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Is modulation of immune checkpoints on glioblastoma-infiltrating myeloid cells a viable therapeutic strategy?

Ruochen Du, Jianzhong Zhang, Rimas V. Lukas, Shashwat Tripathi, Jared T. Ahrendsen, Michael A. Curran, Crismita Dmello, Peng Zhang, Roger Stupp, Ganesh Rao, and Amy B. Heimberger

All author affiliations are listed at the end of the article

Corresponding Author: Amy B. Heimberger, MD, Department of Neurological Surgery, Northwestern University; SQ6-516; 303 E. Superior Street, Chicago, IL 60611, USA (amy.heimberger@northwestern.edu).

Abstract

The field of immunology has traditionally focused on immune checkpoint modulation of adaptive immune cells. However, many malignancies such as glioblastoma are mostly devoid of T cells and rather are enriched with immunosuppressive myeloid cells of the innate immune system. While some immune checkpoint targets are shared between adaptive and innate immunity, myeloid-specific checkpoints could also serve as potential therapeutics. To better understand the impact of immune checkpoint blockade on myeloid cells, we systematically summarize the current literature focusing on the direct immunological effects of PD-L1/PD-1, CD24/Siglec-10, collagen/LAIR-1, CX3CL1/CX3CR1, and CXCL10/CXCR3. By synthesizing the molecular mechanisms and the translational implications, we aim to prioritize agents in this category of therapeutics for glioblastoma.

Key Points

1. Myeloid cells comprise a substantial population in the glioblastoma microenvironment, which is relatively devoid of lymphoid cells.
2. Therapeutic modulation of the myeloid population represents a new opportunity for immune therapeutics.
3. Confounders such as sex dimorphism, age, and target heterogeneity will likely need to be incorporated as companion therapeutic biomarkers.

Gliomas are primary intracranial tumors with a controversial cellular origin.[1] Recent molecular profiling suggests that gliomas may arise from precursor cells, including glial precursor cells, neural stem cells, and oligodendrocyte precursor cells.[1–3] High-grade gliomas (grades 3 and 4) frequently recur and have a poor prognosis, often presenting with seizures, focal neurological deficits, cognitive disorders, or increased intracranial pressure.[4,5] Early diagnosis and intervention are key to prolonging survival and maintaining the quality of life. The classification of gliomas has evolved from a standard histological appearance to include molecular and genetic features.[6] The World Health Organization (WHO) now recommends molecular profiling for more accurate diagnosis and grading of gliomas.[6] Currently, infiltrating gliomas in adults are categorized into either isocitrate dehydrogenase (IDH) mutant astrocytoma, oligodendroglioma, or IDH wild-type (IDH WT) glioblastoma.[6] High-grade gliomas, including glioblastoma, are deemed incurable. For these tumors, standard treatments include surgery, radiation, chemotherapy, and in a subset, alternating electrical fields.[7,8]

Gliomas are highly immunosuppressive, and despite the success of immunotherapies for other cancers, these strategies, for the most part, have not improved survival for glioblastoma patients.[9–11] Glioma-infiltrating immune cells are predominantly myeloid cells, including myeloid-derived suppressor cells (MDSCs), monocytes, microglia, bone marrow-derived macrophages, and dendritic cells (DCs), which are diverse in their phenotypes and functions.[12,13] Historically, macrophages and microglia have been classified as either pro-inflammatory (M1) or immunosuppressive (M2).[14] A lower M1/M2 ratio is linked to worse outcomes in glioblastoma.[15,16] However, this nomenclature is an oversimplification, as these cells exist along the continuum which includes a non-activated M0 state (Figure 1).[17] The molecular characteristics of myeloid

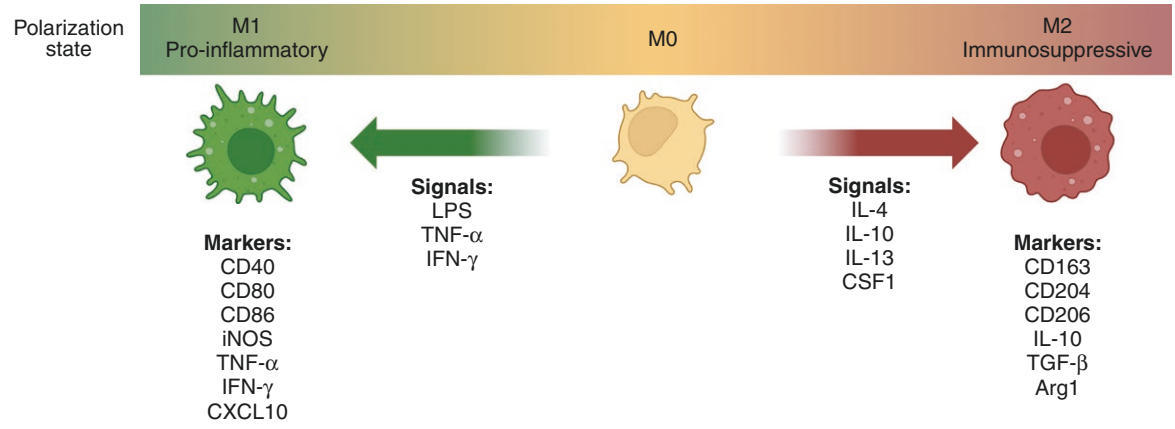

Figure 1. The continuum of macrophage/microglia polarization in response to environmental cues. The polarizing signals and molecular markers (including surface receptors and secreted cytokines) are denoted. Arg1, arginase-1; CSF1, colony-stimulating factor 1; CXCL10, C-X-C motif chemokine 10; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; TGF, transforming growth factor; TNF, tumor necrosis factor.

cells change dynamically as the tumor microenvironment (TME) evolves with glioblastoma progression.[18,19] To enhance the antitumor effects of myeloid cells, strategies to shift them from pro-tumorigenic to pro-inflammatory states are actively being pursued, including the use of immune checkpoint inhibitors.

The failure of established immune checkpoint inhibitors in glioblastoma is not entirely surprising, given the sequestration of T cells in the bone marrow, T cell exhaustion, low expression levels of immune checkpoint blockade targets, and non-immunogenic mutational burden.[20–26] This is likely further compounded by many of the standard therapeutics such as radiation and chemotherapy amplifying the immunosuppressive mechanisms systemically and in the central nervous system (CNS).[27,28] In addition, glioblastoma TME is uniquely characterized by an abundance of myeloid cells, which are highly heterogeneous and profoundly immunosuppressive.[29] The interactions of myeloid cells with T cells and tumor cells are associated with poor prognosis and survival.[12,19] In comparison to the widespread use of immune checkpoint inhibitors to modulate T cell effector responses, modulators targeting myeloid cells have not been advanced as quickly but may be more biologically applicable.[30] We will specifically review immune checkpoint axes contributing to the glioblastoma TME, beginning with those most directly relevant to effector cells of the adaptive immune system that have already been extensively used, and then transitioning to those relevant to antigen presentation and phagocytosis of the innate immune system. We will then discuss the translational challenges for implementation and strategies to potentially improve survival for patients with glioblastoma.

PD-L1/PD-1 Axis

Molecular Mechanisms

Programmed cell death 1 (PD-1; CD279) is a type I transmembrane receptor highly expressed on the surface of T cells, B cells, monocytes, natural killer (NK) T cells, and DCs (Figure 2).[31] Its most studied ligand, programmed cell death ligand 1 (PD-L1; CD274; B7-H1), is a transmembrane glycoprotein of the B7 family that is ubiquitously expressed in inflamed tissues.[32–34] In contrast, the expression of PD-L2 is more restricted and less frequent in human tumors.[34,35] Upon PD-1 engagement with PD-L1, the immune receptor tyrosine-based inhibitory motif (ITIM) and immune receptor tyrosine-based switch motif of PD-1 become phosphorylated and recruit Src-homology 2 domain-containing phosphatase 2 (SHP-2), a regulator of the MAPK pathway encoded by *PTPN11*, thereby inhibiting immune reactivity (Figure 3).[36,37] Although PD-1 is well known to restrain effector responses on T cells, PD-1 is also expressed on tumor-associated macrophages (TAMs) and inhibits phagocytosis and antitumor immunity.[38] In preclinical genetically engineered murine models of high-grade gliomas, treatment with anti-PD-1 in a CD8 knockout background did not ablate the therapeutic effect but did induce reprogramming of the myeloid cells within the TME.[39] This preclinical data would indicate that myeloid manipulation with immune checkpoint inhibitors may be beneficial but is still insufficient in glioblastoma patients, suggesting additional reprogramming of myeloid cells or other complementary approaches will be needed.[40] PD-L1 is typically expressed on myeloid cells but can be appropriated by tumor cells to amplify immune suppression within the TME. This is a dynamic process, and most clinical studies in glioblastoma evaluate expression at one or 2 time points, limiting our understanding of fluctuations in expression and what drives these changes in glioblastoma. Blockade of the PD-L1/PD-1 interaction can maintain the cytotoxic effects of T cells.[41]

Comparison With Other Tumor Types

PD-L1 is expressed across a variety of cancer types and is typically correlated with poor prognosis and the level of infiltrating immune cells. In contrast, PD-L1 expression is low in glioblastoma at initial diagnosis but can be upregulated in glioblastoma-infiltrated myeloid cells with

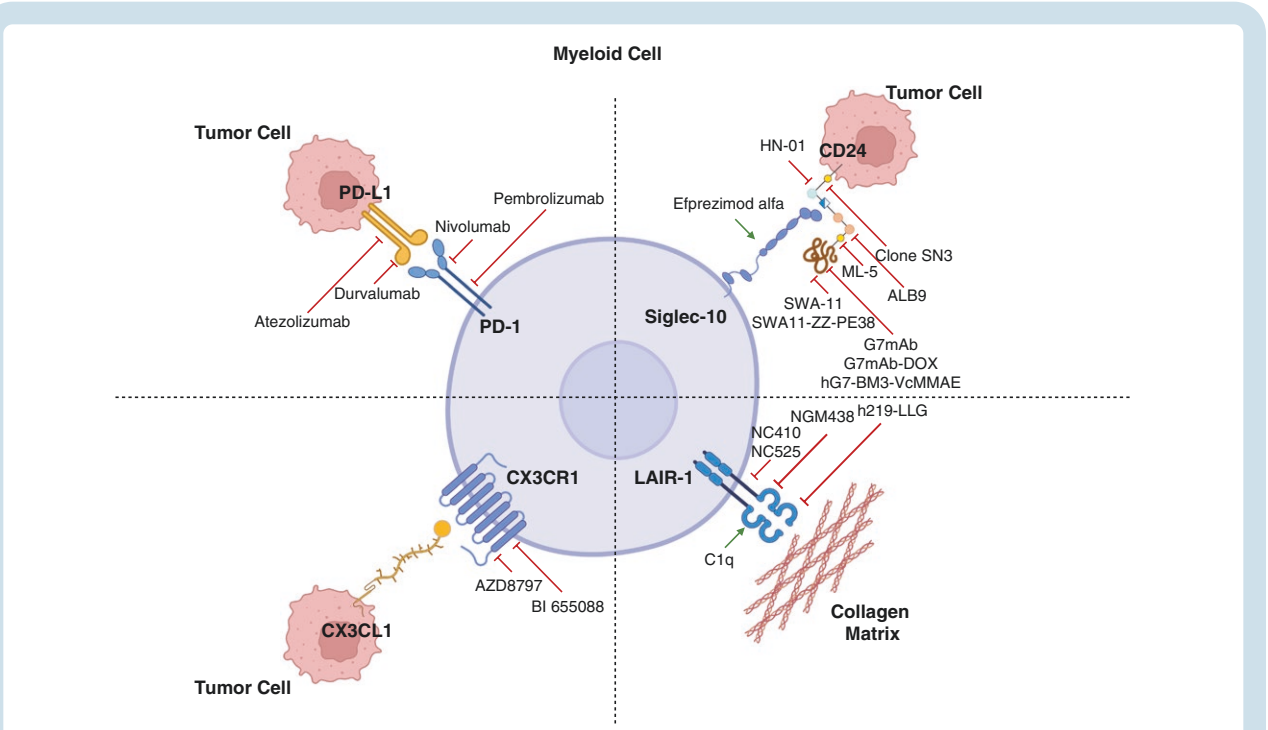

Figure 2. Scheme illustrating the myeloid immune checkpoint axes including the PD-L1/PD-1 axis, the CD24/Siglec-10 axis, the CX3CL1/CX3CR1 axis, and the collagen/LAIR-1 axis as well as immunomodulatory therapeutics targeting these axes. PD-1, Siglec-10, CX3CR1, and LAIR-1 are expressed on myeloid cells, while their ligands PD-L1, CD24, CX3CL1, and collagen are often upregulated by tumor cells. Engagement of the ligand/receptor pair leads to suppression of immune functions in myeloid cells. The key components and associated drugs targeting these axes are denoted in the figure. Receptors are shown in blue color, while ligands are shown in red/yellow color.

radiotherapy.[22,42,43] In contrast to other solid cancers, PD-1-expressing T cells in the glioblastoma TME are infrequent and are irreversibly exhausted.[21,23] In glioblastoma IDH WT patients, neither PD-L1 nor PD-1 is an independent prognostic marker.[44,45] Expression of both markers increases as a function of glioma grade but is still low relative to other cancers.[44–46] As such, their contributions to the immunosuppression of glioblastoma TME may be limited. Monoclonal antibodies (mAbs) targeting this interaction include nivolumab, pembrolizumab, and atezolizumab and have been approved by the FDA based on their therapeutic effects in a wide variety of cancers.[47–57] Immune checkpoint inhibitors are compatible with standard-of-care treatments for many cancers and can be combined with other types of therapies such as vaccines or chemotherapy.[58,59] However, they have marginal clinical benefits in combination with standard-of-care in newly diagnosed and recurrent glioblastoma patients in phase 3 clinical trials.[10,60]

Translational Aspects

Combinatorial strategies have been evaluated in both the preclinical models and the clinical setting of glioblastoma. One study showed that combining a neoantigen vaccine with anti-PD-L1 could prolong survival in a murine glioblastoma model.[61] Another study demonstrated improved tumor control of anti-PD-L1 therapy with radiotherapy in a preclinical model.[62] In a phase 2 study, the combination of durvalumab and radiotherapy showed promising results in newly diagnosed O^6-methylguanine-DNA-methyltransferase (MGMT) promoter unmethylated glioblastoma patients.[63–65] However, the follow-up phase 3 studies were negative. The lack of concordance in response to immune checkpoint inhibitors between preclinical glioblastoma models and glioblastoma patients has stimulated the development of newer models that more closely recapitulate the immune biology of human glioblastoma.[11,66,67] Our group developed lipid nanoparticles loaded with a STING agonist with dual-targeting of PD-L1 and CD47, a widely expressed anti-phagocytosis signal.[68,69] Since these targets are low at baseline in untreated glioblastoma, the tumors are first treated with radiotherapy to up-regulate the targets. Activation of the STING pathway triggers the production of pro-inflammatory cytokines, and this combination elicited a robust antitumor effect in a preclinical glioblastoma model.[68,70] An alternative strategy is to eliminate TAMs using lipid nanoparticles loaded with dinaciclib, a multi-cyclin dependent kinase inhibitor, and targeted to PD-L1, which prolonged survival in 2 preclinical models sensitive to immune checkpoint inhibitors.[71] Notably, glioblastoma utilizes multiple redundant mechanisms of immune suppression, and clonotypic preclinical models lack the heterogeneity of glioblastoma. Future studies are needed to determine if immune checkpoint inhibitors can induce a cytotoxic TAM and/or microglia phenotype alongside the underlying mechanism for this activity.

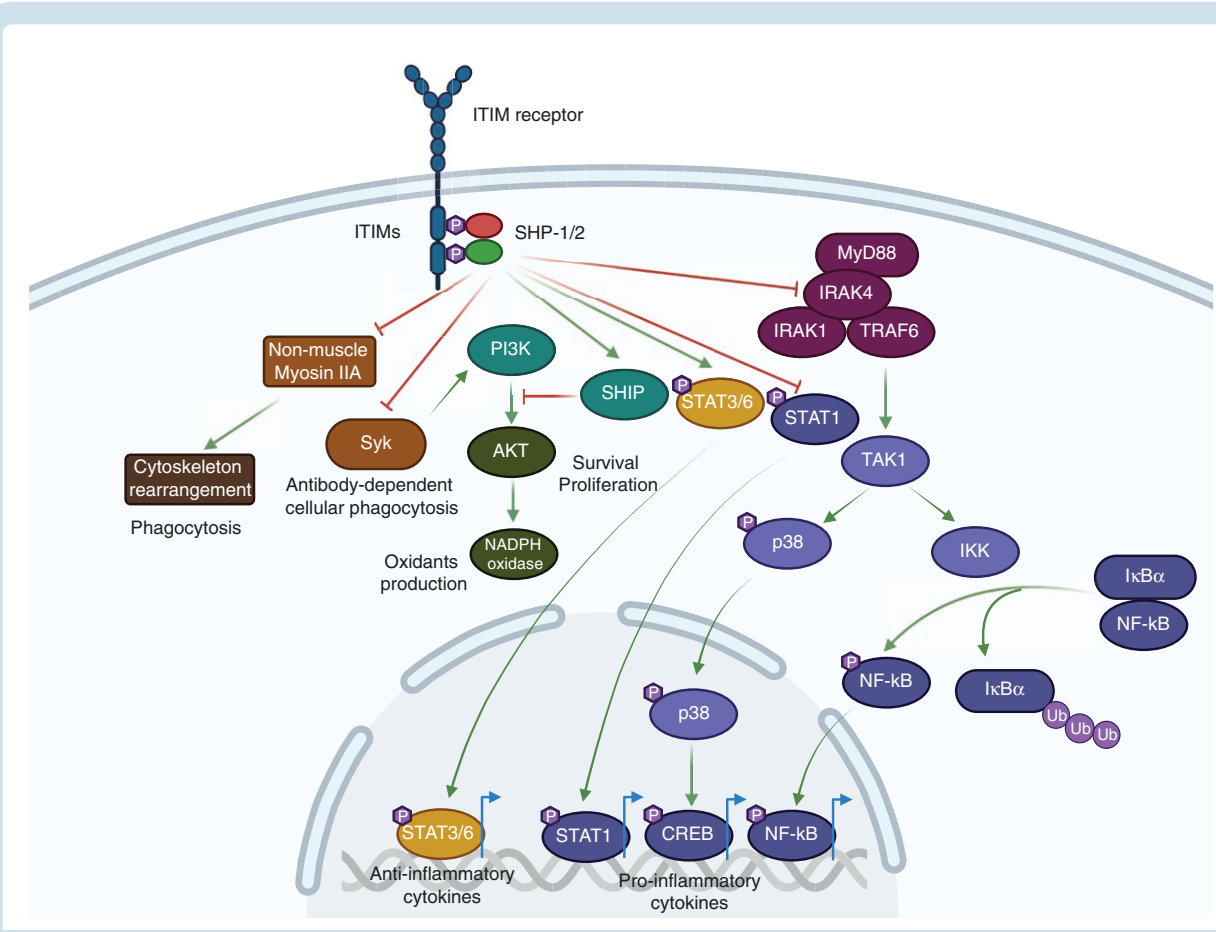

Figure 3. Downstream signaling pathways of myeloid cell surface receptors containing ITIM(s) in their cytoplasmic tails. ITIM has a conservative sequence of S/I/V/L-x-Y-x-x-L/V, where × stands for any amino acids. Ligand recognition leads to tyrosine phosphorylation of ITIM by the Src family kinases, which then leads to the recruitment of SHP-1/2 which mediates inhibitory signaling of myeloid immune functions. ITIM can negatively regulate phagocytosis by inhibiting non-muscle myosin IIA and Syk signaling. ITIM can negatively impact the survival and proliferation of immune cells as well as the production of oxidative species through the PI3K/AKT pathway. ITIM can inhibit immune response by negatively regulating the production of pro-inflammatory cytokines via STAT1, CREB, and NF-κB as well as positively regulating the production of anti-inflammatory cytokines via STAT3/6.

CD24/Siglec-10 Axis

Molecular Mechanisms

CD24 (heat stable antigen, HSA) is a glycosylated protein anchored to the cell membrane by glycosylphosphatidylinositol (Figure 2).[72,73] CD24 is widely expressed by hematopoietic, neuronal, epithelial, and muscle cells.[74] Glycosylation of CD24 is highly variable and specific to cell type, leading to diverse interactions with various cell ligands to perform various functions.[75–81] Sialic acid-binding immunoglobulin-type lectins-10 (Siglec-10) is a type I transmembrane protein, and its intracellular domain contains an ITIM tail and other ITIM-like sequences.[82] Ligand recognition leads to tyrosine phosphorylation by the Src family kinases that trigger SHP-1/2-mediated inhibitory signal transduction (Figure 3).[82,83] In the context of cancer, this interaction elicits an inhibitory signal blocking tumor cell clearance by macrophages.

The CD24/Siglec-10 axis was first described as a myeloid immune checkpoint in ovarian and breast cancer, where CD24 is overexpressed by tumor cells while the inhibitory receptor Siglec-10 is highly upregulated in TAMs.[84] Genetic ablation and antibody blockade of either CD24 or Siglec-10 enhanced phagocytosis of human cancer cells in vivo and prolonged survival in murine models of breast cancer.[84] Silencing CD24 with a small interfering RNA (siRNA) in renal clear cell carcinoma cells increased their phagocytosis during co-culture with macrophages expressing Siglec-10.[85] Similarly, the loss of nucleophosmin, a regulator that binds to the CD24 promoter region to induce CD24 expression, led to decreased CD24 on the cell surface and indirectly promoted macrophage-mediated phagocytosis, while restoring CD24 expression in these cells inhibited phagocytosis.[86]

Comparison With Other Tumor Types

High levels of expression of either CD24 or Siglec-10 are frequently associated with worse outcomes in patients across cancer lineages including brain tumors.[85,87–95] Siglec-10 is an independent prognostic marker in glioblastoma IDH WT patients, and its expression is a function of glioma grade.[44,45]

Translational Aspects

Various treatment approaches targeting the immune inhibitory CD24/Siglec-10 axis have been tested in preclinical studies. The SWA-11 anti-CD24 mAb administered systemically increased CD24 lysosomal degradation and reduced tumor burden in human lung, ovarian, colorectal, and pancreatic xenograft models.[96–98] Other anti-CD24 mAbs such as ALB9, ML-5, and Clone SN3 demonstrated similar antitumor effects but were less potent than SWA-11.[84,99,100] In combination regimens, the anti-CD24 mAb G7mAb significantly enhanced the antitumor effects of cetuximab, an EGFR mAb, in mouse xenograft models of lung, colorectal, and liver cancers by disrupting the STAT3 signaling pathway, which plays a critical role as the "breaks" on the immunostimulatory cGAS/STING pathway.[101,102]

Antibody-drug conjugate (ADC) strategies targeting CD24 have included G7mAb-DOX, a conjugate of G7mAb with the chemotherapeutic agent doxorubicin (cytotoxic chemotherapy with cGAS/STING stimulating properties), and hG7-BM3-VcMMAE, a conjugate of humanized G7mAb with the anti-mitotic agent monomethyl auristatin (MMAE). MMAE has been extensively studied in other ADCs for glioblastoma.[103] Both have demonstrated tumor growth inhibition in hepatocellular carcinoma-bearing mice.[104,105] Similarly, SWA-11 has been conjugated with PE38, a *Pseudomonas* exotoxin derivative that has also been well-studied in glioblastoma.[106] The ADC (SWA11-ZZ-PE38) can induce apoptosis in $CD24^+$ colorectal cancer cells.[107] Finally, a novel conjugate of anti-CD24 mAb with nitric oxide (HN-01) prolonged the survival of hepatocellular-bearing nude mice, although the mechanism of action relied more on increased cellular oxidative stress rather than the activation of an immune response.[108]

Currently, there are no active clinical trials of anti-CD24 mAb for solid cancers, possibly due to the differences between human and murine CD24 sequences and structures.[109] A humanized CD24-Fc fusion protein efprezimod alfa (CD24Fc, MK-7110) has been investigated in the treatment of acute graft-versus-host disease (NCT02663622, NCT04095858) and acquired immunodeficiency syndrome (NCT03960541) and has been well tolerated.[110–112] Alternative strategies like siRNA and chimeric antigen receptor T cell therapy targeting CD24 have shown activity in preclinical mice models of cancer.[98,113] In CNS tumors, $CD24^+$ has been reported as a marker for glioma stem-like cells.[114–116] Yet the role of CD24/Siglec-10 as an immune checkpoint in glioblastoma has not been explored, and no preclinical studies evaluating the effects of its disruption on the immune landscape with CNS tumor models have been published. Since glioblastoma is microglia- and macrophage-enriched, this may be a promising area for future investigation.

Collagen/LAIR-1 Axis

Molecular Mechanisms

Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1; CD305) is a type I transmembrane protein with an Ig-like extracellular domain expressed on human peripheral blood mononuclear cells (Figure 2).[117,118] Like other inhibitory receptors, its intracellular domain contains 2 ITIMs that transmit signals through SHP-1/2, thereby negatively regulating cellular responses.[117,119] Another signaling molecule, C-terminal Src kinase, can also be recruited by LAIR-1 to mediate its negative modulation function (Figure 3).[120] Some studies have pointed out that LAIR-1 interacts with SHP-1 in humans while the mouse homolog interacts exclusively with SHP-2, suggesting a species-specific variation in its immune inhibitory function.[121–124]

LAIR-1 binds to conserved glycine-proline-hydroxyproline collagen repeats with high affinity, making its ligand pool far more extensive and providing it with a unique ability to interact with the extracellular matrix.[125,126] This may have implications for cell migration, cell-to-cell signaling over longer distances, and blood-brain barrier function. Recent studies identified other collagen domain-containing proteins such as C1q, adiponectin, and surface protein D as functional ligands of LAIR-1 regulating the activity of DCs and T cells.[127–131] Palpable stiff tissue is characteristic of many forms of solid tumors, which can be attributed to heightened collagen deposition and has been suggested as a risk factor for breast cancer.[132–134] Due to this abundance of ligands, it becomes necessary to tightly regulate LAIR-1 activity at the protein expression level during development and activation across immune cell populations.[135–138] In addition, secreted forms of LAIR-1 (sLAIR-1) and LAIR-2 (CD306), a soluble homolog of LAIR-1, are unique to humans and not present in the mouse genome. They serve as decoy receptors to antagonize LAIR-1 activity, highlighting the importance of differentiating between the soluble and non-soluble forms in preclinical and clinical studies.[124,139–141]

LAIR-1 is upregulated on monocytes and DCs during the inflammatory phase to inhibit immune responses and returns to normal expression levels during the resolution phase.[142] LAIR-1 activation stimulates M2-polarized macrophages while being downregulated in M1-polarized pro-inflammatory states.[142,143] In the human THP-1 monocyte cell line, LAIR-1 activation reverses the effects of pro-inflammatory IFN-γ treatment.[144] Culturing macrophages on surfaces coated with human collagen III-derived ligand peptide (LAIR1-LP) inhibits the pro-inflammatory program and reduces the secretion of cytokines such as tumor necrosis factor-alpha (TNF-α).[145,146] Furthermore, macrophages in 3D-culture conditions with high-density collagen suppressed $CD3^+$ T cell proliferation and decreased attraction of $CD8^+$ T cells compared to those cultured with a low density of collagen.[147] LAIR-1 can also interact with CD33 (Siglec-3) on monocytes, thereby restricting cell differentiation and activation.[148] When triggered by ligand binding, LAIR-1 on B cells and T cells also leads to immune suppression.[135,149]

Comparison With Other Tumor Types

Elevated levels of LAIR-1 expression have been reported in hematopoietic malignancies, gliomas, renal cell carcinoma, hepatocellular carcinoma, and invasive breast cancer.[150–158] High LAIR-1 expression often correlates with poor prognosis and overall survival. In the case of chronic lymphocytic leukemia, loss of LAIR-1 on B cells directly

contributes to uncontrolled activation and malignant proliferation.[159] Knocking down LAIR-1 in acute myeloid leukemia (AML) cell lines in vitro suppresses tumor growth and promotes cell apoptosis.[160] Mice transplanted with LAIR-1$^{-/-}$ AML cell lines have significantly increased survival relative to those implanted with LAIR-1$^{+/+}$ cell lines.[160] In human breast cancer cell lines, LAIR-1 deficiency reduced cell proliferation and impaired invasion ability.[158]

In gliomas, a recent study revealed the presence of cancer-associated fibroblasts, the major source of collagen expression, are expanded in the TME of high-grade gliomas compared to that of normal brain and low-grade gliomas.[155,161] Indeed, collagen deposition was minimal in the normal brain but increased within the vasculature wall of high-grade gliomas.[155] Potentially, collagen deposition may function to alter the blood-brain barrier, prevent intratumoral trafficking of innate or adaptive immune cells, increase the risk of arterial thromboembolism and venous thromboembolism, and/or impact autoregulation of cerebral blood flow. Moreover, LAIR-1 expression is localized to the tumor and perivascular regions in high-grade gliomas and colocalized with M2 markers on myeloid cells, suggesting a potential mechanism of glioma-mediated immune suppression.[155] One may even speculate that this and the stiffening of the vessels could contribute to the localization of T cells in the perivascular niche. Conversely, in patients with systemic lupus erythematosus, LAIR-1 expression is reduced on B cells and IFN-producing DCs compared to healthy individuals, contributing to their heightened immunoglobulin production and IFN-α secretion.[162,163] The lack of structurally intact transmembrane LAIR-1 that is functionally inhibitory has also been found in patients with mixed connective tissue disease and rheumatoid arthritis.[162,164,165]

Translational Aspects

To manipulate LAIR-1, NC410, a fusion protein of dimeric LAIR-2 and an IgG1 Fc tail, was designed to target collagen-rich areas and block LAIR-1 activity by serving as a decoy while activating T cells.[166] In a humanized mouse model with HT-29 colorectal adenocarcinoma, NC410 promoted T-cell infiltration and reduced tumor size.[166] In immunocompetent mice, NC410 monotherapy was ineffective, probably due to the low homology between mouse and human LAIR-1.[167] However, in a LAIR-1-dependent manner, combining NC410 with anti-PD-L1 effectively reduced tumor burden and prolonged survival.[167] Blocking LAIR-1 with h219-LLG, a humanized antagonistic anti-LAIR-1 mAb, activated immune response in syngeneic human LAIR-1 transgenic mice implanted with B16 melanoma cells, resulting in increased CD4 memory T cells and pro-inflammatory macrophages with decreased anti-inflammatory macrophages and regulatory T cells.[168] Currently, the most clinically advanced LAIR-1 antagonist mAb is NGM438 (NCT05311618), which elicited a strong immune response from both myeloid cells and allogeneic T cells.[169] NC525, another humanized anti-LAIR-1 mAb structurally similar to NC410, is being investigated in advanced myeloid neoplasms in a phase 1 clinical trial (NCT05787496).[170] Reciprocally, LAIR-1 agonistic agents have been used to ameliorate inflammation such as arthritis and asthma in mice.[171,172] There have not been any published studies evaluating anti-LAIR-1 strategies in glioblastoma. As collagens are widely expressed throughout the body, targeting the collagen/LAIR-1 axis may pose issues of safety, but this could be overcome for glioblastoma with direct intratumoral strategies. Given the low homology between human and murine LAIR-1 sequences and the absence of murine LAIR-2, this determination will likely need to use murine models with humanized LAIR-1 receptors.[118,124]

CX3CL1/CX3CR1 Axis

Molecular Mechanisms

C-X3-C motif chemokine ligand 1 (CX3CL1; fractalkine; and neurotactin) has 2 molecular forms with distinct functions: adherence and migration (Figure 2). During synthesis, CX3CL1 is incorporated into the cell membrane (mCX3CL1) primarily to mediate integrin-independent cell-cell adhesion.[173,174] mCX3CL1 can be proteolytically cleaved at the mucin-like stalk by proteases such as a disintegrin and metalloproteinase domain containing 10 (ADAM10), ADAM17, and matrix metallopeptidase 2 (MMP-2).[175–178] The resultant soluble form (sCX3CL1) primarily functions as a chemokine.[175] Its cognate receptor, C-X3-C motif chemokine receptor 1 (CX3CR1), is a G-protein coupled receptor, and binding of CX3CL1 triggers the activation of heterotrimeric G proteins.[179,180] CX3CR1 expression is found in several immune cell populations and varies depending on the specific organ and tissue site.[173,181–191]

The CX3CL1/CX3CR1 axis has important functions in the brain. More specifically, CX3CL1 is constitutively expressed on neurons, while CX3CR1 is expressed on microglia.[192–194] The communication between neurons and microglia enables the removal of damaged neurons, and sCX3CL1 helps to protect healthy neurons.[195] The dysregulation of the CX3CL1/CX3CR1 axis has been implicated in a variety of neurodegenerative disorders.[196] In response to brain injury and inflammation, CX3CL1 acts as a regulator of microglia activation and has an anti-inflammatory role such as inhibiting TNF-α and decreasing major histocompatibility complex class II (MHC-II).[197,198] CX3CR1$^{-/-}$ mice display heightened microglial activation following intraperitoneal LPS injections.[195] In adoptive transfer studies, activated CX3CR1$^{-/-}$ microglia induce more neuronal cell loss than CX3CR1$^{+/-}$ microglia due to elevated IL-1β production.[195] However, there are conflicting reports that suggest CX3CR1 has a pro-inflammatory role in a murine model of stroke.[199] In a study of chronic cerebral ischemia, CX3CR1 RNAi mitigated hypoxic-induced microglial proliferation and secretion of TNF-α and IL-1β.[200] As such, the immunological role of the CX3CL1/CX3CR1 axis is likely contextual. One could hypothesize that the relationship is complex and dynamic with a need to remove injured neurons and protect the adjacent intact neurons.

The CX3CL1/CX3CR1 axis can promote immune suppression in macrophages. An in vitro study showed that CX3CL1 induced M2 polarization of macrophages and increased expression of MMP-9 which degrades the

extracellular matrix and enhances cell motility.[201] In lung cancer, upregulation of CX3CR1 increased M2-polarized macrophages and the migration of cancer cells.[202] In vivo, CX3CR1 knockout promotes M1-polarization of macrophages and prolongs mouse survival.[202] Breast cancer cells attract CX3CR1$^+$ macrophages to enhance angiogenesis.[203,204] In pancreatic cancer, high expression of both CX3CL1 and CX3CR1 is associated with shortened patient overall survival and time to recurrence.[205] Further studies have revealed that CX3CL1 promotes tumor cell motility and invasion, which can be inhibited with CX3CR1 antagonists.[206]

Comparison With Other Tumor Types

The role of the CX3CL1/CX3CR1 axis in mediating immune responses has been studied extensively across many solid tumors. As a facilitator of antitumor responses, CX3CL1 plays a role in recruiting cytotoxic T cells and NK cells into the TME. In non-CNS solid cancers, intratumoral CX3CL1 is positively correlated with the density of tumor infiltration lymphocytes, which is generally considered a marker for better prognosis.[207–209] Tumor cells and DCs engineered to overexpress CX3CL1 have been shown to reduce tumor growth and prolong mouse survival, accompanied by increased infiltration of NK cells, CD8$^+$, and CD4$^+$ T cells.[210–213] Such antitumor effects are generally abolished in NK cell-deficient or CD8$^{-/-}$ mice, indicating that CX3CL1 mediates antitumor effects by mobilizing both NK and T cells.[210,212,213] Similarly, transducing human primary T cells with the cognate CX3CR1 receptor enhances lymphocyte migration and homing in tumor-bearing mice and suppresses tumor growth.[214] This might not be the case for glioblastoma, given the paucity of T cells in glioblastoma TME. scRNA-seq of GL261 glioma-bearing mice and glioma patients has revealed that CX3CL1 is expressed on tumor cells while CX3CR1 is mainly expressed on microglia in the context of glioblastoma.[215,216] In patients, expression of CX3CL1 decreases as glioma grade increases, while that of CX3CR1 remains unchanged.[44,45] This may influence which glioma populations would be optimal for investigating potential efficacy. The expression of CX3CR1 is upregulated at the tumor leading edge and in infiltrating tumors, suggesting its unique role in tumor progression.[46]

Translational Aspects

In CD8$^+$ T cells, CX3CR1 is expressed during differentiation at the effector phase and is a biomarker for cytotoxic memory CD8$^+$ T cells.[217,218] CX3CR1^{int} signifies peripheral memory, and CX3CR1^{high} represents classical effector memory cells.[217] In chronic viral infection, CX3CR1$^+$ cells can express cytotoxic genes that reduce viral load.[219] In the context of cancer, CX3CR1 expression is downregulated during the emergence of T cell exhaustion.[219] In several preclinical cancer models, an increased frequency of CX3CR1$^+$CD8$^+$ T cells is associated with a response to immune checkpoint blockade.[220,221] A similar association has been seen in lung cancer patients treated with anti-PD-1 antibodies.[220] CX3CR1 expression could also be used to predict the response to chemotherapy and anti-PD-L1/PD-1 combination therapy. In a preclinical melanoma murine model, this combination decreased tumor size, prolonged survival, and was associated with a high frequency of CX3CR1$^+$CD8$^+$ T cells.[222] In CX3CR1 knockout mice, this treatment failed to suppress tumor growth but could be rescued with the adoptive transfer of CX3CR1$^+$CD8$^+$ T cells, indicating CX3CR1 is required for the observed tumor rejection.[222] In the peripheral blood of melanoma patients, CX3CR1$^+$CD8$^+$ T cells are enriched in responders before and after anti-PD-1 therapy and especially after chemotherapy.[222] As such, the upregulation of CX3CR1 could be potentially used as a marker to predict patient response to anti-PD-1 blockade, but this has not yet been explored in glioblastoma patients. To date, only one high-affinity small molecular inhibitor of CX3CR1 (AZD8797) and an anti-CX3CR1 nanobody (BI 655088) have been developed but have not yet been evaluated in preclinical glioma models.[223,224]

Should CX3CR1 be classified as a myeloid immune checkpoint molecule? Based on its role as a driver of immune suppression, it could be considered within this category. However, CX3CR1 lacks an immune inhibitory molecular motif like an ITIM that is present in other immune checkpoints. Future mechanistic studies focusing on the intracellular network downstream of CX3CR1 may be able to shed new light on its role in myeloid cells and classification. If its role is fully understood in the context of glioblastoma, targeting CX3CR1 would offer target specificity against microglia activities in the CNS.

Conclusion and Outlook

Therapeutics targeting the PD-L1/PD-1 axis have an established safety profile and a broad selection of available agents with regulatory approval in other cancers. However, most glioblastoma patients do not benefit from their use.[10,11,30,60] Although several biomarkers have been proposed to identify glioblastoma patients that may respond, the commonly used tumor mutation burden biomarker is not predictive.[25,225] Other markers such as activation of the MAPK pathway suggest potential benefit, but this requires further validation.[226–228] Some tumor neoantigens can trigger immunological responses, but most are not immunogenic.[24,229,230] Since MGMT promoter hypermethylation epigenetically silences the DNA repair genes and potentially increases mutations, especially at recurrence following chemotherapy, theoretically recurrent glioblastoma patients may have a greater propensity to respond to immune checkpoint inhibitors.[231–233] However, that has not been the case for most patients as reflected by the phase 3 clinical trial results.[60] In routine clinical practice, the number of patients with a hypermutated tumor at recurrence is rare. Additionally, the therapeutic effect requires the presence of both the target and functional immune cells. In glioblastoma, the expression of both PD-1 and PD-L1 is low.[22–24] Effector T cells are largely absent from the glioblastoma TME, partly due to their sequestration in the bone marrow, and are refractory to being restored to antitumor immune effector functions.[20,21]

Furthermore, MHC expression is downregulated in normal brain parenchyma, leading to a lack of antigen presentation in the glioblastoma TME and limited co-stimulation of T cells locally.[234] Moreover, peripheral immune cells rarely infiltrate the tumor, confining the immune response to the perivascular area.[235] Beyond that, the TME becomes more immunosuppressive with disease progression secondary to increasing cytokines and immunosuppressive myeloid cells, leading to further therapeutic challenges.[236]

Given the abundance of myeloid cells in the TME of glioblastoma and the emerging data that unique subsets may have direct antitumor cytotoxic effects, the question that arises is whether there is a role for myeloid-specific immune checkpoints and, if so, which ones and in what clinical scenarios. The ability to reprogram the TME from immunosuppressive to immune stimulatory/supportive exploiting the most frequent immune population is an attractive endeavor. Mono therapeutics, however, will be insufficient in converting myeloid cells to full cytotoxic functions in glioblastoma. Careful and mechanistically rational pairing with other therapeutic approaches will likely be essential. These other approaches could cover a range of mechanisms, both non-targeted and targeted. Combination with cytotoxic chemotherapeutics could add an element of cytoreduction and would align with what has been observed in other malignancies.[237] Use in conjunction with radiation therapy may facilitate an abscopal effect.[238–240] The addition of tumor-treating fields may help stimulate pro-inflammatory pathways and impede tumor cell motility for potential synergy with checkpoint inhibition.[241,242] Finally, the combination of checkpoint inhibition with oncolytic viruses for both their cytotoxic and immune stimulatory effects could have value.[243]

To date, most efforts have been focused on the inhibition of the PD-L1/PD-1 axis but a strong argument can be made that specifically targeting myeloid-associated immune checkpoints may serve as more fertile ground for success in glioblastoma. One such candidate is the CD24/Siglec-10 axis. Several therapeutics are positioned to enter clinical trials once humanized. Furthermore, the CD24 fusion protein has been evaluated in clinical trials without severe adverse effects, indicating safety in manipulating the CD24/Siglec-10 axis in humans.[110–112] Because CD24 proteins are heavily modified depending on the cellular context, further characterization of structural differences may refine these therapeutics to more specifically abrogate the immunosuppressive effects of CD24 within the TME while sparing other organs and cell types. Because CD24 is also expressed in glioma stem-like cells that give rise to other malignant cells and mediate therapeutic resistance, therapeutically targeting this axis might delay tumor recurrence.[244–247] Future research efforts should be directed to ascertaining the function of the CD24/Siglec-10 axis in the immune landscape of glioblastoma and the effects of CD24/Siglec-10 inhibition in preclinical models including clarification of the mechanism of action.

In addition to directly blocking the inhibitory signaling in myeloid cells, targeting LAIR-1 can influence the recruitment of other immune cells.[248] Alternatively, disrupting the production of collagen by tumor cells or facilitating the degradation of extracellular collagen could be considered. Physically, collagen can form a barrier around tumors, making them less accessible to immune cells and drugs. Reducing such barriers could make tumor cells more vulnerable by exposing them to external perturbations. A key consideration is that collagen subtypes have not been comprehensively characterized in glioblastoma. Preclinical studies on how dysregulated collagen homeostasis impacts immune cell functions and how modulation of collagen/LAIR-1 interactions alters gliomagenesis need to be conducted before implementing clinical trials. As such, amongst the potential myeloid immune checkpoint blockade target candidates, combinations with anti-PD-1 and additional due diligence on the CD24/Siglec-10 axis are more likely to be translationally relevant in the near term.

Several confounders for study design will need to be considered during myeloid cell modulation: IDH status, age, and sex. The presence of an IDH mutation is a positive prognosticator for survival in glioma patients.[249,250] As a key component of the tricarboxylic acid cycle, IDH mutations directly impact tumor cell metabolism, proliferation, progression, invasion, and hypoxia adaptation. IDH mutant gliomas produce the oncometabolite D-2-hydroxyglutarate, a product of mutant IDH1/2 enzymatic activity that is immune suppressive of T cell immunity.[251–253] In IDH mutant gliomas, the differentiation of myeloid cells is blocked and is unable to support T cell responses, but this can be bypassed with pharmacological inhibition of tryptophan.[254] IDH1/2 mutation tumors are more prone to developing hypermutation following alkylating agent treatment due to elevated induced mismatch repair deficiency, which in turn selects tumor cells resistant to chemotherapy.[233,255] The presence of IDH mutations also influences the composition of the glioma TME. In murine models, IDH mutant gliomas have fewer microglia, macrophages, and glioma-infiltrating T cells relative to wild-type.[256] A more recent large-scale scRNA-seq of glioma patient samples revealed that the TME of IDH WT glioblastomas has a higher percentage of macrophages while the TME of IDH mutant gliomas is overwhelmingly dominated by microglia, closely resembling that of normal brain tissues.[257] The microglia population decreases upon recurrence regardless of IDH mutational status. As immune cell populations differ based on IDH status and change dynamically through recurrence, distinct TMEs are created. Consequently, IDH mutant astrocytoma and IDH WT glioblastoma patients, although both classified as grade 4, may respond differently to immune therapeutics. Thus, future studies are urgently needed to understand the longitudinal dynamics of the TME in relation to IDH status to devise more optimal strategies for each patient.

Compared to males, females have a reduced prevalence of glioblastoma and a higher overall survival rate.[258] Sexual dimorphism may influence immune reactivity in glioblastoma, including the biology of myeloid cells. Microglia from male and female mice differ in number, morphology, and function. Male microglia are more predisposed to upregulating inflammatory signatures and MHC-II-associated genes.[215,259,260] Additionally, different subsets of MDSCs are preferentially enriched in the glioblastoma as a function of sex in mice.[261] Estrogen has been shown to modulate regulatory T cells in a murine model of melanoma, but future studies are needed to ascertain the role of sex hormones on myeloid cells in the

context of glioblastoma.[262] Although immune checkpoint inhibitors have not been approved to treat glioblastoma, in cancers where they are used clinically, male patients typically benefit more than females, highlighting the necessity of considering sex when studying immunotherapy.[263] Within glioblastoma, males have a higher frequency of progenitor-exhausted T cells that are responsive to anti-PD-1 treatment indicating that sex influences the fate of T cell function.[264] Other tumor-cell-intrinsic factors, such as the level of MGMT promoter methylation, deactivation of tumor suppressor genes, and stem-like characteristics, also differ between males and females, potentially contributing to differential responses to immunotherapies.[258,265]

Aging is associated with an increased incidence and worse outcome in glioblastoma, likely in part due to a dysfunctional immune system and/or decreased immune surveillance.[266,267] As such, there is a greater propensity for glioblastoma cell initiation and progression and reduced response to immunotherapies.[268,269]There are negligible differences in tumor cell gene expression, DNA methylation, tumor mutation burden, and neoantigen burden between younger and older adult patients, indicating that the differential disease prevalence and therapy response is mainly due to factors in the glioblastoma TME.[267] Indoleamine 2,3 dioxygenase 1 (IDO1), an immunosuppressive metabolic enzyme that facilitates the recruitment of regulatory T cells, is elevated in older adult mice with brain tumors and undermines the efficacy of immunotherapy.[269–271] IDO1 can promote MDSC characteristics but whether this induces an immune checkpoint refractory state is not known. Another contributor is the increased presence of senescent cells in aged brains. Although senescence, hallmarked by permanent cell cycle arrest, is theoretically a defense mechanism against oncogenesis, these cells release pro-inflammatory factors, collectively referred to as the senescence-associated secretory phenotype, into the TME, thereby triggering chronic inflammation and subsequently inducing compensatory augmentation of immune suppression.[272–274]Treating glioblastoma-bearing mice of advanced age with a combination of immunotherapy and senolytics decreased the expression of senescent cell markers and improved survival, shedding light on a promising avenue for elderly patients with glioblastoma.[267] However, the cut points for stratification in human subjects are not yet defined for clinical trial implementation.

A final consideration for myeloid-specific immune checkpoints is their use as biomarkers of treatment resistance for immunotherapy. For example, the immunosuppressive molecule Siglec-9 has been proposed as one such marker since Siglec-9$^+$ monocyte-derived macrophages preferentially accumulate in anti-PD-1 non-responders and targeting Siglec-9 enhanced responses to anti-PD-L1/PD-1.[275,276] However, its predictive power was questioned by a more recent large-scale scRNA-seq profiling of glioma patient samples. Because of the complexity of immunological clearance of cancer, a single biomarker for response is unlikely to rigorously capture therapeutic responders. Therapeutic companion biomarkers should be considered early in the preclinical development of immuno-oncology-targeted strategies, and the heterogenous expression profiles for all of these targets suggest that this would be necessary to identify an appropriate patient population.

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glioblastoma | immune-checkpoint blockade | myeloid cells

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Conflict of interest statement

A.B.H. serves on the advisory board of Caris Life Sciences, the WCG Oncology Advisory Board, Children's National Hospital Brain Tumor Institute, UCSF Neurological and Brain Tumor Program, Duke University Brain Tumor Center, Dana Farber and Brigham and Women's Hospital P01, Cleveland Clinic Sex Difference P01, and UCLA Brain SPORE; serves on the National Cancer Advisory Board; serves as the deputy editor for Journal of Clinical Investigation; receives royalty and milestone payments from DNAtrix for the licensing of the patent "Biomarkers and combination therapies using oncolytic virus and immunomodulation" (no. 11,065,285); receives royalty from Celldex Therapeutics; is supported by research grants from Alnylam, AbbVie, Codiak, and Celularity; receives consulting fees from Novocure, Istari Oncology, Alphasights, and BlueRock Therapeutics; receives drug and equipment from Moleculin, Takeda, and Carthera; has the granted/pending patents titled "miRNA for treating cancer and for use with adoptive immunotherapies" (no. 9,675,633), "Concurrent chemotherapy and immunotherapy" (no. 9,399,662), and "Low-intensity ultrasound combination cancer therapies" (International Applications PCT/US2022/019435 and US 63/158,642); and owns stock or stock options in Caris Life Science. R.V.L. receives grants from NIH; receives consulting fees from Novartis and Servier; receives honoraria from Merck, Novocure, and Servier; receives research support (drug only) from BMS; serves on the advisory board for Cardinal Health, Curio, Telix, and Servier; and receives honoraria for editing for EBSCO, Elsevier, Medlink Neurology, and Oxford University Press. M.A.C. reports grants and personal fees from ImmunoGenesis, Xencor, Agenus, and AstraZeneca outside the submitted work; has a patent titled "Dual specificity antibodies which bind both PD-L1 and PD-L2 and prevent their binding to PD-1" with royalties paid to ImmunoGenesis and a patent titled "Cyclic Dinucleotides as Agonists of Stimulator of Interferon Gene Dependent Signaling" licensed to ImmunoGenesis; and owns stock or stock options in ImmunoGenesis. R.S. receives in-kind or funding support for research from NIH, Agenus, BMS, and Carthera; receives consulting fees from Alpheus, Carthera, and GT Medical; receives honoraria from Novocure and ZaiLab; serves on the advisory board of Sapience, Ipsen, AiMED Bio, and Hemishperian; is a coauthor of an IP filed by Northwestern University related to the content of this manuscript ("CANCER IMMUNOTHERAPIES" No. PCT/US2023/034299); and owns stock or stock options in Carthera, Alpheus, and Modifi Bio. R.D., J.Z., S.T., J.T.A., C.D., P.Z., and G.R., declares no conflicts of interest.

Authorship statement

R.D. and A.B.H. conceptualized the manuscript. R.D., J.Z., R.V.L., and A.B.H. prepared the original draft of the manuscript. R.D. and S.T. contributed to data visualization. R.V.L., J.T.A., M.A.C., C.D., P.Z., R.S., G.R., and A.B.H. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability

No new data were generated for this manuscript.

Affiliations

Department of Neurological Surgery, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA (R.D., J.Z., S.T., C.D., P.Z., A.B.H.); Lou and Jean Malnati Brain Tumor Institute, Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA (R.D., J.Z., R.V.L., S.T., J.T.A., C.D., P.Z., R.S., A.B.H.); Department of Neurology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA (R.V.L., R.S.); Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA (J.T.A.); Department of Immunology, MD Anderson Cancer Center, the University of Texas, Houston, Texas, USA (M.A.C.); Department of Neurosurgery, Baylor College of Medicine, Houston, Texas, USA (G.R.)

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