

Immune surveillance in glioblastoma: Role of the NKG2D system and novel cell-based therapeutic approaches

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Abstract

Glioblastoma, formerly known as glioblastoma multiforme (GBM), is the most frequent and most aggressive brain tumour in adults. The brain is an immunoprivileged organ, and the blood-brain barrier shields the brain from immune surveillance. In this review, we discuss the composition of the immunosuppressive tumour microenvironment and potential immune escape mechanisms in GBM. In this respect, we focus on the role of the NKG2D receptor/ligand system. NKG2D ligands are frequently expressed on GBM tumour cells and can activate NKG2D-expressing killer cells including NK cells and $\gamma\delta$ T cells. Soluble NKG2D ligands, however, contribute to tumour escape from immunological attack. We also discuss the current immunotherapeutic strategies to improve the survival of GBM patients. Such approaches include the modulation of the NKG2D receptor/ligand system, the application of checkpoint inhibitors, the adoptive transfer of ex vivo expanded and/or modified immune cells or the application of antibodies and antibody constructs to target cytotoxic effector cells in vivo. In view of the multitude of pursued strategies, there is hope for improved overall survival of GBM patients in the future.

1 | PATHOPHYSIOLOGY AND CLINICAL FEATURES OF GLIOBLASTOMA

Glioblastoma, formerly known as glioblastoma multiforme (GBM) or grade IV glioma, is the most frequent and most aggressive primary brain tumour in adults. The diagnosis is made based on characteristic molecular and histopathological features.¹ The incidence rate of GBM is 6 per 100 000 per year in Europe, and the disease occurs slightly more often in men than in women with a median age of 55 years. Treatment of patients with newly diagnosed

GBM involves at first surgical resection followed by concomitant application of radiotherapy together with the alkylating agent temozolomide (TMZ). Such treatment results in median overall survival of 14.6 months and progression free survival of 6.9 months.² Median overall survival is slightly longer (20.9 months) when applying low-intensity, alternating electric fields (tumour treating fields, TTFs) following radio-chemotherapy (RCT).^{3,4} Figure 1 illustrates the established therapeutic regimen in GBM. Despite such aggressive therapy, almost all patients relapse as reflected by poor 2 and 5-year survival rates of 18% and 4%, respectively.⁵ The dismal prognosis can be

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explained by the invasive, diffuse growth pattern with disseminated tumour present already at diagnosis which results in hampered surgical efficacy. Furthermore, limited drug delivery across the blood-brain barrier (BBB) and a high degree of tumour heterogeneity also contribute to the overall poor prognosis.⁶

Glioblastoma multiforme is a highly heterogeneous tumour characterized by distinct genetic defects with loss or gain of functions in tumour suppressor or tumour-promoting genes, respectively. These alterations include but are not restricted to isocitrate dehydrogenase 1/2 (IDH1/2), tumour suppressor antigen p53 (TP53), cyclin-dependent kinase inhibitor 2A (CDKN2A), phosphatase and tensin homologue PTEN, epidermal growth factor receptors (EGFR), platelet-derived growth factor receptor alpha (PDGFRA), or loss of transcriptional regulator, an ATP-dependent helicase (ATRX).⁷ The mutation status of IDH1/2 recently gained diagnostic relevance and is used to distinguish between the primary and secondary form of GBM. IDH1/2-mutated GBM (around 10% of cases) defines a subgroup of patients with secondary GBM evolving from low-grade diffuse gliomas. These patients are rather young and have a better prognosis than patients with primary GBM, a form of GBM that is the main focus of this review.^{8,9} Genome-wide association studies have identified three molecular subtypes of primary GBM based on their transcriptome profiles, referred to as the classical, proneural (PN) and mesenchymal (MES) subtypes with respective alterations in EGFR/CDKN2A, PDGFRA/TP53 and NF1 genes.^{10,11} Different genetic aberrations and subtypes are associated with different prognosis. For instance, the classical subtype was found to be most sensitive to intensive therapy, whereas the MES subtype of primary GBM is linked to worse prognosis. The MES subtype is occurring

more frequently at the time of GBM recurrence, suggesting phenotypic plasticity and not a static state of the subtypes over treatment or upon recurrence.¹¹ In addition, the methylation status of the O⁶-methylguanine DNA methyltransferase (MGMT) gene promotor is of particular importance for predicting treatment outcomes to chemotherapy, especially in the classical subtype of GBM.¹² GBM cells with methylated MGMT promotor (around one-third of all GBMs) do not express the DNA repair enzyme MGMT and are more sensitive to alkylating agents such as TMZ.^{13,14} Of note, MGMT enzymatic activity represents one of the resistance mechanisms against TMZ.^{15,16} In line, GBM patients with methylated MGMT promotor and thus without MGMT enzymatic activity have significantly longer median overall survival and respond better to TMZ compared to patients with an unmethylated MGMT promotor.²

Heterogeneity also exists even within the same tumour characterized by the presence of active tumour zones or hypoxic and necrotic zones, and by clonal heterogeneity in terms of immunogenicity or proliferative status of GBM cells.^{7,17} This heterogeneity is believed to be maintained by a rare fraction of cells with stem cell properties (stem-like cells [SLC]). The SLC pool is heterogeneous, and there is no single marker defining the GBM stem cell population. Like classical neuronal stem cells, SLC may express a variety of stem cell markers such as CD133, Musashi-1 and Sex determining region Y-box 2 (Sox-2), CD15, A2B5 and CD44.¹⁸ It is thought that stem cells have tumour-initiating potential, are pluripotent, can self-renew and differentiate into different cell types. This concept is supported by the fact that only CD133⁺ GBM cells with SLC properties can recapitulate brain tumours in model systems^{19,20} and thus are considered GBM-initiating cells. Stem cells in GBM

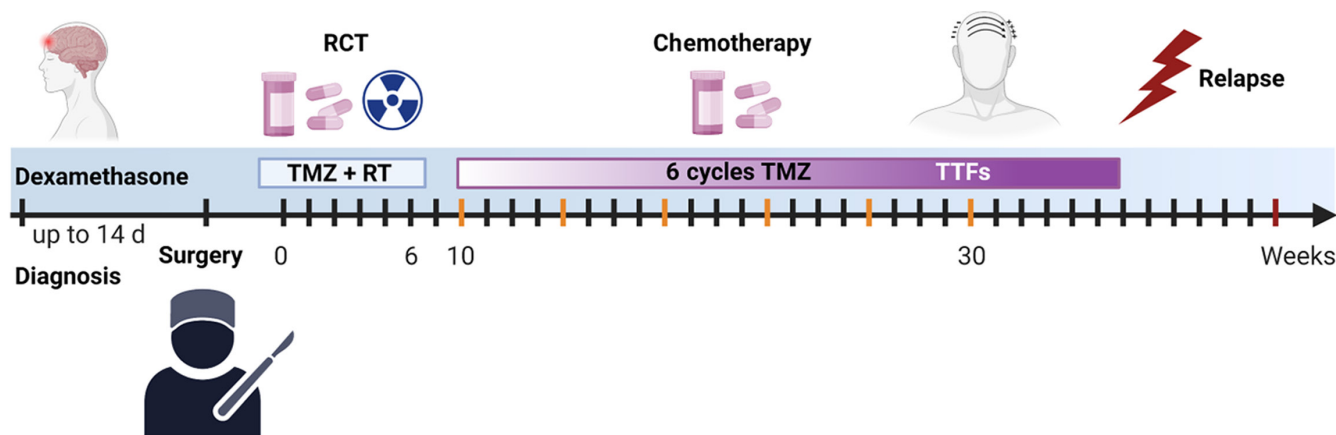


FIGURE 1 Treatment protocol of patients with newly diagnosed GBM. Radiochemotherapy (RCT) is applied according to the Stupp protocol following surgery (60 Gy splitted up on 2 Gy for 5 days a week in 6 cycles plus chemotherapy with temozolomide [TMZ] at 75 mg/m² body surface during radiation). Following RCT, at least 6 cycles of chemotherapy with TMZ at a dose of 150–200 mg/m² body surface on 5 of 28 days are applied. Whenever applicable, tumour-treating fields (TTFs) are administered together with TMZ until reoccurrence of the disease

are controlled at the genomic, epigenetic and metabolic level as well as by the cellular micro-environment and the host immune system.¹⁸ Recent reports indicate that cells expressing stem cell markers do not define clones with distinct functional properties, but rather represent a plastic state which some cancer cells can adopt depending on the environmental conditions.²¹ The proportion of tumour stem cells is closely associated with both aggressiveness and poor prognosis in GBM.²² Due to their low proliferative potential, cancer stem cells can survive standard chemotherapeutic treatment regimens and are therefore key elements in tumour progression and recurrence. Innovative treatment approaches are needed to target these quiescent cells.

2 | IMMUNE SURVEILLANCE IN GBM: THE ROLE OF CD8 CTL, $\gamma\delta$ T CELLS AND NK CELLS

Tumours evolve to evade the immune system, a process referred to as 'cancer immunoediting'. The interaction between the tumour with the immune system can be divided into three phases.²³ In the first phase, tumour cells are eliminated by innate and adaptive immune cells (elimination phase). This is followed by the equilibrium phase where the immune system is unable to destroy the tumour but is still able to control its growth. In the third phase, tumour cells evade the immune control (escape phase) by distinct mechanisms resulting in clinically detectable disease. At the time of diagnosis, tumour cells have already successfully established a niche within the tumour micro-environment (TME) supporting the survival of the tumour which is particularly true for GBM.

The main immune cell populations involved in recognition and potential elimination of GBM cells include conventional $\alpha\beta$ T cells, innate-like $\gamma\delta$ T cells and NK cells (Figure 2). CD4 and CD8 $\alpha\beta$ T cells with tumour antigen (TAG)-specific T cell receptors (TCR) might recognize GBM TAG (for instance EGFRvIII, HER2) presented in the context of HLA molecules, resulting in clonal activation of TAG-specific T cells and the potential elimination of tumour cells by cytotoxic CD8 $\alpha\beta$ T cells (CTL) (Figure 2A). Like most other cytotoxic effector cells, CTL use two main effector mechanisms following TCR-TAG recognition to kill their target cells: (a) the secretory pathway relying on perforin (PFN) and granzyme B (GrB)-dependent granule-mediated cell death; and (b) the death receptor pathway (e.g. Fas [CD95]/Fas-ligand, TRAIL/TRAIL receptor). Both pathways lead to the activation of effector caspases resulting in apoptosis of tumour cells (Figure 2D).²⁴

Accumulating evidence indicates an ongoing but ineffective immune response in GBM. In preclinical glioma

models, immunocompetent wild-type (WT) mice survive significantly longer than immunodeficient mice. While tumours in immunodeficient mice are highly heterogeneous (polyclonal), such tumours in WT mice are immune-edited with reduced clonality and the emergence of immune escape clones.²⁵ Along this line, TCR repertoire signatures significantly differ between GBM-infiltrating and peripheral T cells, reflecting an ongoing (yet ineffective) local T-cell response in GBM.²⁶ Furthermore, the immune cell composition in peripheral blood or infiltration with distinct immune cell types at the tumour site predict treatment outcome. The immune cell composition of the TME plays a key role in tumour immune surveillance and reflects the immunogenic potential of the tumour itself.

The TME in GBM is composed of a higher proportion of myeloid cells and lower numbers of tumour-infiltrating lymphocytes (TILs) compared with extracranial malignancies.^{27–30} T cells among TILs in GBM patients mainly consist of CD4 and to a lesser extent of CD8 $\alpha\beta$ T cells.^{30–32} Higher proportions of total (CD3) and CD8 T cells among TILs have been associated with better outcome in GBM patients,^{31,33} and high numbers of infiltrating CD8 T cells in combination with TAGs quality define a rare subgroup of patients with prolonged survival.³⁴ On the contrary, low CD8 and high CD4⁺/Foxp3⁺ T cell (Treg) signatures were independent predictors of poor outcome.^{35,36} In fact, T cells present in the GBM TME seem to have a severely exhausted phenotype and require blocking of inhibitory pathways with checkpoint inhibitors to regain their effector functions.^{37,38} The immune composition in the peripheral blood also predicts the treatment response.^{39,40} For instance, CD4 T cell counts higher than 200/ μ l blood at diagnosis are linked to improved survival,⁴¹ whereas high numbers of myeloid and CD39⁺ CD4 T cell subsets with an immunosuppressive phenotype predict poor outcome.³⁹

In addition to conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells can effectively target GBM cells in vitro and in vivo (Figure 2B). $\gamma\delta$ T cells comprise a small subset of T lymphocytes in blood (1%–10% of CD3 T cells) and play an important role in the stress response and immune surveillance of cancer and infection.⁴² The main population of human $\gamma\delta$ T cells recognizes non-peptide microbial and eukaryotic pyrophosphates termed 'phosphoantigens' (PAG) and distinct stress-induced molecules expressed at elevated levels in transformed/infected cells.⁴³ Upon activation, $\gamma\delta$ T cells can initiate potent anti-tumour effector functions including the release of cytokines and cytotoxic effector molecules.⁴² $\gamma\delta$ T cells can also kill tumour cells by antibody-dependent cell-mediated cytotoxicity (ADCC) by virtue of expression of the low affinity Fc γ RIII (CD16). Besides direct anti-tumour activities, $\gamma\delta$ T cells can also enhance the anti-tumour properties of other immune cells such as DCs, CD8 T cells or NK cells by secreting

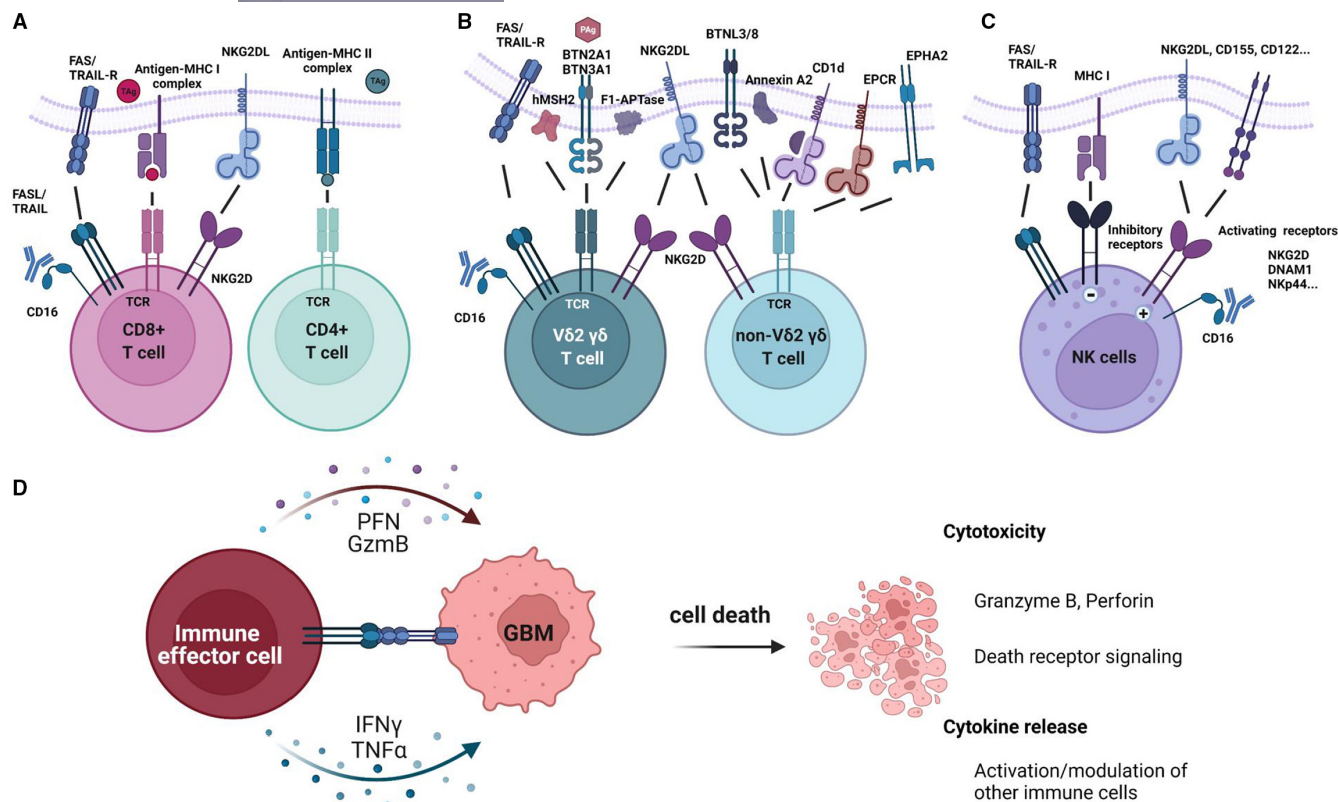


FIGURE 2 Main immune effector cells and the relevant receptor-ligand interactions in GBM immune surveillance. (A) $\alpha\beta$ T cells (both CD4 and CD8) might recognize GBM-associated target antigens (TAG) in the context of MHC-molecules via their clonotypic and highly specific TCR. (B) $\gamma\delta$ T cells and (C) NK cells sense diverse stress-induced molecules upregulated in GBM via the TCR or via NK cell activating receptors including NKG2D. NKG2D provides co-stimulation to the $\alpha\beta$ and $\gamma\delta$ T cells to enhance T-cell effector functions. CD16 (Fc γ RIII) expressed on NK cells, a subset of CD8 $\alpha\beta$ and $\gamma\delta$ T cells recognizes the Fc portion of TAG-specific antibodies and thereby can trigger antibody-dependent cell cytotoxicity (ADCC). (D) Immune effector cells (CD8 $\alpha\beta$ T cells, $\gamma\delta$ T cells and NK cells) eliminate GBM cells by inducing apoptosis via cytotoxic granule-mediated or death receptor-mediated cell death. CD4 $\alpha\beta$ T cells facilitate the cytotoxic response by the release of cytokines. Figure is adapted from Figure 2 in Ref. 44

cytokines, acting as antigen-presenting cells or by delivering costimulatory signals.⁴⁴ Of note, antigen recognition and anti-tumour activity of $\gamma\delta$ T cells does not rely on antigen processing and HLA-dependent antigen presentation. $\gamma\delta$ T cells expressing a TCR with the variable (V) gene V γ 9 paired with V δ 2 (V γ 9V δ 2 $\gamma\delta$ T cells) are the predominant subset in the periphery (50%-95% of $\gamma\delta$ T cells). The TCR-dependent recognition of PAg by the V γ 9V δ 2 T cells requires the presence of members of the Ig-like butyrophilin family of transmembrane molecules, specifically BTN3A1 and BTN2A1. Intracellular PAg bind to the B30.2 signalling domain of BTN3A1, thereby inducing in concert with BTN2A1 a conformational change which is then recognized by the $\gamma\delta$ TCR.^{43,45–51} This process involves additional molecules such as RhoB, a small molecule with GTPase-activity which stabilizes BTN3A1 in the membrane.⁵¹ Activated V γ 9V δ 2 $\gamma\delta$ T cells can effectively kill a broad range of malignant cells including GBM.^{52,53} As endogenous PAg are overproduced in transformed cells,⁵⁴ $\gamma\delta$ T cells can distinguish tumour cells from healthy tissues.

In addition to PAg, other ligands for the V γ 9V δ 2 TCR have been identified, for instance the ectopically expressed and tumour-associated DNA repair enzyme MutS homologue 2 (hMSH2) or the mitochondrial F1-ATPase that can be expressed on the tumour cell surface^{55,56} (Figure 2B).

Other $\gamma\delta$ T cell subsets expressing V δ 1 and to a lesser extent V δ 3 or V δ 5 are more abundant in tissues, especially in the intestinal mucosa and in the liver.^{57,58} These cells contribute to the immune control of malignant and virally infected cells, for instance in the case of cytomegalovirus (CMV) infection, which is frequently associated with GBM development.^{59,60} Some ligands have been identified for non-V δ 2 $\gamma\delta$ T cells, including BTN-like proteins (BTNL) 3 and 8 which have been shown to be recognized by germline-encoded motifs within the TCR V γ 4 chains of human intestinal $\gamma\delta$ T cells.⁶¹ Interestingly, human V δ 5 T cells directly bind endothelial protein C receptor (EPCR), a MHC-like molecule that binds lipids similar to the antigen-presenting molecule CD1d. EPCR-binding to V δ 5 was, however, independent of lipids.⁶² Other stress-associated

molecules including Annexin A2 and ephrin receptor A2 (EphA2) have also been involved in the activation of non-V δ 2 T cells. EPCR and EphA2 are both expressed on CMV-infected endothelial cells and are upregulated in GBM during tumorigenesis.^{63,64} $\gamma\delta$ T cells thus recognize a multimolecular stress signature characteristic for infected or malignant cells. While tumoricidal features of V δ 2 $\gamma\delta$ T cells are well-documented, conflicting data exist regarding the anti-cancer properties of non-V δ 2 T cells (mainly V δ 1 $\gamma\delta$ T cells).⁴³ In fact, pro-tumorigenic properties of some $\gamma\delta$ T cell populations have been described. For instance, TGF- β producing, FOXP3⁺-positive V δ 1 $\gamma\delta$ T cells suppress anti-tumour immune responses. Also, interleukin 17 (IL-17) producing tumour-infiltrating V δ 1 $\gamma\delta$ T cells have been shown to promote tumour development and metastasis by various mechanisms.^{42,65,66} On the contrary, several preclinical studies indicate that non-V δ 2 $\gamma\delta$ T cells also have a high reactivity against certain leukaemia and tumour cell targets including GBM.^{67,68} Both V δ 2 and non-V δ 2 $\gamma\delta$ T cell subsets have been identified among TILs isolated from GBM, V δ 2 at higher frequencies compared with V δ 1 $\gamma\delta$ T cells. Overall, it is clear that different subsets of $\gamma\delta$ T cells can exert potent anti-tumour activity, but the context-dependent potential pro-tumorigenic activity has to be considered.

Apart from T lymphocytes, NK cells also play an important role in tumour immunosurveillance in multiple malignancies including GBM. NK cells recognize transformation (or stress)-induced ligands in the absence of prior sensitization and irrespective of specific tumour antigens (Figure 2C). Such ligands engage NK-cell activating receptors such as natural killer group 2D (NKG2D) and DNAX accessory molecule-1 (DNAM1).⁶⁹ In addition, HLA-A and non-classical HLA-E molecules are recognized by HLA class I-specific inhibitory receptors, primarily killer Immunoglobulin-like receptors (KIRs) and a heterodimer composed of CD94 and natural killer group (NKG)2A. The engagement of HLA molecules inhibits NK-cell cytotoxicity and conversely the lack of engagement activates NK cells. Thus, NK cells can recognize and target tumour and virally infected cells that downregulate MHC class I molecules and/or upregulate stress-induced ligands and the overall activity of NK cells is orchestrated by the integration of activating and inhibitory signals (Figure 2C). NK cells have been demonstrated to recognize and kill human GBM cells with stem cell-like properties through KIR and HLA mismatch and activation of receptor-ligand interactions.^{70,71} Certain KIR alleles have been associated with improved prognosis in CMV-positive GBM patients, indicative of a better immune control of virally infected GBM cells by NK cells.⁷² NK cells also infiltrate the TME in GBM tumour tissue.⁷³ However, NK cells isolated from GBM TILs are functionally compromised and require blockade of inhibitory pathways to regain proliferative

capacity in vitro.³⁸ Furthermore, neutralization of the pro-tumorigenic cytokine TGF- β was shown to restore the anti-tumour function of NK cells infiltrating the TME in GBM.⁷⁴ In addition, the peripheral blood frequency of NK cells has been found to be reduced in GBM patients compared with healthy controls.⁷⁵ In support of a functional relevance, higher frequencies of peripheral blood NK cells with an activated immune phenotype were found to be associated with a better outcome.³⁹

Immune cell composition in peripheral blood or in TME is influenced by GBM treatment which includes RCT and/or application of steroids (Figure 1). Especially steroids, which are often co-administered peri-operatively and during treatment to reduce intracranial oedema, negatively influence the immune response by dampening inflammatory cytokines and reducing lymphocyte counts and their effector functions.^{40,76} Our results show that steroid intake drastically reduces the number of selected immune cell populations (mainly adaptive immune cells, such as CD4 and CD8 $\alpha\beta$ T cells) in the blood of GBM patients compared with steroid-naïve patients and healthy controls. Innate immune populations, however, were detected at lower levels in peripheral blood of GBM patients compared with healthy volunteers, suggestive of compromised innate immune response in GBM patients irrespective of steroid intake.⁴⁰ This aspect is often not considered when reporting the immune cell composition in GBM patients, although is of high relevance, as steroid intake has been negatively associated with outcome in pooled analysis of 22 GBM studies.⁷⁷

3 | THE NKG2D RECEPTOR/LIGAND SYSTEM

The NKG2D receptor/NKG2D ligand (NKG2DL) system plays an important role in immune surveillance because the ligands for the activating NKG2D receptor are expressed on stressed and transformed cells but are generally absent in normal tissue.⁷⁸ Therefore, cell surface expression of NKG2DL allows the recognition of transformed cells and their elimination in an NKG2D-dependent manner.^{79–83} The importance of the NKG2D axis in cancer immune surveillance has been clearly demonstrated in mouse models, where NKG2D-deficiency resulted in a higher incidence of spontaneously arising tumours including prostate adenocarcinoma and B-cell lymphoma compared with wild-type animals. In a prostate cancer model, aggressive tumours arising in NKG2D-deficient mice expressed elevated levels of NKG2DL compared with similar tumours in wild-type mice, suggesting an NKG2D-mediated immune recognition and elimination of NKG2DL-expressing cancer cells.⁸²

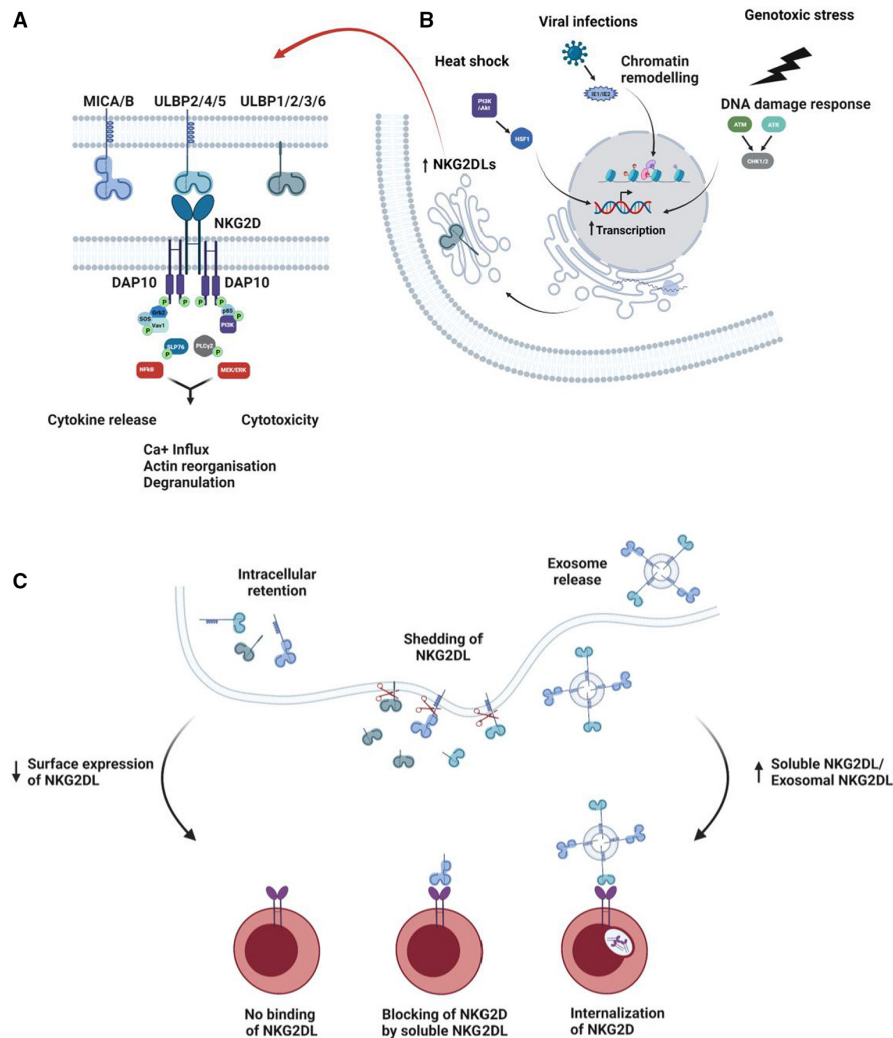


FIGURE 3 The NKG2D receptor/ligand system. (A) MICA/B are transmembrane proteins. The ULBP proteins are linked to the membrane with a GPI anchor (ULBP1,3 and 6) or are transmembrane proteins (ULBP4/5). ULBP2 is either expressed as a transmembrane or as a GPI-anchored protein. All these ligands bind to one NKG2D receptor. In humans, NKG2D is associated with DAP10 containing a YxxM motif. Following receptor ligation DAP10 is phosphorylated, resulting in the activation of PI3K and Grb2 and subsequent activation of SPL76 and of PLC γ 2. Subsequent signalling steps include calcium flux, activation of NF κ B and the MEK/ERK pathways leading to Actin reorganization, degranulation, cytotoxicity and cytokine release. (B) NKG2DLs are induced by various forms of cellular stress including heat shock, infection and genotoxic stress. Heat-shock elements in the promoter region of MIC respond to heat shock factor 1 (HSF1). Viral immediate early proteins (IE1/IE2) from CMV enhance NKG2DL expression by inducing the remodelling of chromatin. The DNA damage response activated under genotoxic stress and initiated by ATM and ATR protein kinases also induces the transcription of NKG2DLs. (C) Intracellular retention, shedding or release of NKGDL contribute to tumor immune escape by decreasing NKG2DL surface expression or increasing the levels of soluble/exosomal NKG2DL

In humans, there are two highly polymorphic MHC class I-related molecules (MIC) A and B and six UL-16 binding protein (ULBP) family members (Figure 3A). MICA/B molecules are type I glycoproteins which structurally resemble MHC class I molecules and harbour three α domains, an α 1/ α 2 super domain and an α 3 transmembrane domain. In contrast to MHC class I molecules, MICA/B are not associated with β 2 macroglobulin and do not present antigens.

The ULBP1-6 proteins harbour two MHC-like super α 1/ α 2 super domains which are linked to the membrane

with a GPI anchor (ULBP1,3 and 6) or possess a transmembrane domain (ULBP4 and ULBP5). ULBP2 has the unique feature of being either expressed as a transmembrane or as a GPI-anchored protein.⁸⁴ Like MHC class I molecules, MIC molecules are highly polymorphic and are co-dominantly expressed. Up to 388 MICA alleles and 237 MICB alleles have reported which encode for 192 and 42 protein variants, respectively. 6 MICA and 25 MICB null alleles have been reported up to date.⁸⁵ In the case of ULBP molecules, 16 transcript variants are known so far.⁸⁶

NKG2DL are upregulated by oxidative stress, malignant transformation, viral infection, chemoradiotherapy, cellular proliferation⁸⁷ and tumour-associated perturbation of miRNA expression⁸⁸ (Figure 3B). Genotoxic stress underlying most of these processes elevates NKG2DL expression via the ATM/ATR pathway.^{89,90} Viral infections such as CMV can initiate the upregulation of NKG2DL by affecting chromatin remodelling. This results from dislocation of histone deacetylases mediated by viral immediate early gene (IE1/IE2) products.⁹¹ In line with this observation, histone deacetylase inhibitors (HDACi) have been shown to induce NKG2DL expression.^{92,93}

Overall, many virally infected cells and transformed cells in a broad variety of cancer types including GBM express NKG2DL, which is further enhanced by chemotherapeutic drugs as a result of genotoxic stress.^{40,94–98} Of note, in tissue samples of 20 newly diagnosed GBM patients, NKG2DL almost exclusively defined the SLC population.⁹⁸ At the protein level, the expression of NKG2DL is absent or very limited in non-malignant tissues, except for the gastrointestinal epithelium^{99,100} and activated immune cells.^{101–103} Expression at the mRNA level does not strictly correlate with protein expression, as the mRNA is detected in a wider range of tissues indicative of post-translational regulation.^{104–107} MIC and ULBP molecules are recognized by a single NKG2D receptor that is expressed on immune cells of both the adaptive and of the innate arm: $\alpha\beta$ T cells (CD8 and a subset of CD4 T cells), $\gamma\delta$ T cells, NK cells and NKT cells.^{69,108} NKG2D is a type II glycoprotein and belongs to the family of C-type lectin receptors. It is expressed as a homodimer and in humans associates with DAP10 molecules for signal transduction and to prevent NKG2D degradation.¹⁰⁹ The cytoplasmic tail of DAP10 contains a tyrosine-based signalling motif (YxxM), which is phosphorylated by Src-family kinases following NKG2D ligation with corresponding NKG2DL. This results in the recruitment of the p85 regulatory subunit of PI3K and of the Grb2-Vav1-Sos1 complex, which in turn leads to the phosphorylation of SPL76, a Src homology 2 domain-containing leukocyte protein of 76 kD and of phospholipase C gamma 2 (PLC γ 2). Subsequent signalling steps include calcium flux, activation of NF κ B and MEK/extracellular signal-regulated kinase (ERK) pathways (phosphorylation of Jak2, Stat5, Akt, MEK1/2 and Erk). This in turn induces actin reorganization, degranulation and finally cytotoxicity and cytokine release (Figure 3A). In line with its central role in signal transduction, inhibitors of PI3K can completely block NKG2D-mediated cytotoxic activity.^{96,108,110–113}

Several mechanisms regulate the surface expression and the release of NKG2DL by proteolytic shedding or exosomal secretion and thus the NKG2D-targeting immunogenicity of tumour cells¹¹⁴ (Figure 3C). Modifications

at the genomic level by DNA hypermethylation limit the transcription of NKG2DL, and recruitment of poly-ADP-ribose polymerase 1 (PARP1) to the MICA/B promoter can selectively repress their transcription.^{93,115,116} Furthermore, post-transcriptional modifications result in miRNA-mediated decrease in NKG2DL expression.^{88,104,117,118} In addition, infection may also result in intracellular retention of certain NKG2DL and ubiquitination with subsequent degradation leading to reduced surface expression of NKG2DL.¹¹⁹ The proteolytic release of NKG2DL results in the accumulation of soluble NKG2DL in the serum which can inhibit NKG2D activation and cytotoxicity by competitive binding.¹²⁰ The tumour-promoting effects of NKG2DL shedding have been clearly demonstrated in mouse prostate tumour models where tumours did not develop when implanted tumour cells expressed a shedding-resistant non-cleavable form of MICB compared to tumour cells expressing wild-type MICB or recombinant soluble MICB.¹²¹ Elevated levels of soluble NKG2DL in the serum of cancer patients are associated with shorter overall survival or with an advanced disease stage and aggressiveness of the tumour.^{120,122,123} Soluble NKG2DL downmodulate NKG2D expression on NK and T cells and inhibit their activation, and thus contribute to tumour immune escape.^{120,124,125} It was also reported that soluble MICA might facilitate the expansion of an immunosuppressive CD4 T cell subset.¹²⁶ Several groups have shown the involvement of a disintegrin- and metalloprotease (ADAM) proteases in the shedding of NKG2DL. Specifically, ADAM10/17 were implicated in the shedding of MIC and ULBP2 molecules as shown by us and others in several cancer types including SLCs/glioblastoma-initiating cells.^{127–130} MMP9 has also been shown to shed MICA in osteosarcoma cell lines.¹³¹ Moreover, certain MICA alleles are not subject to ADAM-mediated shedding but rather are released in association with exosomes. This applies to MICA alleles with additional guanine nucleotide in the transmembrane domain (MICA-TM), resulting in a frameshift and a premature stop codon with shorter cytosolic tail (MICA*008, MICA*023, MICA*028 and MICA*054).¹³² MICA*008, the most frequent allelic variant in the Caucasian population, is enriched in detergent-resistant membrane microdomains and has been shown to be constitutively released in association with exosomes.⁹⁹ Exosomes containing MICA*008 molecules not only trigger downregulation of NKG2D from the cell surface but also provoke a marked reduction of NK cell cytotoxicity that is independent of NKG2DL-expression by target cells.^{128,133} GPI-anchored ULBPs (ULBP1, 2, 3) are known to be processed by phosphoinositide phospholipase C or are also released in association with exosomes.^{84,114} Taken together, it is obvious that the regulation of NKG2DL expression and

secretion is very complex. The expression of NKG2DL on tumour cells renders them visible for cytotoxic attack by NKG2D-expressing effector cells, but this can be blunted by the proteolytic or exosomal release of such ligands.

4 | THE IMMUNOSUPPRESSIVE TUMOUR MICROENVIRONMENT IN GBM

Glioblastoma multiforme like many other tumours establishes an immunosuppressive TME by promoting tumour-associated macrophages (TAM) arising from brain-resident microglia and bone marrow-derived monocytes¹³⁴ and by recruiting myeloid-derived suppressor cells (MDSC) and Tregs.^{135,136} TAMs which can make up to 30%-50% of the TME suppress anti-tumour immune responses by releasing soluble mediators such as arginase and/or inducible nitric oxide synthase (iNOS) which deplete key amino acids relevant for T-cell effector function from the TME. TAMs also act through the expression of interleukin-4 receptor subunit alpha which promotes M2 macrophage polarization and/or through production of TGF- β .^{137,138} Moreover, TAMs can phagocytose dying T cells and in turn release tryptophan metabolites and TGF- β , mediators which enhance the immunosuppressive TME also by recruitment of Tregs.³⁷ Tregs are present at variable numbers in GBM TME (4%-55% of all infiltrating lymphocytes).¹³⁹ They reduce effector T-cell functions for instance by release of TGF- β and IL-10.¹⁴⁰

TGF- β is a key driver of the immunosuppressive milieu in the TME of GBM and plays critical roles in glioma progression through induction of genes implicated in proliferation, angiogenesis and invasion.¹⁴¹ Most importantly, TGF- β impairs T-cell survival and cytotoxic activity by inhibiting the expression of molecules like as interleukin (IL) 6, interferon γ (INF- γ), granzymes A and B, perforin and Fas ligand (FasL).¹⁴² In addition, it drives the differentiation of naive T cells into Tregs.¹⁴³ GBM cells also release TGF- β and this expression positively correlates with the histologic grade of glioma malignancy. Thus, the high expression of TGF- β at diagnosis of GBM correlates with shorter survival.¹⁴⁴ In line, plasma levels of TGF- β are also elevated in GBM patients when compared with healthy controls.¹⁴⁵ This notwithstanding, it is remarkable that TGF- β can also exert immune-stimulatory effects under specific experimental conditions, for instance by enhancing the cytotoxic activity of purified human $\gamma\delta$ T cells.¹⁴⁶

In addition, tryptophan metabolites foster an immunosuppressive TME in GBM. GBM cells take up and metabolize tryptophan by indoleamine-2,3-dioxygenase (IDO) activity which leads to the depletion of tryptophan from the local micro-environment and to the accumulation of

immunosuppressive tryptophan intermediates, such as kynurenine at high micromolar levels. Both the depletion of tryptophan and the accumulation of kynurenine lead to profound suppression of T cell responses by anergy induction and subsequent cell death.¹⁴⁷ Kynurenine also inhibits the killing of tumour cells by activated $\gamma\delta$ T cells.¹⁴⁸

A recent study at the single-cell level provided new insights into the dynamics of evolution of the immune landscape in the TME in in vivo model systems of GBM, which were confirmed in tumour samples from patients with low-grade glioma and GBM, respectively. The results identified a large phenotypic heterogeneity at the early stage of GBM development.¹⁴⁹ A drastic change in the innate immune cell composition during tumour progression was observed, with a preponderance of proinflammatory (M1-like) microglia at the early stage followed by infiltration of protumorigenic MDSC (M2-like) in late-stage tumours.¹⁴⁹ These data highlight the importance of the immunosuppressive TME and its contribution to GBM immune escape and thus tumour progression. Some key features of the immunosuppressive TME are summarized in Figure 4A.

5 | THE IMMUNE PRIVILEGE OF THE CNS

The adaptive and innate immune responses are compromised in the CNS parenchyma compared with peripheral tissues to guarantee the integrity of the brain tissue with limited regenerative capacity.¹⁵⁰ The immune privilege in the CNS used to be explained by the presence of the BBB preventing trafficking of immune cells into the brain parenchyma. However, decades of research into the role of the immune system within the CNS have changed this concept by showing that immune cells can also enter brain tissue. Accordingly, the privilege of CNS is actively maintained as a result of the complex immunoregulation by CNS-resident cells and their surrounding microenvironment.¹⁵⁰ Microglial cells, the most abundant immune cells in the brain, serve as tissue-resident macrophages of the CNS. They are the first line of defence against pathogens and play an important role in the maintenance of the integrity of the brain parenchyma together with macrophages and dendritic cells in the meninges and perivascular spaces.¹⁵¹ There are normally no leukocytes present in the parenchyma of a healthy brain. However, small numbers of immune cells can enter the CNS through distinct region-specific barriers, for example through the choroid plexus where blood vessels are composed of fenestrated endothelia allowing the entry of immune cells and antibodies.^{152,153} Of note, the BBB is disrupted in the majority of GBM patients thus allowing the entry of immune cells into the TME.¹⁵⁴ Along this

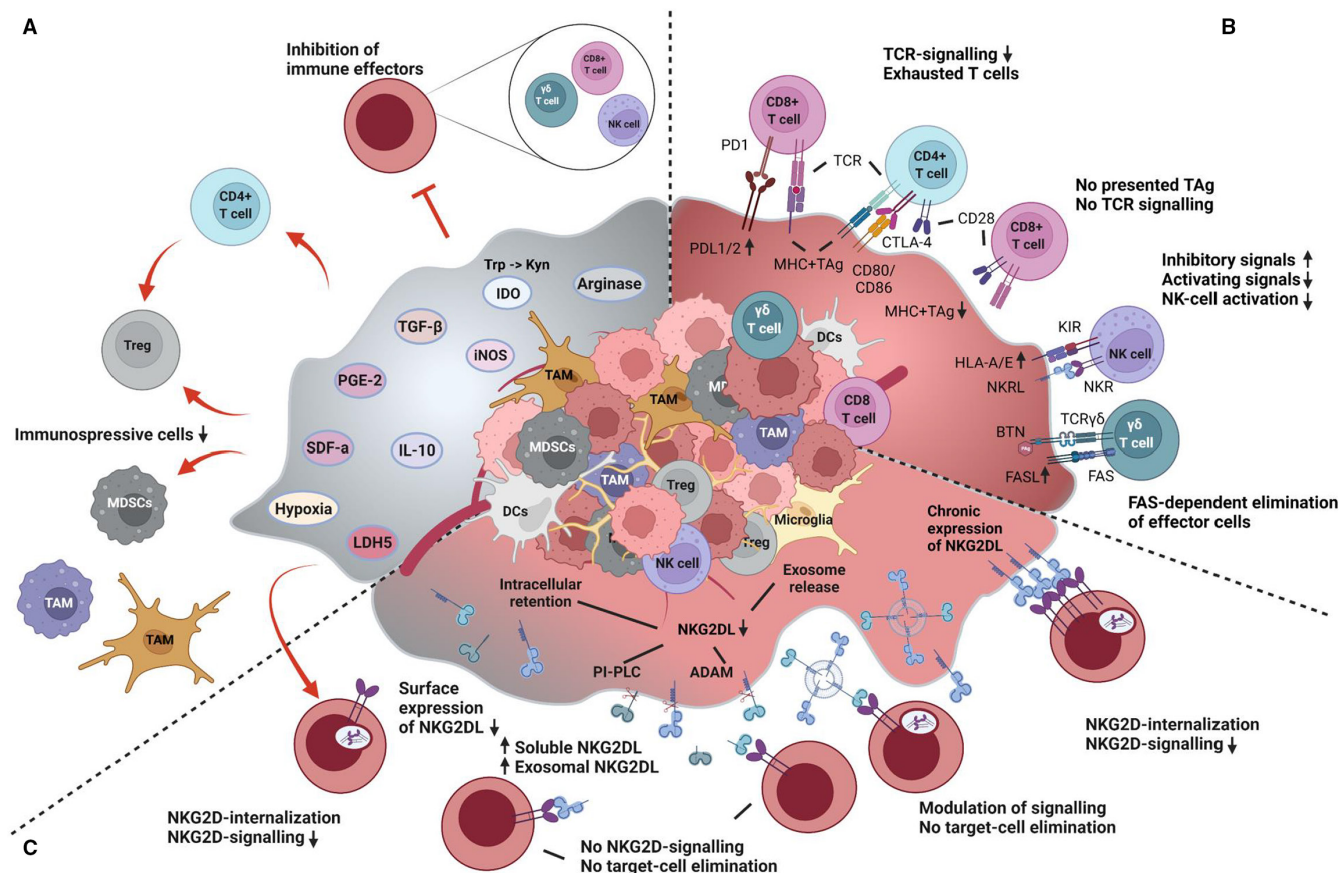


FIGURE 4 The immune escape mechanisms in GBM. (A) GBM establishes an immunosuppressive tumour microenvironment (TME) by producing soluble mediators which negatively influence effector T cell functions and promote immunosuppressive myeloid and T regulatory cells. (B) Low tumour antigen quality, exhausted TILs in the TME, upregulated inhibitory molecules or downregulated MHC class I molecules and expression of death receptor ligands account for immunosuppressive receptor-ligand interactions in GBM. (C) Immune escape from NKG2D-mediated immunosurveillance include the decrease in NKG2DL surface expression due to the release of soluble NKG2DL as shed proteins or in association with exosomes. Soluble NKG2DL and chronic exposure to NKG2DL lead to impaired NKG2D-mediated functions due to downregulation/internalization of NKG2D or due to a competitive binding to NKG2D

line, multiple histological studies show that a variety of immune cells infiltrate the GBM TME. However, the immune cell composition seems to be strikingly different between the CNS and extracranial malignancies,²⁹ possibly reflecting the unique immune regulatory landscape of the brain.

Besides the immune suppressive micro-environment and the immune privileged tumour site, the immunogenicity and thus the immune stimulatory potential of GBM is considered to be generally low (Figure 4B). Immunogenic tumour cells express TAG or tumour-specific neoantigens which can be recognized by the adaptive immune system as ‘non-self’ when presented in the context of MHC molecules and thus elicit antigen specific T-cell responses. GBM has a low level of non-synonymous somatic mutations (also termed as tumour mutational burden, TMB, marker of tumour foreignness) compared with other tumour types,¹⁵⁵ potentially reflecting the low neoantigen load and limited recognition as ‘non-self’ by

the immune system. Moreover, downregulation of MHC class I molecules or defects in antigen presentation might also hamper effector T-cell responses.¹⁵⁶ These changes often occur in GBM and together with the low neoantigen load might explain the limited success of immune checkpoint blockade (ICB) in GBM, which requires tumour antigen presentation in the context of MHC molecules.¹⁵⁷ This view is supported by better outcomes following ICB in a subgroup of patients with hypermutated GBM due to germline mismatch repair deficiency (MMRD).¹⁵⁸ These features (high TMB/neoantigen load, MMRD) along with TME-infiltrating antigen-specific cytotoxic T cells are predictive of favourable outcomes following ICB across multiple cancer types. Of note, recent studies underline the importance of the quality rather than quantity of neoantigen for predicting treatment outcomes to ICB. Results across distinct malignancies suggest that high TMB is only relevant when there is high infiltration of cytotoxic CD8 T cells.¹⁵⁹

As already discussed above, alterations of the NKG2D/NKG2DL axis including decrease in NKG2DL expression levels and/or the release of soluble NKG2L can contribute to immune evasion (Figure 4C).^{124,125} Factors associated with the immunosuppressive TME such as hypoxia, IDO metabolites or cytokines including TGF- β and macrophage migration inhibitory factor (MIF) among others also have a negative impact on NKG2D expression on effector cells.^{78,160} Furthermore, proteases such as ADAM10 and ADAM17 contribute to an immunosuppressive phenotype by cleaving ULBP2, thereby generating soluble ULBP2 in glioma-initiating cells. Here, the inhibition of ADAM10 and ADAM17 enhanced the expression of ULBP2 and thereby immune recognition by NK cells.¹²⁷ Matrix metalloproteases and ADAMs proteases can further promote disseminated growth of GBM and metastasis by proteolytic cleavage of mediators such as N-cadherin¹⁶¹ and neuroglin 3.¹⁶² Along this line, expression levels of ADAM10 were shown to negatively correlate with GBM patient survival.¹⁶³ The immunosuppressive TME also limits the activity of the NKG2D receptor. As an example, it has been recently shown that the expression of NKG2D on $\gamma\delta$ T cells is dependent on oxygen metabolism and is downregulated in the hypoxic TME in GBM thereby limiting NKG2D-mediated effector functions.¹⁶⁴

6 | PATHWAYS TO IMPROVE NKG2D-DEPENDENT IMMUNOSURVEILLANCE AND CELLULAR IMMUNOTHERAPY IN GBM

Based on the above-summarized knowledge of the immune escape mechanisms preventing efficient immunological control of GBM development, a plethora of approaches are undertaken in attempts to improve immune defence and patient survival. Some of the current strategies are illustrated in Figure 5.

6.1 | Modulation of TME and antibody-based strategies

Approaches to enhance NKG2DL expression and to prevent NKG2DL shedding or remove soluble NKG2DL are summarized in Figure 5A. Several agents in clinical use can upregulate the expression of NKG2DL on tumour cells, including PARP1 inhibitors and HDAC inhibitors.^{92,93,116} We also reported that TMZ enhances NKG2DL expression on GBM tumour cells, associated with enhanced susceptibility to $\gamma\delta$ T cell-mediated lysis.⁹⁷ On the contrary, protease inhibitors have been explored to prevent shedding

of NKG2DL. Trials employing the specific hydroxamate-based ADAM10-selective inhibitor GI254023X were discontinued in phase I/II due to hepatotoxicity following systemic administration.¹⁶⁵ Safety studies of another ADAM10/17 inhibitor, referred to as INCB7839, revealed a pro-coagulant effect of this compound in adults although it was otherwise well-tolerated.¹⁶⁶ Currently, INCB7839 is evaluated for its safety in the Phase I trial NCT04295759 in recurrent high-grade gliomas in children.¹⁶⁷ Alternatively, the pro-domain of ADAM10 that inhibits cellular shedding events, or inhibitory antibodies against ADAM10¹⁶⁸ might be useful approaches to inhibit the release of sNKG2DL. Furthermore, shedding sites can be targeted by antibodies which prevents the proteolytic cleavage of NKG2DL, stabilizes surface expression of NKG2DL and improves NK-cell responses.¹⁶⁹

Besides targeting shedding-responsible proteases, there are several strategies to reduce levels of sNKG2DL, for instance the removal of immune suppressive sNKG2DL using neutralizing antibodies¹⁷⁰ or the removal of exosomes by affinity plasmapheresis from the blood flow using a hemopurifier, a device which depletes vesicles of a given size from the plasma.¹⁷¹ This device was recently approved by the FDA as a breakthrough approach and is currently tested in clinical trials with checkpoint inhibitors to deplete cancer-derived immunosuppressive microvesicles and to improve the response to immune therapy in head and neck squamous cell cancer in this way (NCT04453046).¹⁷² Altogether, these strategies might increase the immunogenicity of GBM¹²⁷ and enhance NKG2D-mediated immune recognition.

To improve the reactivity of exhausted T cells, ICB with checkpoint inhibitors might hold the potential to improve the outcome also in GBM (Figure 5B). This might apply to GBM patients with germline MMRD and possibly hypermutated GBM following TMZ treatment occurring in around 24% of GBM patients.^{158,173,174} These tumours are likely more immunogenic and the missense mutations in these GBM cases may generate neoantigens that can be recognized by neoantigen-specific CD8 T cells.¹⁵⁵ However, TMZ-induced hypermutations in MMR-deficient gliomas were characterized by a lack of prominent T-cell infiltrates, extensive intratumoral heterogeneity, poor patient survival and a low rate of response to ICB.¹⁷⁵ Anti-tumour T-cell responses of antigen-specific T cells, that are present in the TME in an exhausted state, can be restored under ICB.¹⁵⁸ ICB application might be more beneficial prior to surgery in a neo-adjuvant setting when more TILs are present in GBM.

Additional major developments are the in vivo targeting of immune cells and the adoptive transfer of ex vivo expanded and/or modified T cells and NK cells (Figure 5C/D). As discussed above, activation of V δ 2 $\gamma\delta$ T cells relies on BTN3A1 and BTN2A1. Activating anti-BTN3A1 antibodies

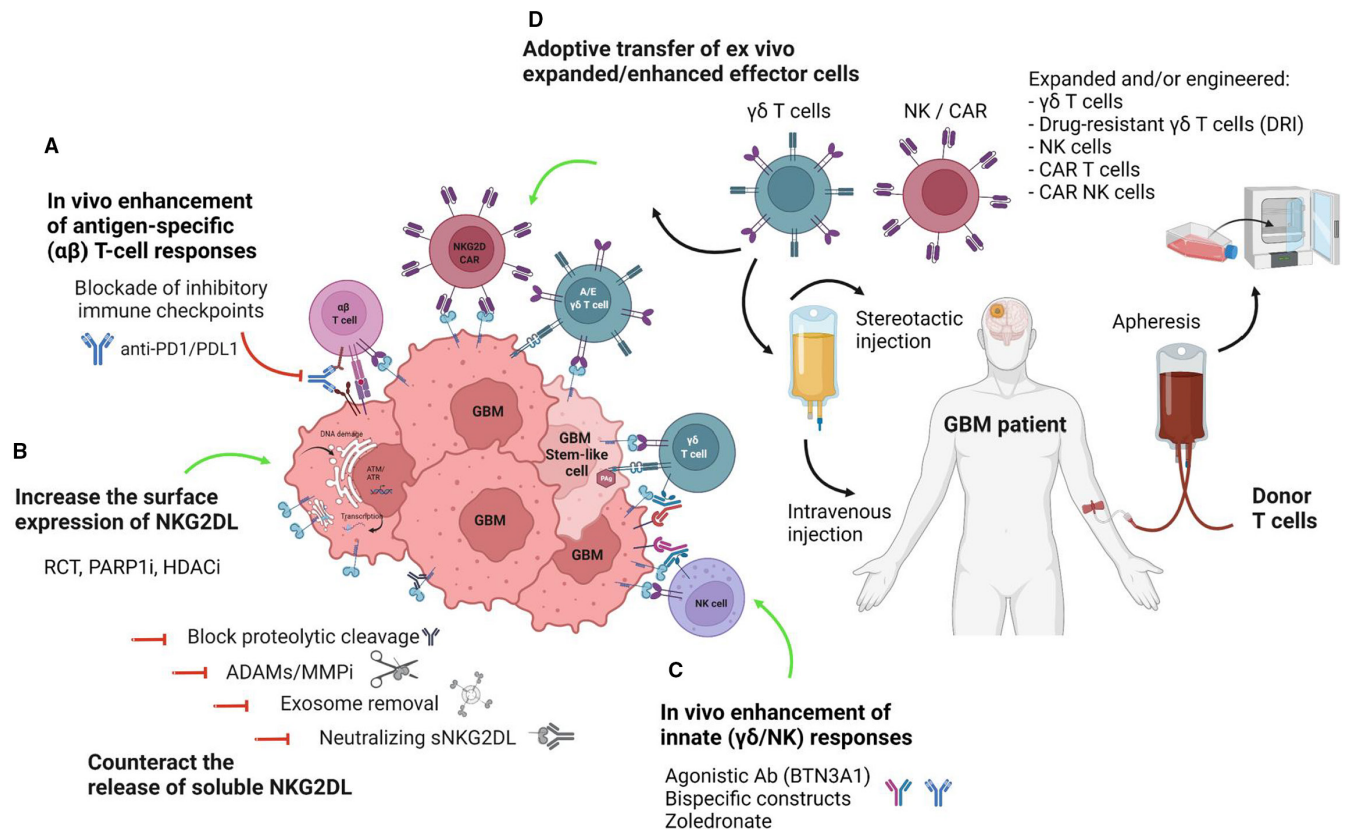


FIGURE 5 Potential clinical pathways to improve NKG2D-dependent immunosurveillance and cellular immunotherapy in GBM. (A) Increasing NKG2DL expression using approved therapeutic strategies, or counteracting negative effects of released NKG2DL by inhibitors of protease-mediated shedding of NKG2DL, neutralizing antibodies or exosome removal, might improve GBM immunogenicity and facilitate immune recognition by immune effectors. (B) Immune checkpoint blockade by monoclonal antibodies. (C) monoclonal antibodies (e.g. anti-BTN3A1) and bispecific antibody constructs target effector cells to tumour cells. (D) Intravenous or intracranial application of adoptive cell therapy with ex vivo activated/expanded and/or genetically modified $\gamma\delta$ T cells or NK cells. Such cells can be modified with CAR constructs, or can be rendered drug resistant (DRI)

have been developed which are very potent and specific activators of V δ 2 T cells.¹⁷⁶ Such antibodies mimic the activity of PAG by inducing a conformational change in the BTN molecules leading to $\gamma\delta$ T cell activation and cytotoxicity.¹⁷⁷ The anti-BTN3A1 antibody ICT01 is tested in clinical trials as first-in-class antibody to activate V γ 9V δ 2 T cell-mediated anti-tumour immune responses for the treatment of advanced solid and haematological malignancies alone or in combination with ICB (Phase I/IIA, EVICTION, NCT04243499).¹⁷⁸ The preliminary results are encouraging and show that ICT01 is well-tolerated and pharmacodynamically active. Furthermore, in melanoma patients, ICT01 seems to promote immune cell infiltration within the TME.¹⁷⁹ It is currently unclear, if ICT01 can also cross the BBB to recruit and activate V δ 2 $\gamma\delta$ T cells at the actual GBM site.

In addition to monoclonal antibodies, bispecific antibody constructs have been developed to target immune cells to tumour cells (Figure 5C). Sun et al. recently designed a bispecific antibody construct which binds to the GBM-associated molecule EGFRvIII and to T cells

via CD3. Anti-EGFRvIII-anti-CD3 eliminated EGFRvIII-positive GBM cells by recruiting and stimulating effector T cells in vitro.¹⁸⁰ Furthermore, anti-tumour immune responses can also be significantly enhanced by using bispecific antibody constructs directing V γ 9V δ 2 T cell responses towards HER2-expressing tumour cells.^{181,182} (tribody, 2 \times HER2, 1 \times V γ 9), a TAG often expressed and also targeted in GBM.¹⁸³ NKG2D-binding bispecific antibodies are already in preclinical development in distinct malignancies and may be of high relevance in future clinical applications also for the treatment of GBM.¹⁸⁴ For instance, the bispecific anti-NKG2D-HER2 construct sensitizes NK cells towards HER2-positive breast carcinoma cells. Furthermore, this construct greatly enhances anti-tumour activity of NK-92/5.28.z and NKG2D CAR T cells engineered to express an NKG2D-CD3 ζ chimeric antigen receptor, which remains unaffected by soluble MICA.¹⁸⁵ While the MAb therapy is promising for many extracranial malignancies, their use for targeting brain cancers may be challenging, particularly due to the presence of the BBB preventing easy access of antibodies to the brain

parenchyma.^{186,187} However, different antibody-delivery platforms are in preclinical development that may help to deliver antibodies at the tumour site across the BBB.¹⁸⁶ Alternatively, intracranial application together with adoptive cellular treatment strategies might also be promising.

6.2 | Adoptive transfer of immune cells

$\gamma\delta$ T cells have recently raised great interest as effector cells in cellular immunotherapy, due to their potent cytotoxic function and their independence of HLA molecules.^{44,188} Although $\gamma\delta$ T cells are compromised in GBM patients,^{40,189} both V δ 2 and non-V δ 2 $\gamma\delta$ T cell subsets can be expanded and potentially eliminate GBM in a TCR- and NKG2D-dependent manner. For instance, ex vivo expanded/activated V δ 1 $\gamma\delta$ T cells as well as V γ 9V δ 2 $\gamma\delta$ T cells effectively eliminate primary GBM cells in preclinical model systems while sparing normal astrocytes, and with higher efficiency than activated $\alpha\beta$ T cells.^{52,53,67,189–192} There are strategies available to easily expand distinct $\gamma\delta$ T cell subsets from peripheral blood under GMP conditions (Figure 5D). $\gamma\delta$ T cells show limited if any alloreactivity which is advantageous in the setting of allogeneic adoptive cell transfer.¹⁹³ The Delta one T (DOT) protocol has been reported to selectively expand V δ 1 T cells from PBMC using anti-CD3 activation and a sequential combination of defined cytokines.¹⁹⁴ Another protocol for expansion of V δ 1 T cells relies on the use of the mitogen Concanavalin A.¹⁹⁵

Similar to V δ 1 T cells, the adoptive transfer of V δ 2 T cells is already in clinical application and tested in several distinct malignancies. Expansion and activation of V δ 2 T cells to clinically relevant numbers are easily achieved with synthetic PAg or with inhibitors of the mevalonate pathway, such as bisphosphonate Zoledronate (Zol), an approved agent for treatment of bone diseases including osteoporosis.^{196,197} There is also a first-in-human phase I trial of adoptive immunotherapy with ex vivo expanded and activated V δ 2 $\gamma\delta$ T cells following haploidentical bone marrow transplantation (BMT) and post-BMT cyclophosphamide administration (NCT03533816).¹⁹⁸ This approach is shown to be safe and well-tolerated; efficacy data of these trials are, however, still pending.¹⁹⁹ Furthermore, allogeneic V δ 2 T cells expanded from healthy donors have been applied to patients with solid cancers, with no major side effects being observed.¹⁹³ The lack of recruitment of immune effectors to the tumour site is an important obstacle in cancer immunotherapy. In the case of GBM, administration of $\gamma\delta$ T cells directly into the tumour-side following surgery (stereotactic administration) is considered a promising approach and has been demonstrated safe and effective in a preclinical model system.¹⁹² Moreover, local administration of allogeneic $\gamma\delta$ T cells does not injure normal brain tissue.^{200,201} Several clinical studies are

currently ongoing to evaluate the safety and tolerability of these approaches. An interesting modification is to render $\gamma\delta$ T cells resistant to the chemotherapeutic drug used to treat the patients.²⁰² $\gamma\delta$ T cells transduced with a MGMT gene are resistant to TMZ and have been shown to act synergistically with TMZ against high-grade gliomas in preclinical models.²⁰³ The intracranially application of drug-resistant V δ 2 T cells (DRI) is currently explored to treat newly diagnosed GBM in adults (NCT04165941).¹⁷²

Besides T cell-based strategies, NK cells expressing NKG2D might also be suitable effector cells in adoptive transfer approaches (Figure 5D). Novel research concepts are emerging and already tested in clinical studies, for instance NK cells endowed with enhanced cytotoxic features. As an example, intracranial injection with an allogeneic NK cell line (NK-92/5.28.z) as off-the-shelf product is currently tested in clinical trials for the treatment of HER2-positive GBM at the time of relapse (NCT03383978).²⁰⁴ Another interesting strategy is to adoptively transfer allogeneic NK cells genetically manipulated and lacking the receptor for the key immunosuppressive molecule TGF- β , so that NK cells are not suppressed in the GBM TME.²⁰⁵ In this study, activated NK cells effectively eliminated GBM stem-like cells in an NKG2D-, NKP30- and DNAM1-dependent manner and significantly improved the survival of GBM-bearing mice relative to untreated controls or treatment with unedited NK cells.²⁰⁵ Interestingly, NK cells can also upregulate the multi-drug transporter Abcc3 under TMZ treatment and thereby are protected from the negative effects of TMZ. Moreover, TMZ promotes the recruitment of NK cells to the brain by releasing IL-18.²⁰⁶ Thus, the ongoing application of TMZ might not be a restricting factor for the adoptive transfer of NK cells.

6.3 | Chimeric antigen receptors (CAR)

CD19-directed CAR T cells revolutionized the treatment landscape of CD19-expressing haematological malignancies.²⁰⁷ This success motivated researchers to develop CAR multiple genetically engineered constructs which could also target solid malignancies including GBM.

NKG2D CAR-modified immune effector cells hold great promise for GBM immunotherapy (Figure 5D). Most of NKG2D CAR constructs consist of extracellular, full-length human NKG2D fused to the intracellular signalling domain of CD3 ζ , which is stabilized through naturally expressed DAP10 (NKG2D CAR, CYAD-01).^{208,209} Some other strategies are also used to enhance the NKG2D response, for instance by incorporating distinct co-stimulatory domains from OX40 or CD137, or a short hairpin RNA which targets NKG2DL on the same cells, effectively preventing self-targeting/fratricide (CYAD-02).²¹⁰ NKG2D CAR cells

are effective in eliminating multiple target cells in pre-clinical model systems and are already in some phase I/II clinical trials to study their safety profile and efficacy in distinct haematological and solid malignancies as recently reviewed by Curio et al.²¹¹ Of note, co-expression of an shRNA targeting MICA/MICB has improved the efficacy of NKG2D CAR T cells (CYAD-02) in patients with relapsed/refractory acute myeloid leukaemia.²¹²

Furthermore, NKG2D-BBz CAR T cells, modified to express a construct containing the NKG2D extracellular region, CD3 ζ , and CD137 signalling domains, efficiently lysed glioblastoma cells and cancer stem cells in vitro and produce high levels of cytokines, perforin and granzyme B.²¹³ The CAR T cells markedly eliminated xenograft tumours in vivo in an NKG2D-dependent manner and did not exhibit significant treatment-related toxicity in mice. CAR expression also did not exert any obvious effects on cell proliferation, apoptosis and genomic stability of T cells.²¹³

CAR T cells can cross the BBB and can also be applied intracranially.^{26,214} For instance, intracranial application of CAR T cells directed against the GBM-associated molecule IL-13aR was well-tolerated and effective over the duration of the response of 7.5 months.²¹⁵ A phase I clinical trial will shortly start recruiting patients with refractory/recurrent GBM to administer NKG2D CAR T cells intracranially via intracerebroventricular injection through an Ommaya catheter (NCT04717999).²¹⁶

Besides conventional T cells as recipients of CAR constructs, $\gamma\delta$ T cells and NK cells can be also considered for CAR modification. $\gamma\delta$ T cells with a CAR directed against GD2, a neuroblastoma-associated antigen, have been found to retain their tropism to TME and exerted enhanced cytotoxicity against GD2-positive tumour cells.²¹⁷ The advantage of innate immune cells such as $\gamma\delta$ T and NK for CAR modification is the absence of alloreactivity. $\gamma\delta$ T cells as recipients of CAR constructs have the additional advantage that they still express their tumour PAG-recognizing endogenous $\gamma\delta$ TCR. The overall goal is to establish off-the-shelf CAR products which would strongly reduce the costs of this expensive therapy. Along this line, allogeneic NKG2D $\gamma\delta$ CAR T cells will be administered in relapsed or refractory solid tumours for intravenous administration (NCT04107142).²¹⁸ Next to $\gamma\delta$ CAR T cells, there are also allogeneic and autologous NKG2D CAR NK cells in the pipeline, however, not for the treatment of GBM (NCT04623944, NCT03415100).^{219–221}

7 | CONCLUDING REMARKS

Even with optimization of treatment protocols in recent years, the overall prognosis of GBM is still very poor. At

the same time, the mechanisms of the immunosuppressive micro-environment and the deficiencies in effective immune surveillance have been extensively elucidated in recent years. Furthermore, innovative immunotherapeutic strategies to target various types of solid cancers are in development, including adoptive cell transfer and CAR therapy. As summarized here, several of these strategies are also under consideration for application in GBM. With the accumulated evidence, we can be hopeful that the dismal prognosis of GBM will improve with innovative immunotherapies.

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