence [320]. It is an anti-apoptotic protein that is frequently expressed during fetal development but not in the normal adult brain tissue [321, 322]. Therefore, making it a prime potential target. In GL261 murine models, surviving peptide vaccines significantly increased CTL and THC responses leading to prolonged survival [323]. Therefore, SurVaxM was then evaluated in 9 rGBM patients in a phase I trial (NCT01250470)

that reported a median OS of 86.6 weeks with 7 patients surviving longer than 12 months [324]. These promising results lead to one completed (NCT02455557) and two ongoing (NCT05163080, NCT04013672) phase II trials evaluating SurVaxM in combination with other therapies. The completed trial administered SurVaxM in combination with TMZ and GM-CSF in 64 patients [325]. They reported median PFS and OS at 11.4 months and 25.9 months from first dose of SurVaxM respectively. Based on these findings, the FDA has granted fast track designation to SurVaxM for nGBM patients.

While many vaccines have been tested in preclinical and clinical models, only some have shown encouraging clinical benefit. The biggest challenges in developing vaccines for GBM include the heterogeneity and downregulation of antigens. However, with further optimization, in either vaccine construction or by combining with other therapies, cancer vaccines could still emerge as a potential therapeutic option for GBM patients.

Oncolytic viruses

Oncolytic viruses (OVs) are another form of Immunotherapy that have been explored to treat GBM. These viruses are designed to selectively replicate in tumors to trigger apoptosis and spread to other oncogenic cells within the tumor without damaging normal cells (Fig 8) [326]. OVs are also capable of enhancing the innate and adaptive immune responses via the released PRR, PAMPs and cytokines as well as increasing the level of TAA within the host [327]. This shifts the immunologically cold nature of tumors to an immunologically hot pathological state. Additionally, non-replicating viruses can be used for efficient targeted delivery of therapeutic agents to the tumor cells [328]. T-VEC, a herpes simplex virus (HSV) OV has been approved for the treatment of metastatic melanoma.

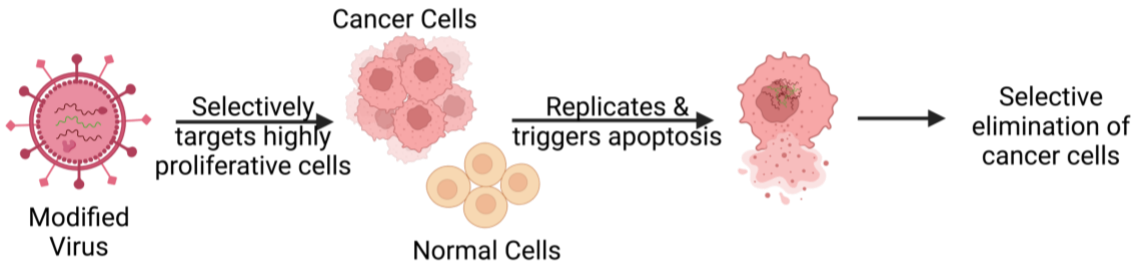

Figure 8: Selecting targeting of Oncolytic Viruses

This figure provides the functional schematics of oncolytic viruses. OVs are modified to preferentially target rapidly proliferating cells. The virus then invades the cell, replicates and triggers cell death.

The HSV type 1 virus has been recently investigated for the treatment of GBM. It is particularly well-suited for therapy due to several factors: (1) its ability to infect a wide range of cell types, making it applicable to various cancer types; (2) a relatively low multiplicity of infection required for comprehensive cell elimination, ensuring high efficacy in clinical settings; (3) the availability of anti-viral drugs, allowing therapy termination if needed; (4) its large genome, enabling the insertion of large and/or multiple transgenes; and (5) the fact that circulating anti-HSV-1 antibodies do not impede the virus's cell-to-cell spread, ensuring efficacy in seropositive patients and permitting repeated dosing without diminishing effectiveness [329]. An HSV virus, T-VEC has been approved by the FDA for treatment of a subset of melanoma patients.

A genetically engineered HSV-1 virus, G207, has deletions in the γ34.5 neurovirulence gene and a disabling lacZ insertion in the UL39 gene, which encodes the large subunit of the viral ribonucleotide reductase [330]. This results in the virus losing its ability to replicate in non-dividing cells and exhibiting a preference for the highly proliferative oncogenic cells, therefore remaining non-pathogenic to normal brain cells [331]. The oncolytic effect of G207 was demonstrated in U87MG and neuroblastoma models. Additionally, there was an increase in cytotoxic T lymphocyte activity against the tumor cells. Two phase I trials (NG1-001, NCT00028158) evaluating Intratumoral inoculations in previously treated GBM patients determined the treatment was well tolerated and no patients developed HSV encephalitis [330, 332]. G207, further evaluated in combination with RT in another phase I trial (NCT00157703) reported partial responses and increased survival in some patients [333].

These trials lead to the development of G47Δ, which introduced a deletion within the α47 gene in G207. The α47 gene downregulates antigen presentation by MHC-I expression in the infected cells and allows the virus to escape immune surveillance [334]. G47Δ showed enhanced tumor targeting and cytocidal activity in U87MG xenograft and Neuro2a syngeneic mouse models compared to G207 [335]. Moreover, it has been demonstrated that G47Δ can effectively eliminate cancer stem-like cells obtained from glioblastoma patients and suppress their ability to self-renew [336-338]. A phase I/II study, G47Δ was administered twice by stereotactic injections into 13 patients with progressive or rGBM [329]. This study reported 3 patients survived over 2 years including one that survived with SD for 11yrs (as of March 2022). None of the long-term survivors had IDH mutations however, the 11yr survival patient had higher peripheral CD4/CD8 than all other patients. This could have contributed to his long-term survival. Another phase II study in 19 GBM patients after TMZ+RT with six Intratumoral doses of G47Δ over a longer

period reported 1 PR and 18 SD at 2 years [339]. This suggests a longer regimen of this intervention could lead to better outcomes and warrants further investigation into different regimens and possible combination therapies. These results led to the approval of G47Δ to treat GBMs in Japan.

M032 is the next generation of G47Δ in which it simultaneously stimulates the tumor cells to secrete IL-12 while exerting cytotoxic effects. In immunocompetent mouse 005 GSC model, G47Δ-mIL12 not only targeted GBM cells but also upregulated IFNγ release, inhibited angiogenesis, and reduced Treg presence within the tumor [340]. The synergistic antitumor effect of G47Δ-mIL12 with anti-PD-1 and anti-CTLA-4 antibodies was dependent on macrophages and $CD4^+$ and $CD8^+$ T cells [341, 342]. Intracerebral administration of M032 was determined to be safe in canine models [343]. Preliminary results from a phase I study (NCT02062827) of M032 used in 21 patients with rGBM report a median post survival time of 9 months with only 2 patients still alive at 1 year [344]. Phase I study (NCT05084430) of M032 with pembrolizumab in rGBM patients is currently ongoing.

Over 30 distinct viral strains have been or are currently being evaluated in preclinical and clinical trials for their effectiveness against GBM (Table 3)[327]. These viruses include Newcastle disease virus, Parvovirus, HSV, adenovirus, measles virus, reovirus, poliovirus, measles, vaccinia and zika virus. One of the limitations of this form of therapy is that effective oncolytic virotherapy response relies on the collaboration between the innate and adaptive immune systems. Therefore, if patients have lymphopenia induced by Dex, OVs may not be efficient as a monotherapy. However, if combined with other immunotherapies such as T cell therapies, the long term and synergistic effects may lead to better outcomes.

Oncolytic Virus	Base Virus	Reference
G207	HSV	[333] (NCT04482933)
*G47Δ	HSV	[329, 339]
M032	HSV	[344]
C134	HSV	(NCT03657576)
DNX-2401	Adenovirus	(NCT02197169) (NCT01956734) (NCT02798406)
ParvOryx	Parvovirus	(NCT01301430)
NSC-CRAd-Survivin-pk7	Adenovirus	(NCT03072134)
**CAN-3110	HSV	(NCT03152318)
Reolysin	Reovirus	(NCT00528684)

Table 3: Oncolytic viruses for GBM currently under evaluation in clinical trials.

*Has received FDA approval in Japan. **Has received FDA fast track designation

One of the major challenges with the evaluation of immunotherapies in these clinical trials is that they were always in combination with either TMZ or RT or both. Therefore, it is difficult to determine whether the lack of therapeutic benefit is due to immunotherapy failure or due to immune suppression by conventional therapies as described earlier. The few trials that evaluated immunotherapies as neoadjuvant therapies observed better outcomes. Therefore,

implementing immunotherapies as a neoadjuvant approach may yield better results and warrants further investigation. Additionally, GBMs evolve and gain evasive/resistance mechanism therefore utilizing varied modes of immunotherapies at different time points to overcome these mechanisms may yield better results.

The rationale for combination therapies with SOC

The SOC treatment protocol consists of surgery followed by TMZ and RT. However, the highly invasive and heterogenous characteristics of GBM, inevitably lead to recurrence and none of the existing treatments have been able to effectively prolong survival upon recurrence.

Patients with hot tumors are prime candidates for immunotherapy however, patients with cold tumors could experience enhanced outcomes from combination therapies due to their synergistic effects. Studies have also shown that direct treatments such as chemotherapy and RT can modulate the immune response to cancer. Both induce cell death of cancer cells and stimulate release of danger signals such as DAMPs or PAMPs but can also have off-target effects on immune cells and other cells therefore dosage is a crucial factor to consider when combining therapies.

Although RT can promote a more immunosuppressive response, due to its lethality on immune cells and triggering the increase of immunosuppressive cytokines and immune cells such as Tregs and MDSCs [345-348], it has also been reported to have systemic "abscopal effects" in which radiation at one site regresses tumors at non-irradiated and remote sites [349-351]. This suggests that brain metastases may benefit from RT at the primary tumor location. RT can amplify antitumor responses through augmenting TA presentation and release [352, 353], promoting the priming and activation of immune cells [354, 355], enhancing T cell recognition

of TA [356, 357], and increasing concentrations of TILs [358, 359]. Additionally, RT can induce the release of proinflammatory cytokines and other inflammatory signals [360-362]. These inflammatory signals along with the immunological, stromal and vascular changes alter the TME, thereby adding to the antitumor response [363, 364]. Reprogramming the TME has been shown to shift "cold tumors" into "hot tumors" [365, 366]. Therefore, making RT an attractive candidate to use in combination with immunotherapies [367]. However, the optimal dose of RT to stimulate immune responses may vary between patients and cancer types and warrants further investigation.

High doses of chemotherapy suppress the immune system however, low doses of chemotherapy have been reported to stimulate the oncogenic immune response [368, 369]. Chemotherapy can stimulate inflammatory responses via similar mechanisms to RT. Cell death induced by chemotherapy increases the release of TA, thereby increasing priming and activation of immune cells [370]. In addition, some chemotherapies can stimulate DCs [371], CTLs [372] and M1-like TAMs [373]. Furthermore, chemotherapies can deplete pro-tumorigenic immune cells, such as MDSCs [374, 375], M2-like TAMs [376, 377] and Tregs, by creating a proinflammatory environments [378]. The selection of chemotherapeutic agents used in combination therapies is crucial as they can have varied target functions.

Combining immunotherapy with RT or chemotherapy can be beneficial and lead to enhanced cancer regression as the RT and chemotherapy can eliminate the tumorigenic cells while increasing immune response and the immunotherapies can provide a prolonged immune response. There are several ongoing trials combining immunotherapies with RT or chemotherapy.

Modeling GBM and the TME:

Modeling GBM tumors and accurately reflecting the immunosuppressive TME is a challenge in understanding of the tumor characteristics and the development of new targeted therapies. Cell lines, mouse models and organoids have all been utilized for research and developmental purposes. While no model perfectly mimics human GBMs, each possess distinctive characteristics that should be taken into account when planning experiments or analyzing preclinical findings. Additionally, the significant number of preclinical treatments that have proven unsuccessful in human trials underscores the limitations of existing models and the significance of choosing the right preclinical model [379].

GL2261 and CT2A are some commonly used murine GBM cell lines [380]. GL261 is relatively immunogenic characterized by elevated MHC I expression and increased neoepitopes, resulting in improved responsiveness to immunotherapies. However, clinical trials of PD-1 immunotherapy did not yield the expected response. On the other hand, CT2A, is more aggressive than GL261 but is not as invasive as human tumors. Patient derived and murine cell lines are susceptible to mutations leading to genotypic and phenotypic alternations thereby differing from in vivo tumor cells. Additionally, simulating the TME in vitro, using coculturing and other techniques, is difficult and not fully representative of in vivo conditions. Furthermore, drugs that show benefit in cell culture, may not translate in vivo due to physical barriers, such as the BBB.

In vivo GBM tumors are routinely investigated using mouse models. There are three main types of mouse models: syngeneic models, genetically engineered mouse models (GEMMs) and xenograft models, each with their own advantages and disadvantages [381]. Syngeneic mouse models involve murine GBM cell lines that are transplanted back into mice

with similar genetics. While this model enables treatments, such as ICBs, to be investigated in intact immune systems, they may not accurately reflect the GBM TME observed in humans. GEMMs are typically used to study the effects of genetic mutations, such as EGFRvIII, in GBM tumorigenesis but can also be used to evaluate therapies, especially those targeting specific alterations. These models typically lack the heterogeneity of GBM tumors seen in humans. Xenograft models use human GBM cells to model the tumor in mice. This can be achieved using human cell lines or patients derived xenografts (PDX). Since this method is typically performed in immunodeficient mice, it is difficult to replicate the TME and some have failed to replicate the primary tumor features. To overcome these challenges, humanized mouse models have been developed. These mice are created by ablating the host's immune system and engrafting human immune cell progenitors. A recent study demonstrated humanized mouse models implanted with patient derived GBM cell lines can exhibit similar histopathological characteristics, TME and response to anti-PD1 therapy to mimic human immune responses [382].

3D organoids can also be created from patient tumor samples. GBM organoids are small, viable, spheroidal structures developed from resected tumor tissues [383]. These tumoroids are able to maintain tumor heterogeneity and simulate the TME. Patient derived tumoroids/organoids (PDOs) have facilitated GBM subtyping and the understanding of drug interactions within the TME [384]. Organoids have also been used to identify new or personalized therapeutic approaches for GBMs [385, 386]. Although GBM PDO xenografts (PDOX) have been successful in immunodeficient mice [387], there are no reports of GBM PDOX in humanized mice. This could be attributed to the time consumption and high costs associated with humanized mice.

Together, these models present a promising approach to preliminary drug screening and for personalized therapeutic strategies. Utilizing these models in preclinical trials could lead to enhanced patient selection for human trials. Furthermore, various immunotherapies can be assessed as monotherapies or combination therapies for each patient, thereby leading to better clinical outcomes.

CONCLUSION AND FUTURE DIRECTIONS

Glioblastoma are the deadliest brain tumor with a very low survival rate at 5 years. Despite the advancements in therapeutic interventions, GBM still have a poor prognosis. Although factors such as age, IDH mutation and MGMT methylation do improve clinical outcomes, the survival of these patients are still low. This warrants the search for new therapeutic strategies. In recent years, Immunotherapies have emerged as an attracted approach due to their success and FDA approval in certain hematologic and solid tumors.

Immunotherapies have been researched and in development for over 100 years. The key events and immune theories that led to the development of cancer immunotherapies have been discussed. Immunotherapies harness the highly intricate immune system consisting of many versatile factors. Additionally, the innate and adaptive immune responses must coordinate strategically to protect the host by preventing autoimmune disorders and eradicating diseases. Here, we have attempted to summarize the roles of the key cells in the innate and adaptive immune systems and how they collaborate to eliminate cancer.

Despite the collaborative efforts of the immune system, malignancies can escape and adopt immunosuppressive/immune evasion mechanisms. GBMs are notoriously difficult to treat due to a variety of factors. GBMs are located in an immune privileged organ with physical barriers, such as the BBB, that make immune cell and drug infiltration difficult. Therefore, the tumors are typically immune cold with low immune cell infiltrates. The immunosuppressive nature of the GBM TME also adds to the immunologically cold nature of the tumors. A hypoxic core with a highly migratory pseudo palisade layer and abnormal vasculature are key

characteristics of these tumors. The various pro-tumorigenic mechanism of GBM, the TME and current therapy options have been discussed.

There are many types of immunotherapies being investigated for GBM in preclinical and phase I – III studies. These include antibody therapies, immune checkpoint blockade, adoptive T cell therapies, cytokine therapies, oncolytic viruses and cancer vaccines. While there have been numerous trials, only one has been FDA approved and a handful have received fast track designation. Bevacizumab is a mAb that targets VEGF and attempts to normalize the GBM TME. There are many trials combining this mAb or other therapies with immunotherapies. Here we have attempted to summarize the various types of immune therapies with an emphasis on those being evaluated in clinical trials, while highlighting their advantages and limitations.

Some of these therapies have shown clinical benefits while others have resulted in undesired effects. In most cases, a combinatory approach has yielded better results than monotherapies. Additionally, immunotherapies as a neoadjuvant treatment are being evaluated due to the potential immunosuppressive nature of SOC treatments. Immunotherapies also have their own set of challenges such as inflammation and cytokine storms. The dual nature of the therapies emphasizes the importance of selecting the appropriate combinatory strategies.

All of the therapeutic options reviewed need further investigation to assess their potential benefits in the fight against GBM. The translation from pre-clinical to clinical trials can be hindered by several factors, ultimately leading to a lack of therapeutic benefit. The different preclinical models have been presented with their advantages and limitations. Additionally, a way to circumvent the challenges by using humanized mice and PDO have been briefly described. To optimize individual therapeutic responses, PDOs can be engrafted into humanized

mice to assess the cumulative effects of the mono- or combination therapies on GBM, the TME and the immune system. The limitations of this method have also been discussed.

A potential future direction could include non-personalized immunotherapies such as ICB, mAb, cytokine and oncolytic viruses administered as a neoadjuvant therapy. A benefit of administering oncolytic viruses, over other options, as a neoadjuvant therapy may result in oncogenic cell death could further stimulate the innate and adaptive immune responses. OVs in combination to therapies targeting the immunosuppressive cells in the TME, such as αGITR, could lead to further enhanced immune responses and could allow for surgical excision of previously inoperable tumors. Surgery followed by an alternative, less immunosuppressive corticosteroid than Dexamethasone may also improve immunotherapeutic outcomes. If there are no alternatives, IL-7 could be used post Dex treatment to reverse lymphopenia. This may also protect the host from lymphopenia caused by subsequent chemo- and radiation therapy. Personalized therapies developed from tumor biopsies can be administered post-surgery. Additionally, BiCAR-T with synNotchs and αGITR or IL-2 can be administered following drug interaction evaluations in PDO or PDOX humanized mice. For inoperable tumors, UCAR-T cells followed by oncolytic viruses could provide better therapeutic outcomes. The UCAR-T cells would target the cells expressing the complementary antigens however, since this method could lead to decreased antigen presentation, the OVs could eliminate the remaining highly proliferative cells with decreased antigen presentation. The constantly evolving nature of the GBM tumors require a multifaceted combinatory therapeutic approach.

Comprehending the underlying foundations, immune dynamics, available treatment modalities, and translational immunotherapies is crucial for discerning the factors influencing the disappointing treatment outcomes and observed toxicities in clinical trials. A profound grasp of

these concepts is pivotal for devising strategies to surmount challenges and enhance survival rates among GBM patients, leading to effective disease resolution.

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