Neuro-Oncology Advances
8(S1), vdaf087, 2026 | https://doi.org/10.1093/noajnl/vdaf087

Redefining the immune microenvironment of gliomas in the era of single-cell genomics

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Abstract

Infiltrating gliomas are the most common primary malignant brain tumor and remain universally fatal. Over the past decade, advances in single-cell technologies, including single-cell RNA sequencing, have significantly advanced our understanding of malignant cells in glioma. These efforts have revealed extensive transcriptional heterogeneity and plasticity within glioma cells, identifying distinct cellular states and developmental programs that are discussed elsewhere. Recognizing that the tumor microenvironment constitutes sometimes more than half of the cells in gliomas, and that it has a profound impact on glioma cellular states, recent research has focused on non-malignant cells and their interactions with cancer cells. The present review reflects on lessons learned from single-cell genomics on the tumor immune microenvironment (TIME) of gliomas, as the dominant component of the tumor outside of the malignant cells and explores implications for developing effective immunotherapies.

Key Points

- Single-cell genomics redefined myeloid and lymphoid cell states that shape glioma progression.
- New analytical methods expand discrete cell types with shared functional programs.
- Insights uncover ways to target immunosuppressive cells and optimize therapies.

Diffuse infiltrating gliomas are the most common primary malignant brain tumors.[1] Depending on characteristic molecular alterations (eg, the presence or absence of isocitrate dehydrogenase [IDH] mutations), these tumors can range from relatively indolent at diagnosis (eg, low-grade IDH-mutant gliomas) to markedly aggressive in their clinical behavior (eg, glioblastoma, diffuse midline glioma histone H3, lysine 27 [H3 K27] altered).[2] Irrespective of their initial clinical behavior, diffuse infiltrating gliomas are progressive, lead to significant disability, and are invariably fatal. Over the last decade, advances in chemistry, microfluidics, and computational biology have led to the development of an array of techniques that facilitate the profiling of tissues at the single-cell level, significantly advancing our understanding of different aspects of cancer and brain tumor biology.[3,4]

Leveraging these techniques, significant progress has been made in understanding the malignant cell heterogeneity of gliomas, revealing the coexistence of multiple transcriptional cellular states within the same tumor as well as uncovering evidence of plasticity enabling transitions between cellular states.[5–9] These malignant cellular states display gene expression programs that resemble those observed in developmental stem-like cells (neural progenitor-like cells, NPC-like; oligodendrocyte progenitor-like cells, OPC-like), as well as those of more differentiated cell types (astrocyte-like cells, AC-like; mesenchymal-like cells, MES-like).[5,6,9,10] Malignant cellular states in glioma have shown plasticity driven by cell-intrinsic mechanisms[7,9,11] and in response to factors in the tumor microenvironment.[12,13]

The non-malignant cells in the glioma tumor microenvironment are diverse and have been an active area of study for decades. The neurons,[14–16] fibroblasts,[17,18] oligodendrocytes,[19] endothelial cells,[20,21] pericytes,[22] and immune cells that constitute the non-malignant component of the tumor can aid and abet the activity of malignant cells. They do this as a result of

inappropriate execution of their standard cellular functions in the setting of cancer or through direct coercion by the malignant cells.[23] The failure of promising immunotherapies to treat glioma in recent years has increased the urgency and intensity of investigation, especially of the immune component of the tumor microenvironment. Aided by advances in single-cell and spatial genomic technologies, there has been significant progress in refining our understanding of tumor-associated immune cells by characterizing their granular cell states and activities at high resolution. This review focuses on lessons learned from single-cell genomics methods in regard to the tumor immune microenvironment (TIME) of gliomas, as this is the dominant component of the tumor outside of the malignant cells, Figure 1. We hope that better integration of the current knowledge in this space might lead to advances in the application of immunotherapies for the treatment of brain tumors.

Evolution in Single-Cell Technologies

Early investigation of glioma cellular composition relied on histology and targeted immunohistochemistry (IHC).[24] Though bulk RNA sequencing permitted higher dimensionality analyses of glioma,[25] its aggregate approach masked the true heterogeneity of cells in the tumor.[26] Flow cytometry and CyTOF (cytometry by time-of-flight) soon enabled high-throughput proteomic analysis of single cells,[27] but these methods were constrained by the low dimensionality of their readouts. By coupling flow cytometry with bulk RNA sequencing, researchers could first sort for populations distinguished by a small set of surface proteins before deeply profiling their transcriptome. However, with this approach, it is difficult to resolve cell-to-cell variation within the sorted populations. The development of single-cell RNA sequencing (scRNA-seq) technologies eventually unlocked a high-dimensional, high-throughput approach to characterize the diversity of cell states, made up of cell types and activities, in the glioma microenvironment. Over the years, technological advances have yielded a wide range of scRNA-seq methods that each vary in how they balance increases in throughput, dimensional complexity, and cost.[3] Researchers can now pick the method best suited for their experimental designs and considerations. Leveraging the high-dimensional readout of scRNA-seq, novel subsets of genes that demarcate specific

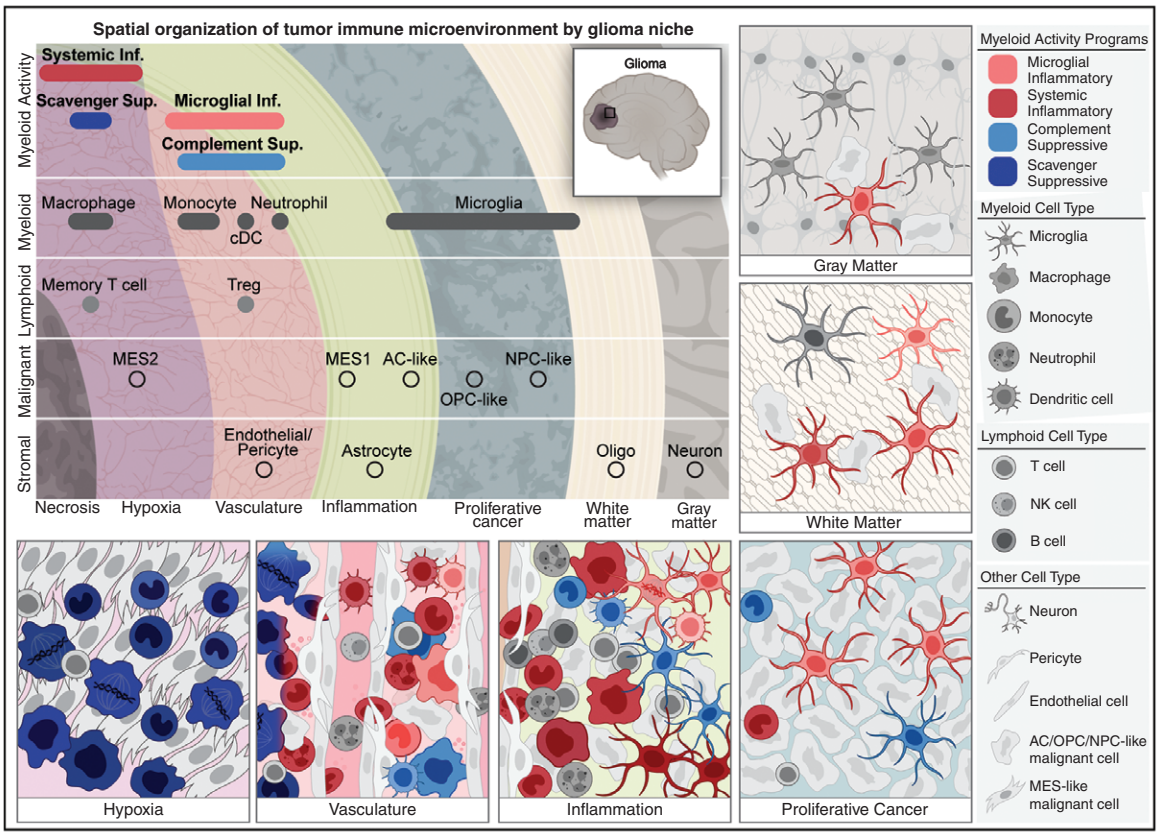

Figure 1. Spatial organization of the tumor immune microenvironment in glioma. Myeloid and lymphoid cells compose the glioma TIME and spatially localize to specific tumor niches. Myeloid cells are abundant and myeloid cell type and myeloid cell activity are enriched in specific niches. T cells and other lymphoid cells are sparse in current data, making it challenging to localize T-cell states to any particular niche. The interactions that immune cells have with one another and other cell types (including cancer and stromal cells) vary based on niche, and it is these interactions and immune phenotypes that help define the overall immune state of the tumor and its response to immunotherapy. NPC, neural progenitor-like cells; OPC, oligodendrocyte progenitor-like cells; AC, astrocyte-like cells; MES, mesenchymal-like cells.

cell types or activities are identified, and can be subsequently applied as panels for flow cytometry or CyTOF for targeted profiling. Advances in epigenomic profiling and 3D genome structure also provide complementary insights at either bulk or single-cell resolution.[28–30] In addition, the development of spatial transcriptomic and highly multiplexed immunofluorescence methods supplement scRNA-seq analyses with spatial context, creating more confident cell-cell interaction predictions and topographically linked phenotypes. Early versions of spatial transcriptomic technologies, such as 10× Genomics Visum, were limited to multicellular regions of the tumor (50 µm spots), requiring indirect computational inferences of cell states and interactions.[31] With spatial methods now approaching true single-cell resolution (<2 microns), the field is likely to benefit from these advances in the near future.[31] Collectively, these multi-omic approaches enable biological investigation at scale and can generate hypotheses to be tested experimentally.

Formation of the TIME

In healthy brain tissue, the immune system's surveillance and effector functions are tightly regulated by the blood-brain barrier, which separates the brain parenchyma from the peripheral immune system.[32] While studies in the last decade have identified meningeal lymphatic vessels that supply peripheral immune cells to survey the central nervous system (CNS),[33,34] it is understood that under homeostatic conditions, most immune functions within the brain parenchyma are carried out by specialized tissue-resident macrophages, known as microglia (MG).[35,36]

In patients with glioma, pro-inflammatory signals from cancer cells and disruptions to the blood-brain barrier cause higher numbers of bone marrow-derived peripheral immune cells, of both myeloid and lymphoid lineages, to infiltrate the brain.[37] Recent studies suggest that some of these immune cells may also originate from skull marrow.[38–40] Alongside resident MG, these infiltrating cells form the immune compartment of the tumor microenvironment.

Though humans evolved mechanisms to limit inflammation in the brain, which is often fatal without medical intervention,[41] these protective mechanisms can enable the progression of glioma. The unique composition of the glioma TIME results in potent immunosuppressive signaling that impairs anti-tumor activity, allowing malignant cells to proliferate.[42] Depending on the molecular profile of the tumor, including IDH mutation or *MGMT* promoter methylation status, the severity of immunosuppression evoked by the TIME varies.[39,43] The interactions of immune cells with one another and malignant cells depend on the spatial niches they occupy within the tumor microenvironment, Figure 1.[44,45] To characterize these immune populations in glioma and decode their immunomodulatory function, high-dimensional scRNA-seq methods have been used to analyze surgically resected samples from patients and tumor-bearing experimental mouse models.[3] By stratifying samples by their metadata, such as age or sex, variation in TIME composition associated with these factors are also identified.

Myeloid Compartment

Myeloid cells in the glioma microenvironment contribute up to 50% of all cells in the tumor[27] and more than 80% of the immune compartment.[46] Traditionally, these cells have been studied through the lens of their specific cell type (eg, MG, macrophage, neutrophil, monocyte, and dendritic cells [DCs]) or developmental origin (eg, endogenous to the brain vs bone-marrow derived), including in nearly all manuscripts focused on the scRNA-seq analysis of myeloid cells in glioma.[6,47–51] Identification of these cell types or origins by scRNA-seq is typically achieved using classical cell type markers and transcriptomic signatures derived from principal component analysis (PCA)-based dimensionality reduction or unsupervised clustering (eg, Louvain, Leiden, etc.),[52] and visualization with low-dimensional embeddings such as Uniform Manifold Approximation and Projection (UMAP).[53] Cell types can be further divided via clustering in an attempt to identify subtypes that have distinct activity profiles, including inflammatory, immunosuppressive, interferon response, and cell cycle. Researchers can then interrogate how the cells belonging to each cluster vary in abundance across conditions and patients to better understand their functions.[3]

More recently, there has been a shift toward factorization-based analytical frameworks that attempt to break down cell states into the component cell type and cell activity programs to better describe cells with states that exist along a continuum.[45,54–57] Isolating these component programs enables researchers to identify functions and cell activities that span across traditional cell-type and cell-origin boundaries. In the glioma TIME, this approach provides a more nuanced understanding of how myeloid cells dynamically respond to tumor and microenvironmental cues.[45] Here, we explore what traditional analytical approaches have revealed about myeloid cell types, and what newer frameworks are unveiling about immunomodulatory activity programs that are shared across these cells.

Characterization by Cell Type

The myeloid compartment primarily consists of MG, monocyte-derived macrophages (MDMs), monocytes, neutrophils, and DCs.[27] As discussed below, these cellular populations are identified by most single-cell studies that investigate myeloid cells in the glioma TIME.

Tumor-Associated Macrophages

Tumor/glioma-associated macrophages (TAMs/GAMs) are the predominant immune cell type in gliomas, comprising a significant fraction of the TIME. These cells are composed of 2 main populations: MG and MDMs. Using CyTOF, Friebel et al identified these TAM populations in glioma samples resected from patients and investigated variations in their abundance on the basis of tumor characteristics.[46] While no differences were associated with *MGMT* promoter methylation, IDH-wildtype gliomas had increased MDM infiltration compared to IDH-mutant tumors.[46] Klemm et al

reached similar conclusions using flow cytometry and bulk RNA sequencing.[27] While these studies identified and enumerated different TAM populations, the rise of scRNA-seq has provided a more nuanced view of their transcriptional diversity and functional plasticity.

Early single-cell analyses used glioma mouse models to reveal that MG and MDMs exhibit distinct gene expression profiles, and confirmed that MG express markers such as *Tmem119*, *P2ry12*, and *Trem2*, while MDMs are distinguished by genes such as *CD49D*, *Gda*, *Sell*, and *Lgals3*.[58,59] Although these markers help delineate MG and MDM populations, functional characterization using scRNA-seq has demonstrated that these cells exist along a spectrum of activation states rather than as discrete cell types. In fact, while earlier mouse model studies demonstrated that resident MG originate from the embryonic yolk sac and are not replaced by bone marrow-derived monocytes,[60,61] recent glioma studies found that peripheral monocytes can adopt resident MG-like transcriptional signatures and cell surface markers.[45,62] This calls into question the ability to use individual cell surface markers or transcriptional signatures to assign resident MG or MDM origin.[45]

Recent studies integrating scRNA-seq with spatial transcriptomics have revealed that MG and MDMs are distributed across different tumor niches, with MG often localized to peritumoral and vascular regions, while MDMs are more enriched in hypoxic and necrotic areas.[44,45,63] Additionally, the ratio of MG to MDMs varies across glioma subtypes and grades. In IDH-mutant gliomas, MG tends to be more dominant, whereas IDH-wildtype glioblastomas exhibit a higher proportion of infiltrating MDMs, suggesting a tumor-intrinsic influence on myeloid recruitment and differentiation.[27]

Functionally, TAMs play both immunostimulatory and immunosuppressive roles, depending on their transcriptional states and microenvironmental cues. Flow cytometry and bulk RNA-seq studies typically categorized macrophages along a binary M1 (pro-inflammatory) versus M2 (immunosuppressive) polarization model.[27,64] However, single-cell approaches have largely deconstructed this framework, demonstrating that glioma TAMs exhibit a spectrum of activation states that cannot be classified into simple M1/M2 categories.[50,65] Instead, TAMs dynamically switch between inflammatory and immunosuppressive programs based on signals from malignant cells, T cells, and other components of the tumor microenvironment.[66] Recent scRNA-seq studies also build upon our existing understanding of sex-dependent differences in immunomodulatory myeloid cells in glioma. Ochocka et al confirm that males have a greater proportion of infiltrating immunosuppressive cells of monocytic lineage, while females are enriched for cells of granulocytic lineage with pronounced pro-inflammatory character.[67]

Importantly, while MG and MDMs maintain distinct transcriptional signatures, they also share common functional programs, highlighting the influence of local environmental signals over ontogeny (see the section "Immunomodulatory Functions of Myeloid Cells" below).

Monocytes

Peripheral monocytes heavily infiltrate glioblastoma and represent a critical component of the glioma immune microenvironment. Despite sharing canonical gene markers with monocytes in circulation, such as CD14, monocytes in the glioma TIME have distinct transcriptomic profiles including enrichment for *EGFR* ligands and pro-angiogenesis markers.[68]

Recently, Kirschenbaum et al developed and utilized "Z-man seq," a new technique that couples scRNA-seq with in vivo fluorescent labeling to trace cell fates over time, to reveal that large numbers of peripheral monocytes infiltrate glioblastoma in mouse models, with many displaying transcriptional signatures indicative of rapid differentiation into macrophages.[69] In parallel, lineage-tracing studies in pancreatic cancer suggest that while some monocytes successfully integrate into the tumor microenvironment and contribute to immunosuppressive TAM populations, a significant fraction fail to establish residency and undergo cell death shortly after infiltration.[70] A similar process may be occurring in glioblastoma, where only a subset of recruited monocytes ultimately contribute to long-term immunosuppressive functions within the tumor.

Spatial transcriptomic studies show that monocytes are particularly enriched in perivascular regions of glioblastomas, where they encounter cues that drive differentiation into TAMs.[45] While much of this process remains to be fully elucidated, these findings suggest that the recruitment and early fate decisions of monocytes may be a key determinant of the overall immune landscape. Understanding the mechanisms that regulate monocyte survival and differentiation could provide new therapeutic avenues to disrupt the continuous replenishment of immunosuppressive macrophages and potentially shift the tumor immune environment toward a more pro-inflammatory state.

Neutrophils

Neutrophils in the glioma TIME, also called tumor-associated neutrophils (TANs), are distinguished by their expression of canonical neutrophil marker genes such as *CD15*, *CD16*, *CD66b*, and *S100A9*.[71] Notably, many scRNA-seq studies detect relatively few or no neutrophils, despite their known presence in tumors. Their low transcriptional activity and fragile nature have posed difficulty for robust characterization by single-cell techniques. Neutrophils are quick to lyse during dissociation, are lost in density gradient centrifuge isolations, and have low RNA content and high RNase activity leading to poor capture. Coupled with preliminary hypotheses that TANs were transient cells with limited function in the glioma TIME, many studies ignored their roles. Despite this, several studies have now revealed that neutrophils in glioma are an important component of the TIME.

Early applications of single-cell RNA sequencing to study TANs used samples from tumor-bearing mouse models of glioma. Notably, Veglia et al revealed that these TANs differ from peripheral neutrophils; they had immunosuppressive features and increased expression of chemotaxis genes.[72] A later study by Maas et al marked the first exhaustive investigation of TANs from patients with glioma using flow cytometry and bulk RNA sequencing.[71] They also identified that TANs and peripheral neutrophils are transcriptionally distinct, validating the findings from the mouse model.[71] In particular, TANs had increased expression of canonical

neutrophil marker genes and the chemokine receptors *CXCR1*, *CXCR2*, and *CXCR4*.[71]

A recent scRNA-seq study by Lad et al characterizes lineages and subtypes of TANs using glioma samples from both patients and mouse models.[38] They reveal that certain populations of TANs may differentiate from the skull marrow, perhaps explaining some of the distinction previously observed when compared to peripheral blood-circulating neutrophils.[38] Among these skull marrow-derived TANs, they identify an antigen-presenting, DC-like subtype that is theorized to promote anti-tumor activity in conjunction with cytotoxic T cells.[38] Remarkably, another recent study that used scRNA-seq to analyze samples from patients and mice also identifies populations of TANs in the glioma TIME with skull marrow lineage.[39] However, Zhao et al primarily find immunosuppressive subpopulations of TANs that promote tumor growth.[39] Further investigation of these TAN populations is needed to resolve their immunomodulatory roles in the glioma TIME.

Dendritic Cells (DCs)

DCs in the glioma TIME are distinguished by their expression of *AREG*, *FCER1A*, *LAMP3*, *CD1c*, and MHC-encoding genes such as HLA-DRA, HLA-DRB1, and HLA-DPB.[47] Despite their limited number, this unique transcriptional profile enables most single-cell studies to annotate DCs.[48–50] Some further classify these cells into more granular clusters corresponding to known subtypes: cDC1 (conventional DC 1), cDC2 (conventional DC 2), cDC3 (a.k.a. migratory DC, LAMP3$^+$ DCs, or mregDCs), and/or moDC (monocyte-derived DC).[47,73] A majority of studies conclude their discussion of DCs at that point, resulting in very few single-cell analyses that have tried to elucidate their roles in the glioma TIME.[47,73] Friedrich et al outline, through scRNA-seq of IDH-wildtype and IDH-mutant mouse models of glioma, that DCs in the TIME have lower expression of genes related to antigen presentation compared to those in circulation.[73] While unclear why, they also observe a more pronounced decrease in expression of these genes among IDH-mutant samples.[73] As DCs are critical for antigen processing and presentation to cytotoxic cells of the adaptive immune system, this dysfunctional phenotype in the glioma TIME likely contributes to the lack of immune response. Further investigation into the DC populations is necessary to understand the implications of these transcriptional changes.

Immunomodulatory Functions of Myeloid Cells

The functional activity of myeloid cells helps define the overall immune state of gliomas and their response to therapy. scRNA-seq studies have consistently identified inflammatory and immunosuppressive myeloid populations in the glioma TIME. Many studies define these populations within the constraints of cell type-centric classifications, often defining clusters first by cell type (such as MG or macrophage) before categorizing them with inflammatory or immunosuppressive descriptors. Another designation that has been widely used is myeloid-derived suppressor cells (MDSCs), which encompass immunosuppressive cells derived from either monocytic (M-MDSCs) or granulocytic lineages (G-MDSCs).[74] A recent study by Jackson et al further distinguished MDSCs into M-MDSCs and early (E)-MDSCs, illustrating the evolving landscape of myeloid state classifications.[51] Indeed, each scRNA-seq study focused on myeloid cells in glioma tends to have their own cell state annotations based on how their specific clusters were divided. Although these classifications have provided many useful insights, cluster-based gene signatures often share functional gene sets, making it difficult to isolate and study cell functions.

We recently led an effort to comprehensively profile the functional gene programs exhibited by myeloid cells in glioma to create a consensus classification for the field.[45] Our effort utilized a recently developed scRNA-seq computational method, consensus non-negative matrix factorization (cNMF)[55,75] With traditional Louvain/Leiden clustering methods, which are typically projected on a UMAP, closely related cells are clustered and gene signatures are created based on average expression of genes across all cells in 1 cluster compared to cells in all other clusters. These gene signatures are then annotated based on prior literature to identify cell states. In contrast, cNMF, which builds on NMF methods used for scRNA-seq,[9,76] uses factorization rather than clustering to identify discrete sets of genes that are coordinately regulated across all cells in an unbiased fashion, labeling their gene expression "programs." These programs can then be annotated based on prior literature and are typically labeled as either a cell identity program, which contains classic cell type genes, or an activity program, which contains genes that indicate a cellular function. Importantly, individual cells can express more than 1 gene expression program, enabling the isolation of activity programs that define cell function from cell identity programs that define cell type.[55]

Among other programs, our work has revealed 4 major immunomodulatory activity programs that are expressed by over 90% of myeloid cells in glioma: 2 immunosuppressive programs (Complement and Scavenger) and 2 inflammatory programs (Systemic and Microglial).[45] These programs are expressed by multiple myeloid cell types, meaning that a cell could co-express the MG identity program and the Systemic inflammatory program while another cell could co-express the Macrophage identity program and the System inflammatory program. In fact, the same Systemic inflammatory program can be expressed by MG, macrophages, monocytes, and DCs. The other immunomodulatory programs could also be co-expressed across different cell types. As such, we found these immunomodulatory programs were expressed by cells across traditional cell clusters defined by Louvain/Leiden clustering, which are typically driven by cell type. This helps explain the mixed gene signatures often seen across myeloid cell clusters. It also means that these 4 programs were previously unable to be studied in isolation because they were mixed in with cell-type signatures during standard differential expression analysis across clusters. We found in our analysis that it was these 4 immunomodulatory programs, rather than the cell type programs, that drove the overall immune state of the tumor and were associated with clinical phenotypes.

The following immunomodulatory program sections outline our perspective of these new findings and harmonize how they relate to prior studies as well as their implications for tumor progression and immunotherapy.

Immunosuppressive Gene Expression Programs - Complement

The Complement Program is the most prevalent immunosuppressive program across the myeloid compartment and is active in nearly all myeloid cell types except for TANs.[45] It is defined by the high expression of opsonization components of the complement system, including *C1QA*, *C1QB*, *C1QC*, and *C3*, which have been associated with tissue remodeling and immune suppression.[77] A pan-cancer (non-CNS tumors) scRNA-seq atlas of tumor-associated myeloid cells identified C1Q$^+$ clusters across nearly all tumor types,[78] and we found our Complement program was expressed in these cells.[45] C1Q$^+$ clusters were also identified in prior scRNA-seq studies of glioma-associated myeloid cells and macrophages in both patients and murine glioma models.[47,59] The activation of this program in gliomas is spatially enriched within vascular regions and correlates with reduced infiltration and activation of cytotoxic T cells.[45] Notably, this program is highly responsive to glucocorticoid receptor signaling, which can be activated by endogenous glucocorticoids such as the stress hormone cortical, but is further activated in glioblastoma patients due to widespread usage of the potent synthetic glucocorticoid dexamethasone.[79] These findings suggest that standard-of-care corticosteroid treatments may inadvertently reinforce tumor-mediated immune suppression, underscoring the need for alternative symptom management strategies.

Immunosuppressive Gene Expression Programs - Scavenger

The Scavenger Program represents a brain-specific immunosuppressive signature that is distinct from the Complement Program and is restricted to monocytes and MDMs.[45] This program is defined by the upregulation of scavenger receptors, including *MRC1* (*CD206*), *MSR1* (*CD204*), *CD163*, *LYVE1*, and *COLEC12*, which are known to suppress T-cell proliferation and promote tumor immune evasion. Unlike the Complement Program, which was excluded from hypoxic niches in the tumor, the Scavenger Program is enriched in hypoxic niches, indicating a strong role for the tumor microenvironment in shaping myeloid-mediated immune suppression.[45] Interestingly, this program can be identified across multiple studies in the cell types or programs that are most significantly associated with poor immunotherapy response and overall survival. Mei et al found that *SIGLEC9* expression in myeloid cells was associated with poor response to checkpoint blockade in glioblastoma patients.[80] Further evaluation of these data demonstrated that *SIGLEC9*$^+$ cells could be stratified by their expression of our 4 immunomodulatory programs and that only those expressing the Scavenger program were associated with poor response. Jackson et. al. also showed that M-MDSCs and E-MDSCs were associated with poor survival.[51] Both of these MDSC populations express the Scavenger program, indicating that this program is associated with poor outcomes across multiple cohorts.

Together, these immunosuppressive programs contribute to T-cell exclusion, checkpoint blockade resistance, and poor patient prognosis. Targeting these programs, either through inhibiting their activation signals, reprogramming myeloid cells toward an inflammatory state, or selectively killing cells that express them, represents a promising avenue for glioma immunotherapy.

Inflammatory Gene Expression Programs - Systemic

The Systemic Inflammatory Program is characterized by the expression of genes associated with acute inflammatory responses and systemic immune activation, including *IL1B*, *IL1A*, *CCL3*, *CCL4*, *TNF*, and *CXCL8*. This program is observed in all major myeloid cell types, including monocytes, macrophages, MG, and DCs, and is likely the default inflammatory program for bone marrow-derived myeloid cells. We found it across all CNS and non-CNS tumors we analyzed[45] and prior scRNA-seq studies of many inflammatory pathologies identify myeloid cell clusters expressing similar genes.[12,50,70,78,81] This program was even found to be expressed by circulating monocytes in glioma patients, which may serve as the starting population for many glioma-infiltrating monocytes.[45] Despite their inflammatory signature, myeloid cells expressing this program in the glioma TIME not only fail to mount an effective anti-tumor response but may instead promote immunosuppression and tumor progression. Chen et al show, through scRNA-seq, that IL1B deletion in glioma-bearing mice reduces tumor growth and improves survival compared to the wildtype glioma model,[82] while Kai et. al. show that IL1B stimulates malignant cell proliferation via Stat3/NFKB signaling.[83] Spatially, the myeloid cells expressing the Systemic inflammatory program are found in hypoxic tumor niches, where the potently immunosuppressive Scavenger program is also enriched.[45] This co-localization, along with several mechanistic studies,[45] suggests that immunosuppressive programs might be activated as a potential negative feedback mechanism in response to inflammatory cytokine secretion. This effect, which may be an evolved mechanism to limit inflammation in the brain, could potentially be therapeutically targeted to improve patient outcomes.

Inflammatory Gene Expression Programs - Microglial

The Microglial Inflammatory Program is a brain-specific inflammatory signature that is likely the default inflammatory program in MG. It is enriched in MG but also observed in a subset of MDMs and DCs that respond to microenvironmental cues.[45] This program is defined by the upregulation of monocyte and lymphocyte recruitment factors (*CXCR4*, *CXCL12*, *CCL3*, and *CCL4*) and immediate stress response genes (*RHOB*, *JUN*, and *KLF2*). Unlike

the Systemic Inflammatory Program, which is present in both circulating and tumor-infiltrating myeloid cells, the Microglial Inflammatory Program is unique to the brain TIME and may be influenced by neural-glial interactions. Spatially, these cells localize to vascular and inflammatory niches, where they may act as a bridge between innate and adaptive immune responses. This program is enriched in lower-grade gliomas and normal brain and may play a tumor-suppressive role.

Implications for Immunotherapy

The identification of these 4 immunomodulatory programs has major implications for the development of next-generation glioma immunotherapies. The immunosuppressive programs represent major barriers to the efficacy of immune-checkpoint blockade, chimeric antigen receptor (CAR)-T therapy, oncolytic viral therapies, neoantigen vaccines, and other immunotherapies. Conversely, inflammatory programs reveal potential strategies for reprogramming myeloid cells toward a pro-inflammatory state to enhance anti-tumor immunity.

Key translational takeaways include:

- Targeting the Scavenger and Complement Programs may improve responses to immunotherapy by preventing myeloid-driven immunosuppression.
- Optimizing dexamethasone usage or developing alternative methods to manage cerebral edema may help prevent the induction of the Complement Program and its immunosuppressive effects.
- Leveraging inflammatory programs could enhance anti-tumor immune responses, though therapeutic strategies must ensure that this does not lead to symptomatic brain edema or inadvertently stimulate immunosuppression through IL1B production.
- Spatial and dynamic profiling of myeloid cells in clinical trials could provide real-time insights into how immunotherapies reshape the TIME, enabling the development of more adaptive treatment strategies.

As the field moves beyond cell type-based classifications to functional program-based analyses, integrating single-cell and spatial transcriptomic data into rational clinical trial design will be crucial for overcoming myeloid-driven immunosuppression and enhancing the efficacy of glioma immunotherapies.

Lymphoid Compartment

T cells, natural killer (NK) cells, and B cells constitute the lymphoid compartment of the TIME. These tumor-infiltrating lymphocytes (TILs) make up a significantly smaller fraction of the glioma TIME.[84] Although myeloid cells in glioma exist in a spectrum with hybrid cell types and shared immunomodulatory programs, lymphoid cells are an evolutionarily younger set of cells with more defined and distinct cell states. Here, we highlight how single-cell methods have enhanced our understanding of TIL cell states and interactions with other cell types.

T Lymphocytes

Among the lymphoid compartment of the glioma TIME, T cells have been the most studied. Analyzing surgically resected patient samples, Mathewson et al first demonstrated that scRNA-seq recapitulates the longstanding subtypes of T cells in the glioma TIME.[85] CD8$^+$ T cells mount cytotoxic immune responses, while CD4$^+$ T cells describe both helper cells that assist immune cell activation and regulatory cells that suppress cytotoxicity (Tregs).[86] Nearly all subsequent scRNA-seq studies that identify TILs in glioma identify clusters of cells that match or expand these subtypes.[47,49,50,81,85,87,88]

CD8$^+$ T cells

Gliomas are described as "immunologically cold" due to poor infiltration of CD8$^+$ cytotoxic T cells into the tumor.[86,89] While most analyzed gliomas do have relatively low numbers of CD8$^+$ T cells, this analysis typically occurs on surgical resections after patients have been treated with corticosteroids, such as dexamethasone, to reduce inflammation and edema in the brain. Corticosteroids are known to ablate T-cell populations[90] and there is evidence that tumors that have not seen dexamethasone can have higher numbers of T cells.[45] Nonetheless, many scRNA-seq studies of glioma detect CD8$^+$ T cells,[47,49,50] and some further cluster them into subsets annotated by their putative cell states.[81,85,87]

Of these, "effector" and "memory" are commonly identified CD8$^+$ T-cell states, while "naive" and "cycling" are also noted in some studies.[45,81,85,87] When effector CD8$^+$ T cells are activated, they mount a targeted cytotoxic response against malignant cells. Although the exact marker genes used to identify these sub-clusters may vary across studies, they are either markers of activation like *CTRAM*, *EOMES*[81] or cytotoxicity-related genes such as *GZMA*, *GZMB*, *GZMH*, *GZMY*, or *GZMK*.[81,85,87] These granzymes enable effector cells to induce apoptotic cell death in target cancer cells. Memory CD8$^+$ T cells are highly specific, long-lived cells that rapidly differentiate into cytotoxic effector cells upon re-encountering their target antigen. Studies of glioma using scRNA-seq further classify these cells into "effector memory" or "resident memory" states[81,85,87] based on whether the TILs recirculate in peripheral blood or not.[91] Transcriptomically, they are distinguished by expression of *IER2*/*BCL2* (effector memory)[81,85,87] or *CD103*/*CD39* (resident memory).[81,85,87]

In other cancers, "exhausted"/"dysfunctional" states that include T cells impaired by immunosuppressive signaling are well described and the target of some immunotherapies. These exhausted T cells are no longer cytotoxic and can be identified by their expression of *PD1*, *CTLA-4*, *TIM3*, *LAG-3*, and *TIGIT*.[81,87] Interestingly, these cells are described in some glioma studies,[81,87] but not found in others.[45,85] No consensus exists on why T cells exhibit markers of exhaustion in the glioma TIME, whether driven by excessive antigen exposure leading to receptor exhaustion (as seen in chronic viral infections and other cancers) or by microenvironmental signals that induce dysfunction even with limited antigen interaction.[92] Ravi et al employ

pseudotime trajectory analysis of scRNA-seq data to describe that the transition from effector to exhausted state might be mediated by IL10.[81] Subsequent spatial RNA-seq yielded a population of *CD163*$^+$/*HMOX1*$^+$ myeloid cells that drive this dysfunctional transition in CD8$^+$T cells.[81] Complementing observations from mouse models and flow cytometry, unbiased scRNA-seq analysis of patient glioblastoma samples by Lee et al identify that CD8$^+$T-cell exhaustion stratifies by sex: males have higher expression of canonical gene markers of exhaustion while females have higher expression of effector cytokines (*IFN*, *TNF*, *GZMK*).[93] Remarkably, Dobersalske et al find that some CD8$^+$T cells in the glioma TIME may have originated from skull marrow, and of which, nearly a third exhibited characteristics of exhaustion—a greater proportion than CD8$^+$T cells that infiltrated from the periphery.[40]

Some studies also delineate a distinct "stressed" state in CD8$^+$T cells that are distinguished by their expression of heat shock proteins such as *HSPA1A*, *HSPH1*, *HSPA6*.[81,85] These cells are suspected to have diminished function as a response to cellular stress, possibly from the hypoxic, nutrient-deficient microenvironment in glioma[92] or the tissue dissociation protocol prior to scRNA-seq.[94]

Mathewson et al also reveal a cytotoxic T-cell subpopulation that expresses canonical markers of NK cells.[85] These "NK-like" cells most notably express *CD161*, an inhibitory receptor that, when bound to its ligand *CLEC2D*, can hamper their anti-tumor activity.[85] Since prior scRNA-seq studies of the glioma TIME identify *CLEC2D* expression in malignant and myeloid populations,[6,9] it is possible that *CD161*-mediated inactivation of these "NK-like" T cells might contribute to immunosuppression.[85]

CD4$^+$ T cells

Early IHC studies of the glioma TIME evaluated the relative abundance and spatial infiltration of CD4$^+$T cells. Han et al suggest a positive correlation between tumor grade and CD4$^+$T-cell abundance, a trend opposite to that observed with CD8$^+$T cells.[95] More recent studies using scRNA-seq characterize the diverse subpopulations within CD4$^+$T cells.

Of the 2 major branches of CD4$^+$T cells, those with "Helper" functions activate other immune cells to mount an effective adaptive immune response. Most scRNA-seq studies subdivide helper CD4$^+$T cells into canonical subtypes based on their expression of inflammatory cytokines including Th1 (IFNγ and TNF) and Th17 (IL-17 and IL-22).[81,85,87] Remarkably, Mathewson et al identify a population of cytotoxic CD4$^+$ cells that express similar cytotoxic gene markers as effector CD8$^+$T cells. This finding motivates future study of shared functional activities in lymphocytes, potentially with the new frameworks currently applied to myeloid cells. Some CD4$^+$T cells in the glioma TIME may also be dysfunctional, with impairment to their conventional, helper functions. As Khan et al observe through their scRNA-seq analysis of experimental mouse models of glioma, there is an association between the abundance of dysfunctional CD4$^+$T cells and exhausted CD8$^+$T cells—further accentuating the immunosuppressive nature of the TIME.[96]

On the other hand, Regulatory CD4$^+$T cells (Tregs) impair anti-tumor activity in "effector" TILs. Distinguished by their expression of CD4 and FOXP3, Tregs function by secreting immunosuppressive cytokines such as IL-10 and TGF-β.[97] Through flow cytometry and bulk RNA sequencing, it was revealed Treg abundance may be higher in IDH-wildtype glioma than in IDH-mutant tumors.[27] In scRNA-seq studies, Tregs are grouped into at least 1 cluster,[81,85,87] but due to their limited number, sub-classifying them into specific subtypes remains challenging. Despite this, scRNA-seq has enabled correlation analyses that reveal associations between Tregs and other cell types. Notably, we identify a spatial association between Tregs and populations of immunosuppressive myeloid cells in the glioma TIME.[45]

NK Cells

NK cells, also capable of cytotoxic activity, are a prominent, growing area of study in the glioma TIME. As innate immune cells of lymphoid origin, they recognize and target malignant cells that lack MHC expression. Identified by their expression of canonical marker genes *CD56* and *CD16*, NK cells are thought to more effectively infiltrate glioma than T cells.[98] However, through scRNA-seq studies of both mouse models and patients, studies reveal that NK cells in the glioma TIME are dysfunctional.[99,100] Compared to NK cells in the periphery, those in the TIME have decreased expression of cytotoxicity markers, lower IFN-gamma levels, and display markers of NK cell exhaustion such as *KLRG1*.[98–100] Remarkably, comparing the peripheral NK cells of glioblastoma patients with healthy donors using functional assays also indicates decreased cytotoxicity.[101] Nonetheless, further investigation of NK cells using single-cell methods is necessary to further define their cell states to inform future therapeutic strategies.

B Lymphocytes

There is still little consensus about the roles that B cells play in the TIME of glioma. In most single-cell studies of TILs in glioma, B cells are of little abundance and are subsequently not the focus of analyses. That said, preliminary findings by Hou et al suggest that B cells in the TIME may be more activated than donor-matched peripheral B cells and may play a role in response to PD-1 blockade in glioblastoma.[102] Evaluating how to design single-cell experiments to capture and study a greater number of B cells can inform future investigation of their roles in the glioma TME and motivate potential therapeutic applications.

Discussion

The use of single-cell technologies has transformed our understanding of the immune microenvironment in gliomas, revealing the cellular diversity and functional plasticity of immune populations within the TIME. Glioblastoma and other diffuse infiltrating gliomas present unique challenges to effective immunotherapy due to their highly immunosuppressive microenvironment, which actively limits

immune cell infiltration and functionality. Historically, characterization of the glioma immune microenvironment relied on histological techniques and bulk RNA sequencing, which provided only a partial view of immune cell diversity and their roles in immunosuppression. The advent of high-dimensional single-cell technologies—including scRNA-seq, spatial transcriptomics, and highly multiplexed proteomics—has provided new insights into how immune cells interact with glioma cells and how these interactions contribute to tumor progression, therapeutic resistance, and immune evasion.

A key focus of recent scRNA-seq studies has been on characterizing myeloid cells, which dominate the immune landscape in gliomas and play critical roles in shaping the immunosuppressive TIME. These cells, including the major populations of MG and MDMs, have been traditionally studied through the lens of their developmental origins and classic cell-type surface markers. Though this approach has identified new cell states and subpopulations, it also revealed that myeloid cells exist on a continuum of states that make individual cells difficult to classify with traditional scRNA-seq clustering methods.[45,54] Importantly, these studies have shown myeloid states are not static; they shift dynamically in response to cues from malignant and other cells in the microenvironment, as well as in response to treatment, underscoring the remarkable plasticity of myeloid cells in gliomas.

Recent computational approaches, such as cNMF, have enabled more precise analyses of these functional programs across glioma samples. Unlike traditional clustering methods that categorize cells in discrete clusters heavily influenced by underlying cell type, cNMF is better at understanding cells that exist on a continuum of cell states. It allows researchers to deconstruct total cell states into component gene expression programs that, in combination, determine the cell state. This approach has led to the identification of key immunosuppressive programs that operate across different myeloid cell types. It allows for the isolation and study of functional activity programs, such as the Scavenger and Complement programs, which are critical to glioma immune evasion. These programs are associated with worse patient outcomes and resistance to immune-checkpoint inhibitors, underscoring the need to develop therapies that target these specific immunosuppressive pathways. Additionally, scRNA-seq analysis by cNMF has revealed that the use of glucocorticoids such as dexamethasone, a frequently administered treatment for glioma patients, strongly induces an immunosuppressive gene program in myeloid cells. These insights suggest that clinical trials should consider the impact of glucocorticoids on the immune microenvironment and explore alternative strategies to reduce peritumoral edema without compromising anti-tumor immune responses.

Much of the current focus in glioma immunotherapy has been on T-cell-based therapies, namely immune-checkpoint blockade[103] and CAR-T cells,[104] and single-cell technologies are increasingly facilitating more refined approaches to optimizing T-cell therapies in gliomas. For example, scRNA-seq analyses of TILs have revealed that T cells within the glioma TIME often adopt dysfunctional, exhausted states that limit their cytotoxic potential. Recent single-cell studies have highlighted key inhibitory pathways contributing to T-cell dysfunction in gliomas. For example, *CD161*, an inhibitory receptor expressed in T cells, interacts with its ligand *CLEC2D*, which is expressed in glioblastoma cells.[85] Blocking this interaction with an antibody has been shown to reinvigorate dysfunctional T cells and restore their anti-tumor activity.[85] Future CAR-T cell therapies could leverage single-cell data to identify novel antigens expressed by glioma cells, thereby expanding the repertoire of potential CAR targets beyond conventional antigens such as *EGFRvIII* and *IL13Ra2*. Moreover, scRNA-seq could be used to design CAR-T cells that are armored against the immunosuppressive TIME, either by engineering resistance to myeloid-derived inhibitory signals or by including additional functions to modulate the myeloid compartment directly. For example, dual-function CAR-T cells that both target glioma cells and secrete cytokines to reprogram myeloid cells toward a pro-inflammatory state could overcome some of the barriers to successful T-cell therapies in gliomas.

Another emergent approach to effectively modify the glioma TIME is based on the use of oncolytic viruses. These modified neurotropic viruses can have a dual anti-tumor effect by both directly targeting glioma cells and promoting T-cell infiltration into the tumor.[105] This approach was recently demonstrated in a phase 1 study of an oncolytic herpes simplex virus (oHSV) for progressive glioblastoma, where repeated oHSV injections led to increased $CD8^+$ and $CD4^+$ T-cell infiltration, as well as a decrease in regulatory T cells, a shift in T-cell populations consistent with TIME reprogramming toward a more pro-inflammatory and anti-tumor state.[106] The design of this study also allowed for longitudinal tumor sampling during treatment administration, enabling assessment of anti-tumor response and repeated TIME characterization.[106] Another phase 1 study co-administered adenovirus-expressing the conditionally cytotoxic HSV1 thymidine kinase, HSV1-TK (Ad-hCMV-TK), and adenovirus-expressing the FMS-like tyrosine kinase ligand 3, Flt3L (Ad-hCMV-Flt3L), which aids with the recruitment of DCs to the TIME, both under the control of the major immediate early human cytomegalovirus promoter to the post-resection cavity followed by oral valacyclovir in combination with radiation and chemotherapy in patients with newly diagnosed high-grade glioma.[107] In this study, analysis of post-treatment samples also demonstrates increased anti-tumor immune response as evidenced by an increase in $CD8^+$ T-cell infiltration.[107] Lastly, personalized neoantigen vaccines provide another avenue to improve the specificity of the anti-tumor response to diffuse infiltrating gliomas,[108] with prior studies providing evidence of cytotoxic T-cell responses targeted to tumor neoantigens.[109]

Beyond T-cell-based approaches, recent insights into the molecular drivers of myeloid immunosuppressive programs provide potential therapeutic targets for modulating the glioma immune microenvironment. For instance, small molecules that disrupt the Scavenger or Complement programs could reduce immune suppression and enhance the efficacy of T-cell-based therapies. These strategies could be particularly effective when combined with checkpoint inhibitors or CAR-T cells, as reprogramming the myeloid compartment may enhance the ability of cytotoxic T cells to infiltrate and kill tumor cells. Additionally, recent

trials with agents such as colony-stimulating factor 1 receptor (*CSF1R*) inhibitors[110] or *CD47* blockers,[111] which aim to reduce myeloid-driven immune suppression, may benefit from integrating single-cell analyses to more precisely target specific subsets of myeloid cells that contribute to tumor progression.

Looking ahead, future clinical trials could focus on reprogramming the glioma immune microenvironment to shift the balance from immunosuppressive to pro-inflammatory states. Single-cell technologies could be used in real-time during trials to monitor how immune cell states evolve during treatment, enabling adaptive trial designs that account for dynamic changes in the TME. For example, scRNA-seq could identify emerging immunosuppressive states in response to therapy, allowing for the timely addition of combination treatments to overcome resistance. This adaptive approach could improve outcomes in both primary glioblastoma and recurrent disease settings.

Beyond gliomas, these insights into the immune microenvironment have broader implications for other CNS pathologies, including brain metastases, multiple sclerosis, and Alzheimer's disease, where myeloid cells play key roles in disease progression.[45,112] Applying single-cell technologies and program-level analyses across these conditions could help identify shared and disease-specific immune programs, paving the way for novel therapeutic strategies targeting myeloid cells in a range of CNS diseases. For example, insights gained from glioma studies could be leveraged to design immunotherapies that modulate the immune microenvironment in brain metastases, which share similar immunosuppressive features.

In conclusion, single-cell technologies have revolutionized our understanding of the glioma immune microenvironment, shifting the focus from cell types to functional programs that define immune cell behavior. By identifying key immunosuppressive programs and their molecular drivers, these studies lay the groundwork for developing therapies that reprogram the immune microenvironment to enhance anti-tumor immunity. The next generation of clinical trials should integrate these insights to design more effective combination therapies and explore novel strategies to target the glioma TME. Ultimately, the ability to modulate immune programs at a functional level may hold the key to overcoming the immunosuppressive barriers that have limited the success of immunotherapies in glioblastoma and other CNS malignancies.

Keywords

glioma | single | cell RNA sequencing | tumor immune microenvironment (TIME)

Conflict of interest statement

T.E.M. is an equity holder and advisor to Reify Health and Care Access Research. L.N.G.C. receives consulting fees from Elsevier, Oakstone Publishing, Prime Education, Medscape, BMJ Best Practice and Servier, and research funding from Merck & Co (to the Dana-Farber Cancer Institute). M.L.S. is an equity holder, scientific co-founder, and advisory board member of Immunitas Therapeutics. V.S.K. has declared no conflict of interest.

Funding

T.E.M. is supported by the UK Brain Tumour Charity's Future Leaders Awards (GN-000722) in partnership with the Jake McCarthy Foundation and the Ria Melvin Fund, the SITCBristol Myers Squibb Postdoctoral Cancer Immunotherapy Translational Fellowship, and the NCI K08 award K08CA276819. L.N.G.C. is supported by the Robert Wood Johnson Foundation.

Supplement sponsorship

This article appears as part of the supplement "Single-Cell Technologies," sponsored by the Princess Margaret Cancer Research Centre and the Toronto Western Hospital Division of Neurosurgery.

Author contributions

All authors participated in consensus discussions, contributed to writing, and approved the manuscript.

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