Review

Gamma-delta T cells in glioblastoma immunotherapy

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Abstract

Conventional immunotherapy in the treatment of glioblastoma (GBM) has essentially produced no significant advantage over the use of chemotherapeutic drugs. A strongly immunosuppressive tumor microenvironment and lack of antigen-presenting major histocompatibility expression on tumor cells have made GBM a poor immunological target. Molecular heterogeneity of GBMs, both within the tumor and across patients, results in the immunological escape of tumors that do not express target antigens. Therefore, the development of nonconventional immunotherapy for GBM is continuously being sought. γδ T cells are a minor subset of the human T-cell repertoire with unique antitumor properties that have been shown to be functionally superior to conventional αβ T-cell receptor expressing T cell-based immunotherapy for cancer, including GBM. Unlike, the more abundant αβ T cells, γδ T cells do not require major histocompatibility proteins for activation. In addition to the γδ T-cell receptor, these cells express a plethora of other antigenic receptors that recognize external stimuli, as well as several self-peptides, which make these cells a strong candidate for the development of cancer immunotherapeutics. A higher threshold of activation-induced cell death and resistance to inducing graft-versus-host disease are also characteristics of these T cells. In this review, we discuss the biology and immunological characteristics of γδ T cells and review current research using γδ T cells in GBM immunotherapy to explore whether these cells can be the potential next-gen immunotherapeutic candidate for this dreadful disease.

Keywords: Cancers, glioblastoma, immunotherapy, T cells, tumors, Vγ9 Vδ2 T cells, γδ T cells

Introduction

Glioblastoma (GBM) is the most aggressive form of malignant brain cancer. Despite continuous advances in the development of therapies, prognosis remains dismal.[1] Extensive recent sequencing studies of GBM have uncovered novel immunological targets and provided rationale for immunotherapy to be developed along with surgery, radiation, and chemotherapy as the fourth arm of GBM treatment. Due to extensive molecular heterogeneity[2] in GBMs and severe immunosuppressive conditions in the tumor,[3] classical immunotherapeutic approaches for the treatment of GBM have been less than promising. Therefore, new approaches to immunological management of GBM are continuously being tested. Recent developments in understanding the role of γδ T cells in immuno-oncology have kindled interest in these T cells for GBM immunotherapy.

γδ T cells are a group of "unconventional" T cells with distinct γ and δ chain T-cell receptors (TCRs) on their surface, as opposed to classical CD4+ and CD8+ T cells that express α and β TCRs.[4] γδ T cells make up no more than 10% of T-cell repertoire in peripheral blood,[5-7] but are enriched in epithelial and mucosal tissues such as skin, gut, and liver where they are thought to serve as the first line of defense against immunogenic challenge.[8] In addition to these unique TCRs, γδ T cells also express natural killer (NK) group 2D (NKG2D), an NK receptor,[9] and a variety of natural cytotoxicity receptors such as NKp30 and NKp44.[10] Functionally, this subgroup of T cells plays a unique role in immunological responses as they function at the interface of innate and acquired immune responses.[11,12] They can induce cytotoxic activity by production of cytokines (interferon-γ [IFN-γ], interleukin [IL]-17, tumor necrosis factor-α [TNF-α]) and cytolytic enzymes (perforin and granzymes)[13-15]; or through the activation of innate immune responses by interacting with epithelial cells, monocytes,[16] dendritic cells (DCs),[17] B cells[18] and also by priming $CD4^+$ and CD8+ T cells.[19] In cancer, γδ T cells have been reported to

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play a dichotomous role. Depending on how the naïve γδ T cells are activated, these cells can exhibit either pro-tumor or anti-tumor activity.[8,20] There are abundant reports of γδ T cells that secrete IL-17, express forkhead box P3 (FoxP3) and recruit immunosuppressive myeloid-derived suppressor cells (MDSCs) in tumors,[21,22] the majority of studies have confirmed significant antitumor effects of γδ T cells. These T cells can recognize cancer stress-related proteins such as Major histocompatibility complex (MHC) class-I chain-related proteins (MIC-A/B) and human cytomegalovirus (CMV) membrane glycoprotein-binding proteins (ULBPs) by TCRs and NKG2D in an MHC independent fashion,[23,24] leading to abundant cytokine secretion and sufficient cytotoxic responses. These characteristics make γδ T cells an attractive candidate for cancer immunotherapy.[25] Moreover, resistance to activation-induced T-cell death (AICD) makes γδ T cells preferable to conventional T cells for sustained antitumor responses.[26] Recent developments in methods for robust expansion of γδ T cells[7] and discovery of activation with phosphoantigens and T-cell cytokines such as IL-2 and IL-15[27] has made it easier to produce sufficient cells for cancer immunotherapy. In the majority of clinical trials conducted in several cancers, such as renal cell carcinoma, malignant leukemia, and advanced lung cancer, γδ T cells have been shown to be well tolerated and safe.[8]

Although circulating γδ T cells were purified and expanded from the blood of GBM patients as early as 1997,[28] immunotherapeutic potential of these cells was reported only in 2009.[29] Since then, some exciting research has been performed to identify the potential of these cells in GBM immunotherapy. In this review, we will discuss the biology of γδ T cells, its role in cancer immunotherapy and state of the research on the use of these cells in GBM immunotherapy.

Biology of γδ T Cells

Background

γδ T cells are a select subgroup of T cells that are defined by the expression of heterodimeric TCRs composed of γ and δ chains. They represent <10% of peripheral blood immune cells but are present in significantly higher proportions in epithelial and mucosal tissues. There are two major subsets of γδ T cells that are identified by their Vδ chain. Vδ1 T cells are predominant in the normal human epithelia, skin, liver, and spleen, while Vδ2 T cells are mostly present in the blood.[30] Two other lesser known subsets include Vδ3 and Vδ5.[8] These δ chain TCRs form combinational heterodimers with several subsets of γ chain to form γδ T cells.[31] Majority of γδ T cells in human peripheral blood express Vγ9 and Vδ2 TCRs and are commonly referred to as Vγ9 Vδ2 T cells[32] [Table 1]. Vγ9 Vδ2 T cells can inhibit cancer cell proliferation, angiogenesis, lymphangiogenesis, and also increase cancer cell apoptosis.[8,33] Vδ1 T cells are associated with distinct innate recognition and regulatory properties, possess powerful tumoricidal activity, and do not preferentially pair with any specific Vγ chain.[26] Vδ1 T cells show reduced susceptibility to AICD, and tumor-reactive T cells have been shown to persist in the circulation.[34] Vδ1 T cells can also exhibit immunosuppressive and regulatory properties.

Table 1: Subsets of γδ T-cells

Structure subset	Paired Vγ gene	Distribution
Vδ1	*Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9*	Peripheral blood, skin, gastrointestinal tract, spleen, liver
Vδ2	*Vγ9*	Peripheral blood
Vδ3	*Vγ2, Vγ3*	Peripheral blood, liver
Vδ	*Vγ4*	Peripheral blood

Adapted from Zhao *et al*.[8]

These T cells recognize phosphoantigens, in a γδ TCR-dependent but MHC-independent manner.[20] Most potent phosphoantigens are (E)-4-hydroxy-3-methyl-butenyl pyrophosphate generated by bacteria and parasites. Vγ9 Vδ2 T cells can also be activated by metabolic intermediates of the mevalonate pathway (such as isopentenyl pyrophosphate [IPP]) that accumulate in stressed or transformed vertebrate cells.[35,36] Due to metabolic dysregulation, IPP is often accumulated by cancer cells. Vδ1 T cells cannot be activated by IPP but recognize markers of cellular stress, resulting from infection (ULBPs), or tumorigenesis (MIC-A/B).[23,24] Stress surveillance performed by γδ T cells is thought to depend not only on TCRs but also on costimulatory signals from NK receptors, especial NKG2D.[37] Vδ1+ T cells, but not Vγ9 Vδ2 T cells, notably enhance expression of natural cytotoxicity receptors, especially NKp30, NKp44, and NKp46 on following *in vitro* stimulation with strong TCR agonists and cytokines.[10] CD94/NKG2A and CD94/NKG2C expressed on γδ T cells can be activated by nonclassical MHC class I molecule human leukocyte antigen (HLA)-E, their only known ligand. HLA-E is massively overexpressed in GBMs.[38] Finally, γδ T cells also recognize lipid antigens presented by CD1d.[39] Significant expansion of pure cultures of γδ T cells can be achieved by incubation with IL-2 and commercially available bisphosphonates such as zoledronate or pamidronate. Bisphosphonates act through induced accumulation of IPP in the target cells, which are in turn recognized by the γδ T cells. These γδ T cells that are activated by the effect of bisphosphonates on the target cells are cytotoxic against a variety of tumors.[27,40]

Plasticity of γδ T-cell function

Human γδ thymocytes are functionally immature, but depending on the stimuli these cells have the plasticity to differentiate into either pro-inflammatory or immunosuppressive phenotypes upon stimulation by distinct cytokines[41] which have profound effects on the role of γδ T cells in cancer immunotherapy.

Protumor roles of γδ T cells

Tumor microenvironment (TME) plays an important role in the protumor polarization of γδ T cells.[42] TME can disable antitumor components of the immune system through recruitment of immunosuppressive cells or secretion of immunosuppressive factors.

Peripheral γδ T cells can polarize after exposure to IL-1 β, IL-6, IL-23, and transforming growth factor-β (TGF-β) in the TME, toward IL17+ IFNγ− T17 γδ T cells, which play an immunosuppressive role in cancer and promotes cancer progression.[43] γδ T cells can transform into FOXP3$^+$ γδ T regulatory cells (γδ Tregs) under immunosuppressive stimulation.[22] These γδ Tregs function similarly as CD4$^+$CD25$^+$FoxP3$^+$ αβ T regulatory cells and suppress the proliferation of activated peripheral blood mononuclear cells.[44] γδ Tregs have been shown to suppress DC maturation in cancer vaccination experiments.[45] Vδ1 γδ T cells have more regulatory potential than αβ T regulatory cells.[46] High levels of TGF-β have been reported to be secreted by Vδ1 T cells.[47] Depending on IL-17 production, Vδ1 T cells are involved in inflammation-induced cancer progression.[48] In the presence of IL-4, Vδ1 T cells secrete significantly higher IL-10, and express lower NKG2D, compared with Vδ2 T cells.[49]

γδ Tregs cells can inhibit DC maturation and their antigen-presenting cell functions and induce DC senescence, thus impairing naïve αβ T-cell activation and differentiation into effector T cells.[50] It has also been discovered that tumor-derived γδ T regs can suppress naïve and effector T cells by inducing cell cycle arrest of responder T cells.[8]

T17 γδ T cells also promote tumor progression by IL-17-mediated recruitment of immunosuppressive MDSCs in TME[51] leading to MDSC-mediated CD8+ T-cell exhaustion,[52] and angiogenesis.[21,43] MDSCs also suppress antitumor functions of Vδ2 T cells.[53] Intratumoral exosomes, under hypoxic conditions, induce HSP-70 dependent enhancement of immunosuppressive effects of MDSC on γδ T cells.[54] Coculture of IL-3 and CpG-activated plasmacytoid DCs with Vγ9 Vδ2 T cells induces immunosuppressive polarization of the T cells.[55]

Neutrophils have been shown to synergize with T17 γδ T cells to create an immunosuppressive TME. T17 γδ T cells can stimulate the expansion and polarization of tumor-induced neutrophils, which acquire the ability to suppress CD8$^+$ T lymphocytes facilitating tumor metastases, and also suppress peripheral Vγ9 Vδ2 T-cell function.[56,57]

Cancer-associated fibroblasts, which are major components of TME, are also known to induce pro-tumoral polarization of γδ T cells.[58]

Antitumor functions of γδ T cells

Vγ9 Vδ2 T cells can secrete cytolytic enzymes perforin and granzyme that can directly lyse cancer cells.[59,60] T1/T17 γδ T cells, produced from stimulation of naïve γδ T cells with stimulatory cytokines, can also eliminate cancer cells through the ligands TRAIL and FasL.[61-63] In addition, γδ T cells kill cancer cells directly through CD16-mediated antibody-dependent cellular cytotoxicity of malignant B cells. CD16 can also be up-regulated on γδ T cells, depending on the precise biological situation.[64] Cytotoxic type 1 (T1 γδ) T cells can be generated from naïve T cells upon stimulation through TCR and NKG2D ligands along with IL-12 and IL-15. T1 γδ T cells can also enhance antitumor immunity by secreting IFN-γ and TNF-α.[65-67]

In a recent review, Siegers and Lamb discussed in detail the antitumor effects of Vδ1 T cells.[26] Circulating Vδ1 T cells have been shown to target leukemia and Non-Hodgkin's lymphoma through the expression of ULBPs on the tumor cells.[68] Circulating Vδ1 T cells have been also shown to be cytotoxic to neuroblastoma and epithelial colon tumor cells.[69,70] Polyclonal Vδ1 T cells derived from melanoma with an effector phenotype that secrete TNF-α and IFN-γ have been shown to kill melanoma cell lines.[71]

The stimulation of Vγ9 Vδ2 T cells with TCR ligands and IL-21 polarizes the cells toward IL-4, IL-10 and CXCL13 expressing follicular B-helper γδ T cells (γδ T_{fh} cells) that helps B cells to boost antibody *in vitro*.[72] γδ T cells can also perform the role of antigen-presenting cells for priming αβ T cells. Levels of CD69, HLA-DR, and T-cell costimulatory molecules are increased in stimulated γδ T cells.[19] High levels of CD36, a scavenger receptor usually present on macrophages, helps in the uptake of apoptotic tumor cells by γδ T cells and through their antigen-presenting cell function induce a cancer antigen-specific CD8$^+$ T-cell response.[73] γδ T cells can also trigger DC maturation.[17] Matured DCs can induce the activation and proliferation of γδ T cells, exhibiting that both DC and γδ T cells can either act on their own or interact synergistically to remove cancer cells.[8] γδ T cells can also induce anti-cancer immunity through NK cell-mediated cytotoxicity by engagement of CD137 on NK cells and enhance NK cell cytotoxicity to NK-resistant cancers[74] [Figure 1].

γδ T Cells in Glioblastoma Immunotherapy

Immunotherapeutic approaches to manage GBM have shown a mixed response, generally disappointing. The unique immunological landscape of the brain coupled with the immunosuppressive microenvironment of GBM presents a substantial challenge to effective immunotherapy.[3,75] Immunosuppressive cytokines such as TGF-β and IL-10,[76,77] overexpression of indoleamine 2,3-dioxygenase,[78] signal transducer and activator of transcription 3,[79] and programmed death-ligand 1 on tumor cells and tumor-infiltrating lymphocytes, infiltration of MDSCs[80,81] and other factors limit immunological responses by preventing DC maturation, antigen presentation, and cytotoxic T lymphocyte cell activation. Moreover, inherent antigenic heterogeneity in GBM, both between patients and within individual tumors, has made it difficult for adoptive T-cell strategies, especially chimeric antigen receptor T-cell (CAR-T) therapy, which otherwise has been shown to be useful in hematological tumor management, to replicate its success against GBM.[82] Therefore, any immunotherapeutic approach to GBM management must overcome these challenges.

γδ T cell-based immunotherapy may be perfectly positioned to fit into the void left by traditional GBM immunotherapy.

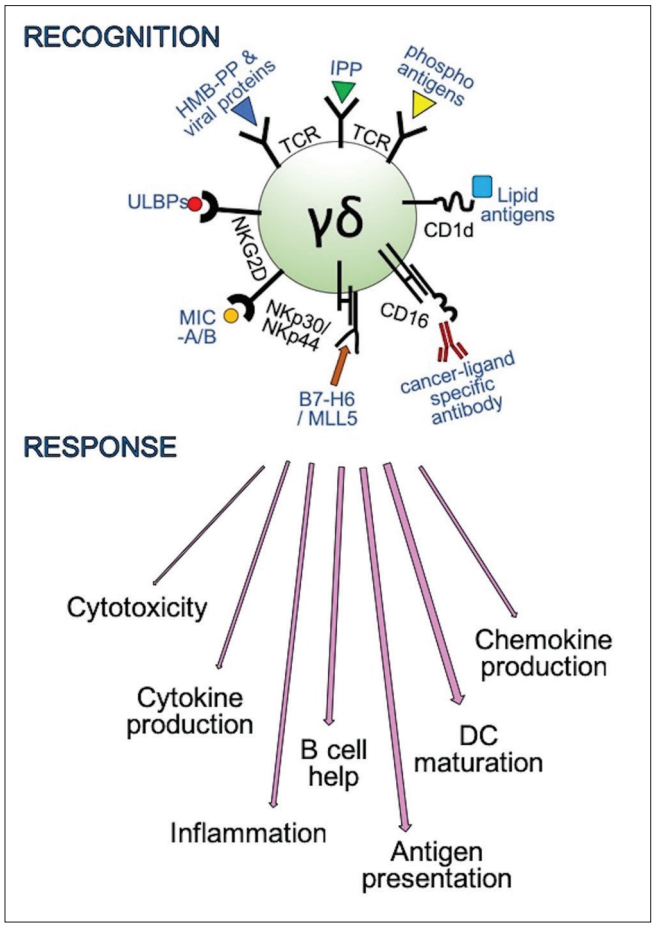

Figure 1: Receptor-ligand recognition and antitumor response of γδ T cells. γδ T cells express a variety of receptors recognizing a wide-range of ligands inducing a wide-range of antitumor immune response. HMB-PP: (E)-4-hydroxy-3-methyl-butenyl pyrophosphate, IPP: Isopentenyl pyrophosphate, ULBP: Glycoprotein-binding protein, NKG2D: Natural killer group 2D, MIC: Major histocompatibility class-I chain-related protein, NKp30: Natural cytotoxicity triggering receptor 3, NKp44: Natural cytotoxicity triggering receptor 2, B7-H6: Natural cytotoxicity triggering receptor 3 ligand 1, MLL5: Mixed lineage leukemia 5, TCR: T cell receptor, DC: dendritic cell

Malignant gliomas, including GBM stem cells, are known for their constitutive expression of self-antigens or stress-response antigens such as MIC-A, MIC-B, and ULBP proteins.[83] γδ T cells can recognize these molecules by their γδ TCR as well as through NKG2D expressed on these cells using MHC-independent mechanisms.[23,24,84] Long-term human glioma cell lines, as well as Grade IV GBMs, have been reported to express massive over-expression of nonclassical MHC Class I molecule HLA-E, the only known target for CD94/NKG2A and CD94/NKG2C expressed on γδ T cells.[38] Moreover, stress-induced ligands for γδ T cells are not expressed in brain tissues (healthy as well as irradiated or temozolomide [TMZ] treated) which makes γδ T-cell therapy safe for brain tumor patients.[85] These factors, together with the increased resistance to AICD and long-term persistence of Vδ1 T cells,[26] and resistance to dexamethasone-mediated lymphopenia,[86] may render this unique group of T cells suitable for GBM adoptive T-cell therapy.

The presence of γδ T cells in GBM was reported as early as 1997 by Fujimiya *et al*.[28] Peripheral blood γδ T cells from GBM patients, activated in cultures in the presence of IL-2, IL-12, as well as IL-15 and solid phase anti-CD3 significantly increased the cytotoxicity of γδ T cells against autologous GBM targets *in vitro*.[7,28,87] Years later, in 2009, Bryant *et al*.[29] showed that γδ T cells from GBM patients exhibited less potential than those from healthy donors in proliferation and killing GBM cells, suggesting the importance of this subgroup of T cells in allogeneic immunotherapy. They also showed that the absolute count of Vδ2 T cells, but not Vδ1 T cells, declined progressively in GBM patients throughout the treatment period, due to their sensitivity to AICD. In another study, stereotactic injections of allogenic Vγ9 Vδ2 T cells in orthotopic xenograft GBM model showed that these cells participated in immunosurveillance and eliminated infiltrative GBM cells.[88] In an intracranial GL261 syngeneic mouse model, circulating γδ T-cell count showed an initial increase followed by a sharp decline as the tumor progressed. Circulating γδ T cells showed neither regulatory nor cytotoxic phenotype.[89]

Ex vivo expansion of γδ T cells activated by traditional methods with OKT-3[29,90] or by the effect of zoledronic acid on glioma cells[91] resulted in expression of effector/memory phenotype and mediated killing of new or established GBM xenografts and reduced tumor progression through recognition of ULBPs on or accumulation of IPPs in glioma cells respectively. The effect of zoledronic acid on enhancing the cytotoxicity of γδ T cells against GBMs was further confirmed by Nakazawa *et al*.[92] Expansion of allogeneic Vγ9 Vδ2 T cells with IL-21 showed increased elimination of GBM tumor cells in an orthotopic GBM model.[93]

γδ T cells were first used for immunogene therapy by Friese *et al*.[83] Human glioma xenografts as well as syngeneic tumors over-expressing MIC-A (plasmid-mediated or adenovirus-mediated overexpression) were treated with γδ T cells. However, loss of MIC-A expression and progression of MIC-A negative tumors suggested strong selection against MIC-A overexpression *in vivo*. Furthermore, rejection of MIC-A overexpressing tumors in the syngeneic model resulted in protective immunity. However, vaccination of syngeneic animals with MIC-A overexpressing tumor cells and subsequent challenge with wild-type tumors resulted in inhibition of tumor growth and activation of both NK cells and γδ T cells. Similar results were observed when *ex vivo* expanded Vδ1 T cells from CMV seropositive and seronegative patients were tested for their cytotoxic efficacy against GBM cells since malignant glioma often contains CMV genetic material.[94] It was observed that independent of serological status, expanded Vδ1 T cells killed wild-type tumor more efficiently than tumors that were artificially infected with CMV.[95] The authors observed that artificial infection reduced

the expression of NKG2D ligands ULBP and MIC-A/B in GBM cells.

TMZ, the primary chemotherapeutic agent used for GBM, increases expression of stress-associated NKG2D ligands on GBM cells rendering them vulnerable to γδ T cells.[96] TMZ is highly toxic to lymphocytes, especially T cells.[97] Lamb *et al*.[98] engineered TMZ-resistant γδ T cells which showed increased cytotoxicity to TMZ-resistant GBM cell lines treated with TMZ, suggesting that TMZ-resistant γδ T cells can be generated without impairing their antitumor functions in the presence of high concentrations of TMZ. More recently, it has been shown that combination therapy with checkpoint inhibitors augments the cytotoxic effects of TMZ-resistant γδ T cells in a patient-derived GBM xenograft model.[99]

Conclusion

Unique immunological properties of γδ T cells have made these cells distinctive from the more abundant αβ T cells. Pro-inflammatory antitumor effects of these cells far outweigh the immunosuppressive properties of γδ T cells, making them a favorable tool, where conventional immunotherapy has failed or shown lower efficacy. Nonrequirement of MHC-mediated antigen presentation to the γδ TCR and the opportunities of using several non-TCR receptors for activating these T cells are added advantage for using these cells in cancer immunotherapy. A higher threshold of AICD and the ability to expand these cells in culture conditions using cytokines and phosphoantigens like zoledronates has also made these cells favorable for immunotherapy. Further, the resistance of γδ T cells to induce graft-versus-host diseases has made these cells important in the development of allogeneic T-cell therapy.[100] γδ T cells have been shown to be highly responsive in GBM immunotherapy, especially where chemotherapy has failed. TMZ-resistant GBMs are known to produce several stress-related molecules such as ULBPs and MIC-A/B which are novel ligands to γδ T cells. Combination therapies of γδ T cells with TMZ and/or checkpoint inhibitors have shown superior tumor killing efficacy over the use of traditional immunotherapies. More recently, γδ T cells have been engineered to express CARs against hematological cancer antigens. Considering the availability of several antigen receptors to modify on these cells, unique γδ CAR-Ts have been designed showing more substantial effects over the traditional second of third generation CAR-Ts.[101-103] Conventional immunotherapies, including CAR-T cells, are yet to show significant potency in GBM management because of the immunosuppressive TME and antigenic heterogeneity, which mediate immune escape. Due to its inherent properties of resistance to immunosuppression, and its allure to stress-induced self-ligands, γδ T cells can fill in the positions where traditional immunotherapy has failed to treat GBMs.

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Conflicts of interest

There are no conflicts of interest.

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