

## Review

## Incorporating Tumor-Associated Macrophages into Engineered Models of Glioma

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## SUMMARY

**Tumor progression is profoundly influenced by interactions between cancer cells and the tumor microenvironment (TME). Among the various non-neoplastic cells present, immune cells are critical players in tumor development and have thus emerged as attractive therapeutic targets. Malignant gliomas exhibit a unique immune landscape characterized by high numbers of tumor-associated macrophages (TAMs). Despite encouraging preclinical results, targeting TAMs has yielded limited clinical success as a strategy for slowing glioma progression. The slow translational progress of TAM-targeted therapies is due in part to an incomplete understanding of the factors driving TAM recruitment, differentiation, and polarization. Furthermore, the functions that TAMs adopt in gliomas remain largely unknown. Progress in addressing these gaps requires sophisticated culture platforms capable of capturing key cellular and physical TME features. This review summarizes the current understanding of TAMs in gliomas and highlights the utility of *in vitro* TME models for investigating TAM-cancer cell cross talk.**

## INTRODUCTION

The term “glioma” encompasses a broad group of primary brain tumors that arise from glial stem or progenitor cells. Glioblastoma (GBM) accounts for 70% of all gliomas and is the most malignant form of primary brain cancer in adults, with a median survival of less than 15 months (Goodenberger and Jenkins, 2012; Stupp et al., 2007). Current standard therapy for GBM includes maximal surgical resection followed by radiotherapy and temozolomide chemotherapy and can include bevacizumab and tumor-treating fields. Although standard treatment may prolong patient survival, it is not curative, and the majority of patients experience tumor recurrence. In an effort to identify new therapeutic targets that are not cell-autonomous, the tumor microenvironment (TME) has attracted much attention as a key regulator of glioma progression and therapeutic resistance (Charles et al., 2011; Quail and Joyce, 2017; Tomaszewski et al., 2019). Gliomas contain various non-neoplastic cells, such as vascular and immune cells, that co-evolve with cancer cells to create an environment that fosters tumor growth.

Although the importance of investigating the individual and collective contributions of each stromal population is undeniable, the striking rise in US Food and Drug Administration-approved cancer immunotherapies has motivated studies investigating the glioma immune microenvironment. Malignant gliomas are one of the most immunosuppressive solid tumors (Thorsson et al., 2018). GBM tumors contain very few infiltrating T cells and instead are dominated by brain-resident microglia and infiltrating monocytes/macrophages, collectively termed tumor-associated macrophages (TAMs) (Giering et al., 2017) (Figure 1A). To date, immunotherapy has been shown to be most beneficial in patients with tumors containing high numbers of infiltrating T cells, such as melanoma and lung cancer (Bonaventura et al., 2019). The relative paucity of T cells along with the low mutation burden of GBM tumors has made the development of effective T cell-based immunotherapies challenging (McGranahan et al., 2019). TAMs are the most abundant non-neoplastic cell type in the GBM TME and can comprise up to 40% of the total cells in gliomas (Hambarzumyan et al., 2015). Clinical studies have shown a correlation between TAM accumulation and poor patient prognosis for multiple cancer types, including brain tumors (Mantovani and Allavena, 2015; Müller et al., 2017; Shih et al., 2006). There has been considerable interest in targeting TAMs for glioma immunotherapy, but despite encouraging results from preclinical studies, clinical success has been limited.

The slow translational progress of TAM-targeting therapies is, in part, due to an incomplete understanding of the phenotypes and functions that macrophages adopt in gliomas (Pires-Afonso et al., 2020). Although

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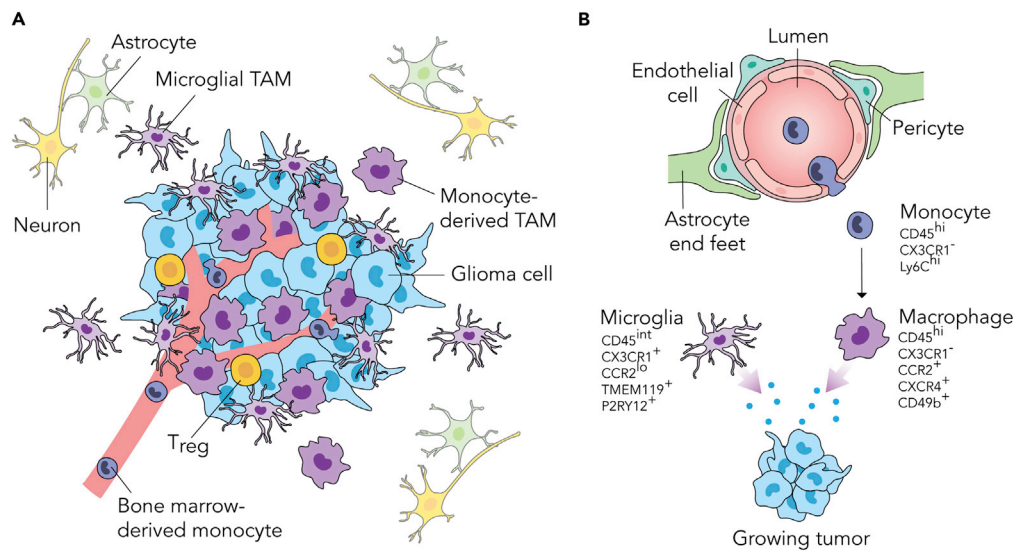
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**Figure 1. Tumor-Associated Macrophages (TAMs) in Gliomas**

(A) The cellular microenvironment of gliomas is composed of many unique cell populations including brain-specific cell types such as astrocytes and neurons, as well as immune and vascular cells. TAMs are the most abundant non-neoplastic cell population in gliomas, the majority of which are blood-derived infiltrating monocytes/macrophages that localize primarily in the perivascular and necrotic regions. Microglial TAMs are more abundant in the peritumoral regions.

(B) TAMs arise from two distinct sources: bone marrow-derived monocytes and brain-resident microglia. In the tumor-bearing brain, the blood-brain barrier is impaired, resulting in the infiltration of blood-derived monocytes into tumors, where they differentiate into macrophages. Resident and infiltrating TAMs are recruited to the growing tumor in response to a variety of tumor-secreted cytokines, including CCL2, GM-CSF, EGF, CXCL3, SDF-1, and CSF-1.

investigating intricate cell-cell interactions *in vivo* remains difficult, the emergence of novel *in vitro* tumor models offers the ability to perform detailed mechanistic discovery and screening in a controlled environment. Engineered *in vitro* models have proven instrumental in identifying chemical and physical microenvironmental factors that regulate glioma invasion and therapeutic resistance, and with the addition of multiple cell types, they offer the possibility to study the contribution of stromal cell populations, such as TAMs, on tumor progression (Bahlmann et al., 2020; Civita et al., 2019; Herrera-Perez et al., 2018).

In this review, we summarize the current understanding of TAMs in gliomas, with a focus on the regulation of TAM polarization. We discuss current strategies to interrogate TAM-cancer cell interactions and outline the advantages and limitations of each approach. Last, we highlight the potential of three-dimensional (3D) experimental platforms to provide a deeper understanding of the precise role of TAMs throughout glioma progression that will ultimately facilitate the development of novel TAM-targeting therapies.

## Tumor-Associated Macrophages in Gliomas

### TAM Ontogeny

TAMs in gliomas are believed to arise from two distinct sources: brain-resident microglia and bone marrow-derived monocytes (Hambardzumyan et al., 2015) (Figure 1B). Microglia are specialized immune effector cells that populate the central nervous system (CNS) during early embryogenesis. They are the sole resident immune population in the healthy adult brain and perform various functions related to immune defense and CNS maintenance (Ginhoux et al., 2013). In gliomas, the blood-brain barrier (BBB) is disrupted, and there is an infiltration of inflammatory monocytes into the tumor where they differentiate into macrophages (Laviron and Boissonnas, 2019).

There have been mixed reports of the abundance and composition of TAMs in brain malignancies relative to other infiltrating leukocyte populations such as T cells. Two recent studies used a multi-omics approach to characterize the TME of patients with gliomas or brain metastases and found that whereas TAMs comprise up to 80% of infiltrating leukocytes in gliomas, the majority of infiltrating leukocytes in brain metastasis are T cells (Friebel et al., 2020; Klemm et al., 2020). In the same studies, TAM composition was shown to depend on IDH mutational

status; more aggressive gliomas (IDH1<sup>WT</sup>) displayed a greater abundance of infiltrating bone marrow-derived TAMs than IDH1<sup>mut</sup> gliomas, which contained a higher proportion of resident microglial TAMs. In a patient-derived xenograft (PDX) GBM model, immunofluorescent staining of CX3CR1 (microglia marker) and CCR2 (bone marrow-derived monocyte/macrophage marker) revealed that the majority of TAMs in GBM are infiltrating bone marrow-derived macrophages (Zhou et al., 2015). Similar results were found using a genetically engineered mouse (GEM) model of GBM and combinatorial fluorescently activated cell sorting analysis (Chen et al., 2017). Consistent with data obtained in glioma mouse models, histological examination and single-cell RNA sequencing (RNA-seq) of primary human gliomas have also reported an abundance of monocyte-derived macrophages over microglia (Müller et al., 2017; Venteicher et al., 2017). In addition, patients with mesenchymal GBM, the most aggressive molecular subtype, have the greatest accumulation of infiltrating TAMs compared with less aggressive GBM subtypes and low-grade gliomas (Sørensen et al., 2018; Wang et al., 2017; Zeiner et al., 2019). Resident and infiltrating TAMs have been observed to preferentially colonize different regions within GBM tumors. Microglia are predominately found in peritumoral regions, whereas bone marrow-derived monocytes/macrophages occupy perivascular and necrotic regions (Chen et al., 2017; Darmanis et al., 2017). Elucidating the dynamics of TAM composition and localization throughout brain tumor development remains an active area of investigation.

### TAM Phenotypic Diversity

Macrophages, in particular, are known for their ability to alter their behavior in response to distinct micro-environmental cues through a process known as polarization. They are highly plastic cells that integrate input from cytokines, growth factors, and other stimuli, to adopt a diverse range of activation states and cellular functions. Macrophage polarization has traditionally been described using the M1/M2 classification system in which M1 and M2 represent two extreme phenotypes along a continuum of activation states (Murray, 2017; Sica and Mantovani, 2012) (Box 1). TAMs in gliomas are commonly portrayed as M2 macrophages due to their high expression of anti-inflammatory cytokines, scavenger receptors, pro-angiogenic factors, and extracellular matrix (ECM)-related proteins, all of which are often associated with M2 macrophages (Hambardzumyan et al., 2015). Although the M1/M2 polarization model has proven to be valuable for *in vitro* studies, its relevance to the *in vivo* functional states of macrophages in homeostatic and pathological settings is an ongoing topic of debate (Guilliams and van de Laar, 2015; Nahrendorf and Swirski, 2016; Sankowski et al., 2019). The maturation of high-dimensional characterization techniques including single-cell RNA-seq and time-of-flight mass cytometry (CyTOF) has provided an unprecedented opportunity to investigate the spatial and temporal dynamics of macrophage polarization in the CNS and other tissues (Mrdjen et al., 2018; Sankowski et al., 2019; Sevenich, 2018). Over the past 5 years, several groups have harnessed a combination of these techniques to phenotypically characterize TAMs isolated from human and murine gliomas, and their findings have raised questions regarding the relevance of the M1/M2 classification system. Genome-wide transcriptomic analysis of TAMs isolated from GL261 glioma-bearing mice identified distinct TAM activation states that only partially overlap with canonical M1/M2 gene signatures (Maas et al., 2020; Szulzewsky et al., 2015). In human gliomas, single-cell RNA-seq analysis identified individual TAMs displaying both pro-inflammatory (M1) and anti-inflammatory (M2) gene signatures (Darmanis et al., 2017; Müller et al., 2017). Furthermore, several *in vivo* studies have highlighted TAM ontogeny (resident versus infiltrating) as a predictor of TAM activation, which may explain the heterogeneous phenotypes reported in previous bulk TAM characterization studies (Bowman et al., 2016; Gabrusiewicz et al., 2016).

Macrophages are known as “professional phagocytes” for their ability to recognize and engulf foreign and host-derived debris (Arandjelovic and Ravichandran, 2015). Interestingly, whereas all macrophages display phagocytic activity, M2 macrophages exhibit enhanced phagocytosis of multiple substrates, including silica nanoparticles (Hopstädter et al., 2015) and antibody-opsonized target cells (Leidi et al., 2009). Recent studies have demonstrated that macrophages are capable of phagocytosing glioma cells (Hutter et al., 2019; Saavedra-López et al., 2020); however, phagocytosis may also play a pro-tumoral role in the TME. Phagocytosis is accompanied by the upregulation of matrix remodeling enzymes, including cysteine proteases, serine proteases, and matrix metalloproteinases (MMPs) that render the TME more conducive to cancer cell invasion (Porter et al., 2013). Phagocytosis-driven debris clearance and matrix degradation may exceed the anti-tumor effects occurring when phagocytosis kills individual tumor cells; however, this has not yet been explored in detail.

Given the multitude of chemical and biophysical signals within the TME, the phenotypic diversity and complexity of TAMs is not surprising. It is likely that TAMs are transcriptionally driven along a spectrum

of polarization states by integrating cues from their microenvironment that are reflective of tumor type, location, and stage (Yang et al., 2018). It is also likely that even within an individual tumor, TAMs exhibit regional variability in polarization with equally variable phenotypes and functions (Castro et al., 2017). Despite recent progress in phenotypically characterizing glioma TAMs, the factors responsible for pro-tumor TAM activation and maintenance are still unclear. Unraveling the complexity of macrophage polarization throughout tumor initiation and development will be instrumental in the design of novel TAM-targeted therapies.

Numerous studies have revealed that TAMs are critical for tumorigenesis and continued tumor growth in both low- and high-grade gliomas (Gutmann and Kettenmann, 2019). Tremendous progress has been made over the last decade to elucidate some of the molecular pathways and signaling molecules orchestrating TAM pro-tumor function. In brief, TAMs are active contributors to tumor growth, glioma cell invasion, ECM reorganization, the generation of an immunosuppressive TME, and neo-angiogenesis (Figure 2). TAMs have also been proposed to regulate tumor response to standard anticancer therapies (De Palma and Lewis, 2013) and have been shown to participate in glioma resistance to radiation (Wang et al., 2013) and anti-angiogenic therapy (Lu-emerson et al., 2013; Piao et al., 2012).

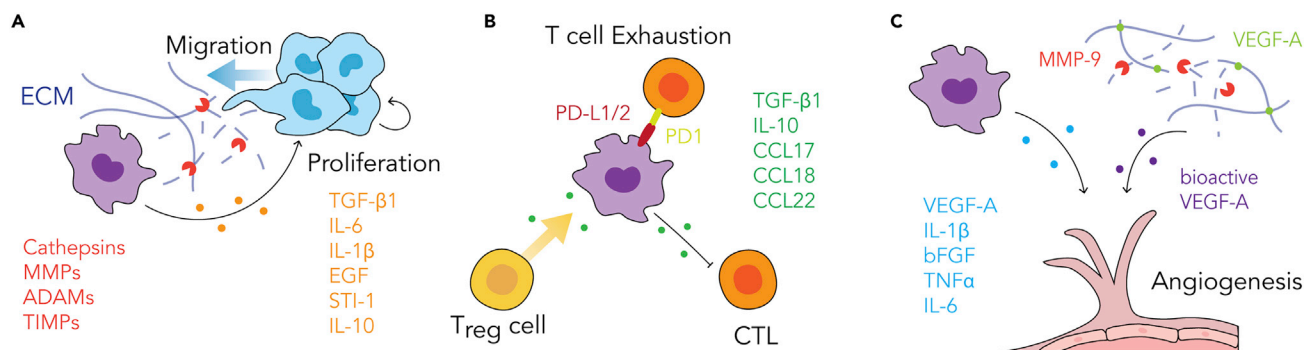
### *TAMs as Therapeutic Targets*

Considering the multifaceted roles of TAMs in tumor development and their correlation with poor overall survival, these cells are emerging as promising therapeutic targets. Major strategies targeting TAMs within the glioma TME include inhibiting TAM recruitment, depleting accumulated TAMs, and reprogramming pro-tumoral TAMs (Choi et al., 2018; Grégoire et al., 2020; Pires-Afonso et al., 2020). Multiple TAM-targeting agents are currently being investigated in early-phase clinical trials for patients with GBM (Table 1).

A common strategy of TAM-targeted therapies is to block the accumulation of monocytes and macrophages into the tumor by inhibiting pathways involved in macrophage recruitment, differentiation, and survival. A notable example is the inhibition of colony-stimulating factor 1/colony-stimulating factor 1 receptor (CSF-1/CSF-1R), a critical signaling axis in the differentiation and survival of macrophages (Stanley and Chitu, 2014). Administration of a small molecule CSF-1R inhibitor (BLZ945, PLX3397) blocked glioma progression and improved overall survival in multiple preclinical GBM models, including a PDGF-B-driven transgenic murine model and intracranial xenografts (Pyonteck et al., 2013; Yan et al., 2017). Surprisingly, the beneficial effect of CSF-1R inhibition was mediated by TAM reprogramming rather than TAM depletion. The authors subsequently found that TAMs within the glioma microenvironment survived CSF-1R inhibition and were reprogrammed into anti-tumor TAMs through glioma-secreted factors including GM-CSF and IFN- $\gamma$ . In a Phase II study of recurrent GBM, PLX3397 was well tolerated and readily crossed the BBB but showed limited efficacy compared with standard-of-care treatment (NCT01790503) (Butowski et al., 2016). Despite its modest clinical benefit as monotherapy, CSF-1R inhibition is gaining interest as combination therapy and is currently under clinical evaluation in combination with immune checkpoint inhibitors in patients with GBM (NCT02829723, NCT02526017) (Cannarile et al., 2017; Wesolowski et al., 2019).

Another signaling pathway that has gained recent attention for the treatment of multiple types of cancer is the C-C Motif Chemokine Ligand 2 (CCL2)/C-C Motif Chemokine Receptor 2 (CCR2) axis (Lim et al., 2016). CCR2 is expressed by monocytes and facilitates recruitment to tumors in response to tumor-secreted CCL2 (Deshmane et al., 2009). CCR2 inhibition has yet to enter glioma clinical trials, but has shown promise in preclinical models as well as in clinical trials for other types of cancer (Nywening et al., 2016). CCR2 inhibition in combination with immune checkpoint blockade (anti-PD-1) was recently shown to increase the accumulation of tumor-infiltrating lymphocytes and extend overall survival in immunocompetent mice bearing intracerebral KR158 and GSC005 tumors (Flores-Toro et al., 2020).

Although TAM-targeting agents have largely focused on TAM depletion, it is becoming increasingly evident that TAM reeducation may be a more effective anticancer strategy. TAM reprogramming can be achieved through multiple strategies: by suppressing tumor-promoting functions, stimulating anti-tumor functions, or directly converting pro-tumor TAMs into anti-tumor TAMs (Kowal et al., 2019). The signal transducer and activator of transcription 3 (STAT3) pathway has been identified as a potent regulator of pro-tumor TAM polarization and has emerged as a potential therapeutic target to enhance antitumor immune responses (Lee et al., 2011; Tugal et al., 2013). STAT3 blockade using the BBB-penetrant inhibitor



**Figure 2. Pro-tumor Functions of Glioma TAMs**

(A) TAMs facilitate glioma invasion through the secretion of various proteases and matrix-degrading enzymes including cathepsins, matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs), and a disintegrin and metalloproteinase (ADAM)-family proteases, which create a microenvironment conducive to cell invasion. TAMs also secrete various cytokines that promote glioma proliferation.

(B) TAMs foster an immunosuppressive TME through the secretion of anti-inflammatory cytokines, including IL-10, TGF- $\beta$ 1, and CCL17/18/22, which inhibit cytotoxic T lymphocyte (CTL) survival and recruit immunosuppressive regulatory T cells ( $T_{reg}$ ). TAMs also directly participate in T cell exhaustion through the PD1/PD-L1 signaling pathway.

(C) TAMs secrete pro-angiogenic cytokines and matrix-degrading enzymes, particularly MMP-9, which release matrix-bound cytokines and growth factors, such as VEGF-A, to promote angiogenesis.

WP1066 has been shown to induce pro-inflammatory activation in monocytes isolated from patients with GBM (Hussain et al., 2007). In a GL261 orthotopic immunocompetent murine model, combination treatment of radiotherapy (RT) and STAT3 inhibition reversed the immunosuppressive glioma environment, increased cytotoxic T cell effector functions, and led to enhanced median survival relative to treatment with RT or WP1066 alone (Ott et al., 2020). STAT3 inhibition with WP1066 is currently being investigated in phase I clinical trials for patients with GBM (NCT01904123).

One major hurdle in the development of TAM-targeted therapies is to minimize the occurrence of negative side effects in patients. Macrophages play multifaceted roles in tissue homeostasis and host defense, and as a result, systemic depletion may lead to increased risk of infection, tissue damage, and organ failure (Cannarile et al., 2017). In patients with a compromised immune system, such as those undergoing chemotherapy, these harmful implications are exacerbated (Poh and Ernst, 2018). The identification of novel markers and molecules exclusively expressed by pro-tumor TAMs will enable the development of therapies specifically designed to target pro-tumoral TAMs while preserving systemic macrophage function. An additional challenge is determining the optimal reprogramming strategy to generate effective tumor-fighting TAMs. A clearer understanding of the mechanisms underlying TAM polarization and function in gliomas is critical in the design of effective TAM-targeted therapies.

## EXPERIMENTAL MODELS TO INVESTIGATE TAMs

Both *in vitro* and *in vivo* studies have consistently demonstrated that TAMs accumulate within gliomas and are educated by TME factors to adopt tumor-supportive phenotypes (Hambardzumyan et al., 2015). TAM-targeting agents are emerging as promising anticancer therapies, but advancing these treatments to obtain maximal clinical benefit requires a detailed understanding of glioma-TAM interactions. In the remaining sections, we outline experimental models that have been used to study cancer-macrophage cross talk ranging from traditional cancer models to novel engineered *in vitro* platforms, specifically highlighting models of glioma.

### Traditional Experimental Models

Current preclinical glioma models include glioma cell line xenografts, PDXs, and GEM models (Miyai et al., 2017; Xiao et al., 2017). GBM xenografts are often fabricated using commercially available GBM immortalized cell lines U87, U251 T98G, and A172. Although relatively easy to grow, these cell line xenografts lack some characteristic GBM features, most notably the heterogeneity and highly infiltrative character of the tumor (Huszthy et al., 2012). PDXs utilize biopsied patient tumor tissue or cultured tumor spheres and, compared with GBM cell line xenografts, better recapitulate the genetic and histological features of the primary patient's tumor (Wakimoto et al., 2012). To prevent tumor graft rejection, xenograft models

Macrophage Target	Modality	Drug Name	Additional Treatment	Phase	Study Identifier
CSF-1R inhibitor	TAM Depletion	Cabiralizumab	Nivolumab (anti-PD-1)	I	NCT02526017
CSF-1R inhibitor	TAM Depletion	BLZ945	PDR001 (anti-PD-1)	I/II	NCT02829723
CXCR4 inhibitor	TAM Depletion	USL311	Lomustine	II	NCT02765165
PD-L1 inhibitor	Molecular Target	Avelumab	MRI-guided LITT therapy	I	NCT03341806
PD-L1 inhibitor	Molecular Target	Avelumab	RT + TMZ	II	NCT03047473
STAT3 inhibitor	TAM Reprogramming	WP1066		I	NCT01904123
GM-CSF	TAM Reprogramming	VBI-1901		I/II	NCT03382977
GM-CSF	TAM Reprogramming	Leukine	Poly I:C + RT	I	NCT03392545
GM-CSF	TAM Reprogramming	Leukine	ERC1671 + bevacizumab	II	NCT01903330
MIF inhibitor	TAM Reprogramming	Ibudilast	TMZ	I/II	NCT03782415

**Table 1. Current Clinical Trials Targeting TAMs in GBM**

Patterned after Pires-Afonso et al. (2020).

CSF-1R, colony-stimulating factor-1 receptor; CXCR4, C-X-C chemokine receptor type 4; PD-L1, programmed death-ligand 1; STAT3, signal transducer and activator of transcription 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; MIF, macrophage migration inhibitory factor; RT, radiotherapy; TMZ, temozolomide; LITT, laser interstitial thermal therapy; poly(I:C), polyinosinic-polycytidylic acid

typically utilize genetically immunocompromised mice and therefore do not fully recapitulate cancer-immune cell interactions that occur in humans. GEM and syngeneic mouse models use immunocompetent mice and are thus superior for analyzing potential anti-tumor activity of novel therapeutics (Noorani, 2019; Oh et al., 2014).

Mouse models have greatly facilitated the field's understanding of TAM ontogeny, localization, and gene expression in gliomas (Bowman et al., 2016; Chen et al., 2017). Despite these significant advances, there is increasing concern over the utility of mouse models to predict clinical outcomes (Tao and Reese, 2017). Previous studies have reported discrepancies in innate and adaptive immune responses between mice and humans, including leukocyte composition, cytokines and cytokine receptors, as well as costimulatory molecule expression and function (Mestas and Hughes, 2004). Additionally, *in vivo* models are often impractical for mechanistic investigation of cell-cell interactions due to the influence of heterogeneous cell populations and additional TME complexity.

*In vitro* experimental models provide the opportunity to investigate molecular mechanisms governing cell behavior in a relatively simple and controllable environment. A traditional and widely used approach to study macrophage/glioma interactions involves culturing one cell type in the presence of conditioned media from another cell type, typically on a two-dimensional (2D) substrate, such as plastic or glass. Using this approach, researchers have demonstrated that glioma-derived factors are capable of polarizing macrophages into immunosuppressive M2-like macrophages characterized by increased transforming growth factor (TGF)- $\beta$ 1 and interleukin (IL)-10 expression and aberrant phagocytic capacity (Walentynowicz et al., 2018; Wu et al., 2010). Although conditioned media experiments have facilitated our understanding of some of the soluble and molecular mechanisms governing glioma-TAM interactions, this culture system is limited in its ability to recapitulate dynamic and reciprocal cell-cell interactions found *in vivo*. Additionally, there is growing awareness that traditional 2D models do not accurately reflect the dimensionality and mechanics of *in vivo* cellular microenvironments.

Modified versions of the Boyden chamber or Transwell assay are also commonly used to investigate macrophage-glioma cross talk. In this co-culture model, macrophages and cancer cells are separated by a porous membrane that allows the diffusion of soluble factors between the upper and lower chambers while maintaining physical separation between the two cell populations. To assess the influence of paracrine signaling on cell invasion, the membrane can be coated with a layer of ECM, often Matrigel or collagen, to facilitate cell migration to the underside of the membrane for a quantifiable readout of chemotaxis. This co-culture platform has been used to demonstrate that glioma invasion is stimulated by microglia-derived factors such as EGF (Coniglio et al., 2012), TGF- $\beta$ 1 (Ye et al., 2012), IL-10 (Qi et al., 2016), and IL-1 $\beta$  (Lu et al., 2020). Transwell assays have also been used to identify glioma-secreted factors regulating TAM

recruitment and polarization, including immunity-related GTPase family M protein (IRGM) (Xu et al., 2019) and periostin (POSTN) (Zhou et al., 2015). Although the physical separation of cell types is useful when studying paracrine signaling, this system is less suited to investigate macrophage-cancer cell juxtacrine interactions. Additionally, Transwell assays offer limited control over the size, number, and distribution of cellular compartments and are challenging to use for live imaging of cell-cell interactions or cell migration.

### 3D Engineered Models

3D culture platforms have emerged as useful paradigms to bridge the gap between conventional 2D systems and animal models. Current 3D *in vitro* tumor models differ in their complexity, advantages, and limitations and have been comprehensively reviewed elsewhere (Katt et al., 2016; Lv et al., 2017). Advanced glioma models have been developed that include various cell types, ECM proteins, and soluble factor gradients to recapitulate multiple aspects of the TME (Wolf et al., 2019). Although the development of 3D TME models is an area of rapid expansion, the integration of macrophages and other immune cells in these platforms is still in its infancy (Di Modugno et al., 2019). In the following sections, we review current 3D engineered platforms to study macrophage-cancer interactions and outline the contributions that these models have made to our understanding of TAMs, emphasizing glioma models.

#### Hydrogel-Based Systems

Hydrogels, cross-linked polymer networks with high water content, are commonly employed as matrices for 3D cell culture. They can be assembled from a broad range of natural and synthetic polymers, enabling the fabrication of scaffolds with a broad spectrum of chemical and physical properties. Hydrogel scaffolds based on biological polymers typically use mammalian ECM components such as collagen, fibrin, hyaluronic acid (HA), or non-mammalian structural components such as chitosan and alginate (Tibbitt and Anseth, 2009). Perhaps the majority of scaffold-based tumor models utilize collagen I or Matrigel, a heterogeneous basement membrane matrix derived from Engelbreth-Holm-Swarm mouse sarcoma (Bahlmann et al., 2020). Although these commercially available hydrogels are relatively simple to use, the resulting composition and mechanics of Matrigel and collagen hydrogels differ significantly from that of brain tissue. Brain matrix is relatively soft (300–3,000 Pa), has a low abundance of fibrous collagens, and is rich in glycosaminoglycans, proteoglycans, and glycoproteins (Novak and Kaye, 2000). HA, a polyanionic glycosaminoglycan, is the most abundant component of brain ECM, and as with many tumors, its abundance is increased in high-grade gliomas compared with healthy tissue (Liu et al., 2019). HA hydrogels have been used to identify microenvironmental cues driving glioma invasion and therapeutic resistance (Cha and Kim, 2019; Chen et al., 2018; Nakod et al., 2020; Wolf et al., 2018; Xiao et al., 2020); however, there are very few studies investigating macrophage-glioma cell interactions within an HA-rich environment.

Accumulating evidence suggests that macrophages are sensitive to matrix-mediated cues, such that the choice of material may strongly influence macrophage phenotype (Jain et al., 2019). In fact, matrix composition (Delcassian et al., 2019; Kim et al., 2019a), stiffness (Adlerz et al., 2016; Blakney et al., 2012; Sridharan et al., 2015), and cross-linking density (Hsieh et al., 2019) have all been shown to influence macrophage polarization. A previous study cultured macrophages in 3D matrices of low- and high-density collagen and found that high-density collagen induced gene expression profiles in macrophages resembling immunosuppressive TAMs (Larsen et al., 2020). The transcriptional changes also had functional consequences, as macrophages cultured in high-density collagen were less efficient at recruiting cytotoxic T cells and more capable of inhibiting T cell proliferation compared with macrophages encapsulated in low-density collagen hydrogels. Another study exploited mixed collagen-HA 3D matrices to investigate the influence of HA on macrophage phenotype (Hyebin Kim et al., 2019a). Compared with cells embedded within collagen hydrogels, macrophages in mixed collagen-HA hydrogels exhibited increased expression of traditional M2 markers, suggestive of an immunosuppressive, tumor-supportive phenotype.

**Tumor Spheroids.** Tumor spheroids are 3D cellular aggregates of uniform or heterogeneous cell populations and have emerged as promising *in vitro* platforms for disease modeling and drug screening. Spheroids have been shown to mimic some histological features of human gliomas, such as their multicellular structural organization, hypoxic core, and gradient distributions of oxygen, nutrition, pH and metabolic wastes (Tevis et al., 2017b; Xiao et al., 2017). Glioma cells can be cultured as tumor spheroids using multiple approaches, such as agitation- and microwell-based systems, liquid overlay techniques, or the hanging-drop method, all of which can be adapted to form heterotypic spheroids (Nunes et al., 2019). Tumor spheroids can be maintained in suspension culture, placed upon an ECM-coated surface, or fully

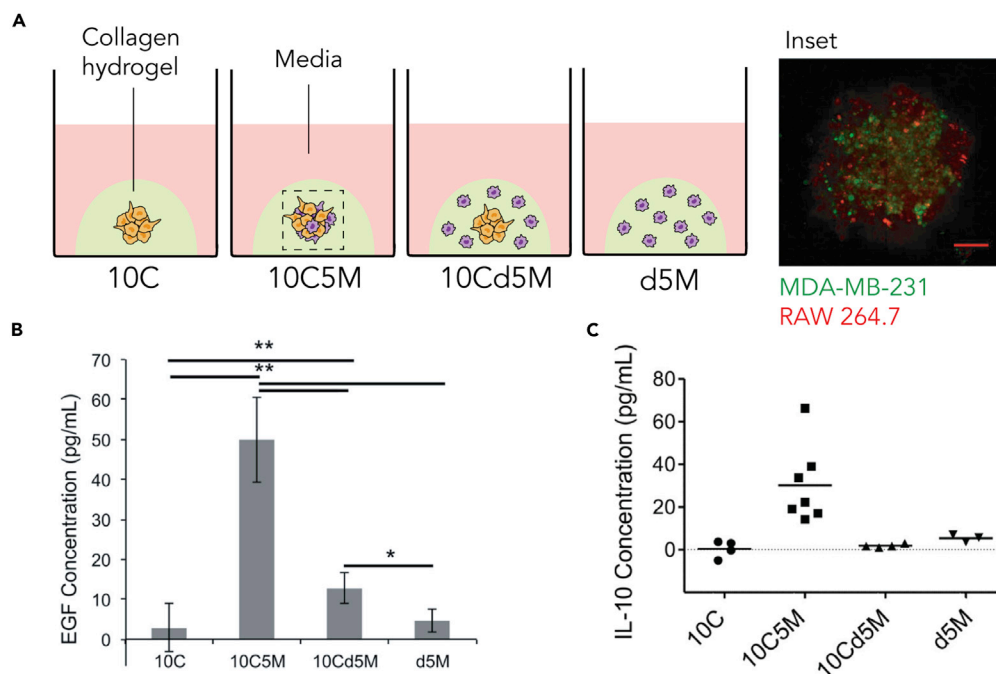
embedded in a 3D ECM scaffold (Tevis et al., 2017b). Macrophages can be incorporated into tumor spheroids by directly forming spheroids from a mixed macrophage/cancer cell suspension. Alternately, macrophages have been reported to spontaneously infiltrate tumor spheroids, offering an arguably more physiologically relevant method of macrophage incorporation (Herter et al., 2017; Pang et al., 2016). The use of embedded spheroids offers additional flexibility, as macrophages can be encapsulated within the matrix as either single cells or spheroids, or seeded on top of the matrix. The modularity of this approach provides the opportunity to develop multiple application-specific models.

Using mixed microglia/glioma cell spheroids embedded in collagen, Mora et al. demonstrated that upon pro-inflammatory stimulation (lipopolysaccharide [LPS] + interferon [IFN]- $\gamma$ ), primary murine microglia acquire cytotoxic potential and kill tumor necrosis factor (TNF)- $\alpha$  and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant glioma cells (Mora et al., 2009). The authors subsequently found that this anti-tumor effect was mediated by microglia-secreted factors that induce autophagy-driven cell death in glioma cells but have no toxicity toward primary cultures of astrocytes or neurons. A related study reported that inactivated primary murine microglia, when seeded as single cells within a 3D collagen matrix, increased the invasiveness of a mixed microglia/GBM cell spheroid (Cisneros Castillo et al., 2016). However, despite exhibiting a traditional anti-tumor cytokine profile, classically activated microglia (LPS + IFN- $\gamma$ ) had no effect on spheroid invasion compared with mixed spheroids embedded in a cell-free collagen matrix. Using a similar platform, Tevis et al. investigated the influence of direct macrophage-cancer interactions on macrophage polarization (Tevis et al., 2017a). Macrophages were either incorporated directly within tumor spheroids or diffusely seeded throughout the 3D collagen hydrogel (Figure 3). Interestingly, only the macrophages included in the tumor spheroids displayed tumor-promoting phenotypes. Compared with cultures in which the macrophages were embedded in the surrounding matrix, mixed spheroids exhibited increased expression of the M2-associated cytokines IL-10 and EGF, rapid oxygen consumption, and resistance to the chemotherapeutic drug paclitaxel. These results reflect the importance of direct macrophage-cancer cell contact on TAM activation, tumor progression, and resistance to treatment. Although this study was designed to investigate TAM-breast cancer cell interactions, GBM cells and macrophages can be co-cultured in a similar platform to study TAM-GBM cell dynamics. It should be noted, however, that brain and breast tissue have slightly different mechanics and compositions, the latter displaying a higher abundance of fibrillar collagens (Insua-Rodríguez and Oskarsson, 2016; Rauch, 2004). Thus it is crucial to consider both cell-cell and cell-matrix interactions when designing and validating *in vitro* experimental models.

**Tumor Organoids.** Tumor organoids are self-assembled 3D aggregates generated from pluripotent stem cells or fresh tumor specimens. GBM organoids are commonly fabricated by dissociating and culturing fresh human tumor specimens or PDX tumors, often in the presence of Matrigel and exogenous growth factors. Although this approach can generate heterogeneous organoids with gradients of stem cell density and hypoxia (Hubert et al., 2016), it results in a limited preservation of immune and stromal cell types. Additionally, the low and regionally variable growth rates of tumor organoids require time-consuming procedures, which may be impractical for high-throughput screening assays. Recently, dissociation-free methods have been developed to generate patient-derived GBM organoids from fresh tumor specimens (Jacob et al., 2020). These organoids captured the cellular diversity, gene expression, and mutational profiles of their parental tumors and contained both microglia and macrophages, noting a decreased abundance of immune cell populations over time. The preservation of immune cell quantity and phenotype in GBM organoids will require a careful tuning of organoid culture conditions, which have likely been optimized to maintain glioma cell viability, growth, and invasiveness (Neal et al., 2018).

To overcome the limited expansion potential of primary tumor-derived immune cells, organoids can be co-cultured with autologous peripheral immune cells (Dijkstra et al., 2018). Similar to heterotypic spheroid fabrication, macrophages can be directly incorporated into organoids during fabrication, allowed to infiltrate established organoids, or seeded into the surrounding matrix of embedded organoids (Baker, 2018). Despite the successful implementation of these approaches for other cancer types (Neal et al., 2018), patient-derived organoids have not been extensively used to investigate glioma cell-TAM cross talk. The rapid development of induced pluripotent stem cell (iPSC) technology has provided an additional model system, termed cerebral organoids, to study brain development and disease (Qian et al., 2019). To specifically model GBM tumors, cerebral organoids can be genetically manipulated to develop oncogenic properties (Bian et al., 2018; Ogawa et al., 2018), or co-cultured with tumor spheroids to study tumor cell invasion





**Figure 3. Embedded Cancer Cell/Macrophage Spheroid Model**

(A–C) (A) Four model systems were used to assess the influence of TAM localization on TAM function and cancer progression. A tumor-only model consists of MDA-MB-3231 breast cancer spheroids embedded in a collagen gel (10C). RAW 264.7 macrophages were incorporated into the spheroid at a ratio of 2:1 (cancer cells: macrophages) to form a heterospheroid (10C5M) or were seeded in the collagen gel surrounding the cancer spheroid (10Cd5M). In the macrophage-only model, macrophages were distributed within the 3D collagen gel (d5M). Inset shows fluorescently labeled cancer cells (green) and macrophages (red) in a heterospheroid model (scale bar, 100  $\mu$ m). The heterospheroid (10C5M) model exhibited the highest (B) EGF and (C) IL-10 concentrations detected by ELISAs, indicative of an M2 activation pathway associated with TAMs. Reprinted with permission from Tevis et al. (2017a). \*\*p < 0.005; \*p < 0.05.

(da Silva et al., 2018; Linkous et al., 2019). To date, iPSC-derived cerebral and glioma organoids containing microglia and macrophages have not yet been established, although the generation of both cell types from iPSCs has been described (Rajab et al., 2018). We anticipate that multicellular glioma organoid technology will provide exciting opportunities to study TAMs during multiple stages of cancer development.

### Microfluidic Systems

Microfluidic devices rely on the use of micron-sized channels to handle small fluid volumes (Whitesides, 2006). Using this technology, *in vitro* culture platforms can be designed to recapitulate complex physiological microenvironments due to the ability to precisely control cellular, biochemical, and physical components. Microfluidic platforms have been successfully used to study cell migration, angiogenesis, and other cancer-associated phenomena, and are increasingly being leveraged to model cancer-immune cell interactions (Boussommier-Calleja et al., 2016; Ma et al., 2018; Sung and Beebe, 2014).

**Models of Monocyte Extravasation.** To enter the glioma microenvironment, monocytes must first extravasate the BBB. The BBB is an endothelial permeability barrier that serves as the brain's first line of defense against blood-borne pathogens and is composed of multiple cell types, including microvascular endothelial cells, pericytes, and astrocytes. This complex structure has remained difficult to model faithfully *in vitro*; however, the available models of microvasculature are rapidly expanding. Engineered microvessels are commonly fabricated by casting a polymer or hydrogel around a steel needle that, upon removal, forms a channel that can be seeded with endothelial cells (Polacheck et al., 2019). Needle-based methods can form microchannels with varying diameters (20–400  $\mu$ m) depending on the geometry of the needle (Linville et al., 2016; Zhang et al., 2013). Using this approach, researchers investigated monocyte-endothelial cell interactions by perfusing monocytes throughout an endothelialized channel embedded in polymethyl

siloxane (PDMS) (Zhang et al., 2013). Monocyte attachment increased in channels that were pre-treated with TNF- $\alpha$ , a pro-inflammatory cytokine that has been shown to facilitate monocyte transmigration by increasing the expression of surface adhesion proteins in endothelial cells (Sedgwick et al., 2000). Microfluidic microvessel platforms can be modified to include various ECM components, soluble factors, or additional cell types to investigate monocyte adhesion and transmigration in disease-specific conditions. For example, a 3D vascularized microfluidic device containing five channels was deployed to study the effects of monocytes on cancer cell extravasation (Boussommier-Calleja et al., 2019). The central channel contained a fully vascularized 3D hydrogel, fabricated by cultivating endothelial cells and fibroblasts together within a 3D fibrin gel. A monocyte suspension was introduced into one of the two reservoirs adjacent to the hydrogel to create a transient pressure drop and to facilitate monocyte extravasation through the vascular network. The platform recapitulated multiple hallmarks of transmigration observed *in vivo*, including endothelial wall permeability and extravasation patterns of distinct monocyte subsets. In particular, this study reported that inflammatory monocytes had an increased tendency over patrolling monocytes to extravasate the vascular network and differentiate into macrophages, a phenomenon that has been shown to occur during infection (Shi and Pamer, 2011) and glioma progression (Chen et al., 2017, 2019). Researchers have begun to incorporate additional cell types, primarily pericytes and astrocytes, into microfluidic designs to fabricate a more realistic BBB model (Brown et al., 2015; Xu et al., 2016). These platforms provide additional opportunities to investigate immune cell trafficking into the CNS; however, the increased complexity is accompanied by additional validation and standardization challenges (van der Helm et al., 2016).

**Models of Cancer Invasion.** High-grade gliomas are characterized by a rapid and highly invasive growth in which glioma cells individually or collectively migrate away from the central tumor into the surrounding healthy brain tissue. Targeting invasion has been proposed as a potential therapeutic strategy for GBM; however, translational progress has been slow primarily due to an incomplete understanding of the mechanisms driving invasion. Conventional cell migration and invasion assays, such as Boyden and Transwell chambers, are helpful to understand broad trends in cell migration but are unable to provide detailed information about individual cell behavior. Researchers have harnessed the power of microfluidics to visualize and identify cancer cell-autonomous and microenvironmental factors driving glioma invasion (Lin et al., 2018; Rianna et al., 2020). A previous study used a 3D microfluidic cell invasion assay to demonstrate that the presence of macrophages enhanced the speed and persistence of cancer cell invasion through a collagen I matrix in an MMP-dependent fashion (Li et al., 2017). Further investigation revealed that macrophage-secreted TNF- $\alpha$  and TGF- $\beta$ 1 synergistically induce nuclear translocation of nuclear factor- $\kappa$ B in cancer cells, leading to increased cancer cell MMP-1 expression and enhanced cancer cell invasion persistence. Macrophage-released TGF- $\beta$ 1 was also shown to promote cancer cell invasion speed by upregulating MT1-MMP expression in cancer cells. An engineered 3D pre-metastatic microfluidic device illustrated a role of macrophage-driven MMP signaling in facilitating cancer cell invasion (Hyunho Kim et al., 2019b). Monocytes were seeded in a microchannel layered with endothelial cells and surrounded by a 3D collagen matrix to mimic the process of monocyte extravasation and entry into the surrounding ECM, which was found to be mediated by MMP-9 (Figure 4). Furthermore, the pre-invaded monocytes and macrophages enhanced cancer cell invasion within the collagen matrix by forming tunnel-like structures, termed “microtracks,” which enabled persistent invasion of subsequent cancer cells. One advantage of this microfluidic design is the ability to temporally and spatially control the introduction of multiple cell types within a single platform. A similar approach can be taken to investigate macrophage-dependent and independent mechanisms of glioma cell invasion through brain-relevant matrices, including HA hydrogels.

**Models of Tumor Angiogenesis.** Angiogenesis, the formation of novel blood vessels from pre-existing vessels, is indispensable for tumor growth beyond a critical size and has been an ongoing therapeutic target for solid tumors (Lugano et al., 2020; Viallard and Larrivée, 2017). The ability to precisely control fluid flow, soluble factor gradients, and cellular compositions has positioned microfluidics as useful *in vitro* models to study interactions between glioma cells and blood vessels (Lewis and Gerecht, 2016; Truong et al., 2020; Wolf et al., 2018). 3D angiogenesis platforms are typically fabricated by seeding endothelial cells (ECs) into an open vessel-like channel embedded within an ECM-like matrix (Nguyen et al., 2013). To investigate macrophage-mediated angiogenesis, macrophages can be embedded within the matrix surrounding the EC channel, seeded within the EC channel, or seeded in an adjacent parallel channel. In one such study, a 3D microfluidic platform was utilized to investigate the proangiogenic capacity of glioma TAMs by fabricating two parallel channels embedded within a collagen matrix (Cui et al., 2018). The study found that co-culturing uncommitted macrophages (M0) with GBM tumorspheres steered macrophage

polarization toward an M2-like phenotype characterized by the secretion of the anti-inflammatory cytokines TGF- $\beta$ 1 and IL-10. To characterize the functional consequences of M2-like TAMs on angiogenesis, pre-polarized macrophages (M1, M2a, M2b, M2c) were seeded in different locations relative to ECs within the microfluidic device (Figure 5). Similar to previous studies, secreted factors from both M1- and M2-polarized macrophages resulted in increased capillary area and sprouting length of ECs compared with untreated controls (Spiller et al., 2014). However, when M1-like macrophages were seeded within the hydrogel directly in contact with the ECs, they inhibited angiogenesis, suggesting that the anti-angiogenic capacity of M1-like macrophages depends on direct macrophage-EC interactions. The study found that M2-like macrophages promote angiogenesis through soluble secreted factors, primarily TGF- $\beta$ 1, as well as direct  $\alpha_v\beta_3$  integrin-dependent EC-macrophage interactions. These findings highlight the possibility of targeting TAMs to arrest GBM angiogenesis and improve anti-angiogenic therapeutic efficacy.

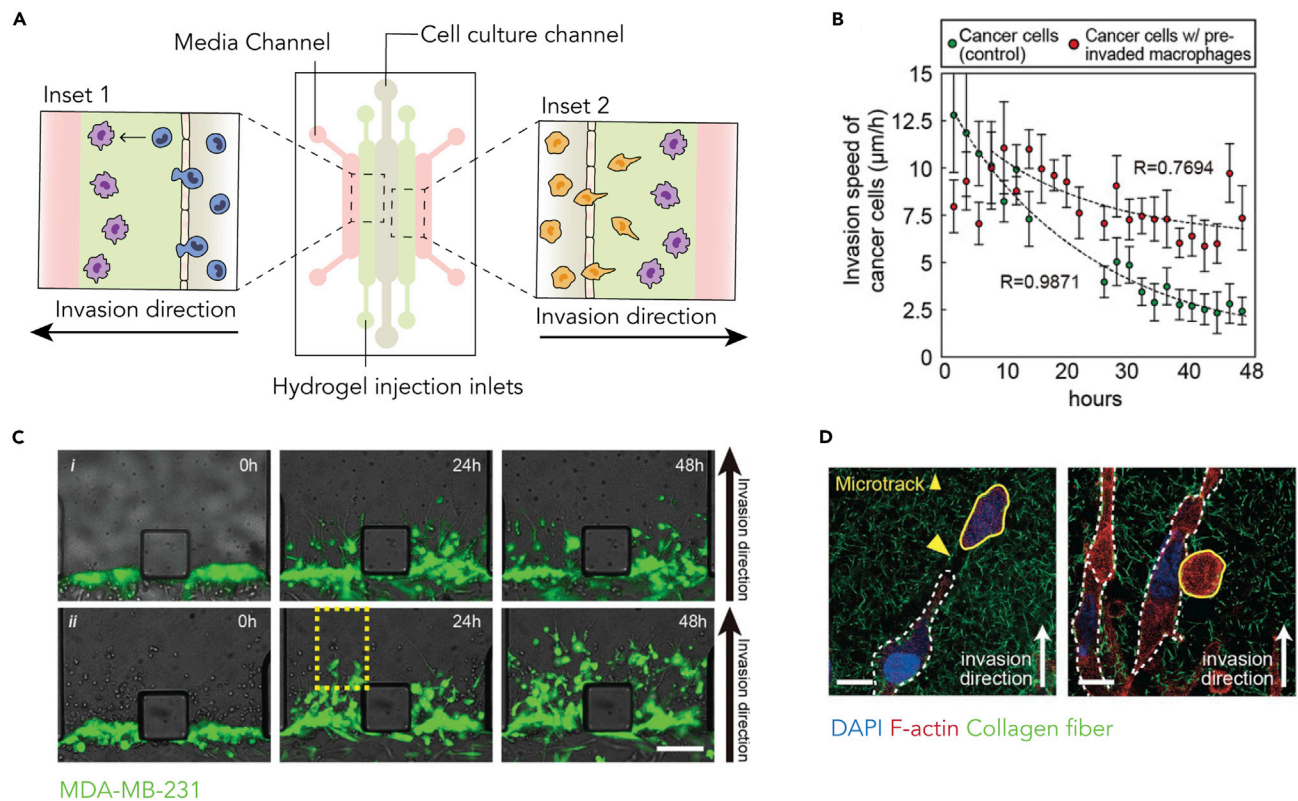
## CONCLUSIONS AND PERSPECTIVES

Although the development of 3D glioma TME models is expanding rapidly (Wolf et al., 2019), the integration of macrophages and other immune cells in these platforms is still in its infancy (Di Modugno et al., 2019). Multicellular culture systems are gaining increasing popularity, and when combined with relevant ECM-like scaffolds, these platforms allow for the investigation of heterotypic cellular interactions in near-physiological environments. Hydrogels better capture the 3D geometry of a brain tumor than 2D culture or Transwell assays, but they too lack many of the components found in the TME and therefore are still relatively simplified model systems. A reductionist approach in developing TME models is useful to mimic *in vivo* tumor dynamics while minimizing cost and complexity. However, it remains unclear which minimal components are needed to recapitulate *in vivo* mechanisms. Despite great efforts focused on designing novel scaffolds to mimic various aspects of brain tissue, the majority of models utilize Matrigel or fibrillar collagen-based matrices. Although commercially available and relatively easy to use, these matrices do not sufficiently mimic the brain microenvironment and could limit the predictive power of discovery and screening platforms (Bahlmann et al., 2020; Di Modugno et al., 2019).

Access to patient-derived TAMs is limited; therefore the majority of *in vitro* studies use primary or immortalized macrophages to model TAMs. Macrophages can be derived from multiple sources, most commonly human peripheral blood mononuclear cells (PBMCs), the leukemic cell line THP-1, murine bone marrow mononuclear cells, or murine RAW 264.7 cells. Many studies have equated TAMs to M2 macrophages and thus have generated *in vitro* TAMs using M2 macrophage polarization protocols, sometimes in the presence of cancer cell conditioned media (Benner et al., 2019). The field is currently lacking a reliable and standardized protocol to generate *in vitro* TAMs, which will likely differ between tumor location, stage, and grade.

There is growing awareness that *in vitro* macrophage behavior and response to stimuli may differ between cells obtained from different sources. Previous studies have reported differences in gene expression profiles between mouse and human macrophages after pro-inflammatory (M1) and anti-inflammatory (M2) activation (Martinez et al., 2013; Schneemann and Schoeden, 2007; Schroder et al., 2012). Furthermore, there are notable differences between human PBMC-, THP-1-, and iPSC-derived macrophages, although in a previous study macrophages derived from PBMCs and iPSCs behaved more similarly to one another than they did to those derived from THP-1 cells (Spiller et al., 2016). Some limitations of PBMC-derived macrophages include low proliferative capacity, limited accessibility, and donor-to-donor genetic variability. Although iPSC-derived macrophages also contain donor-to-donor variability, they could potentially yield unlimited numbers of human macrophages and are amenable to genetic perturbation (Gutbier et al., 2020; Zhang et al., 2015). It is therefore of great importance to consider macrophage source and polarization protocol when designing experiments and interpreting results from previous studies.

As the field's understanding of TAM phenotypic complexity and heterogeneity expands, it will be important to incorporate these advances in experimental TME models. TAMs in gliomas arise from two populations, resident microglia and bone-marrow derived macrophages. Although there have been multiple studies investigating the role of each population in glioma progression, both TAM populations are not regularly incorporated into a common experimental model. The standard method of characterizing macrophage phenotype has primarily relied on the M1/M2 polarization model. Over the years, this nomenclature has led to an extensive list of cytokines and surface receptors that are generally considered to define M1 or M2 macrophages. Researchers typically characterize macrophage polarization by selecting a few markers



**Figure 4. Microfluidic Model of TAM-Mediated Cancer Cell Invasion**

(A) Schematic of microfluidic design containing media, cell culture, and hydrogel injection inlets. Inset 1 shows monocyte migration through EC monolayer and subsequent macrophage differentiation within the collagen hydrogel. Inset 2 shows cancer cell invasion following pre-invaded macrophages.

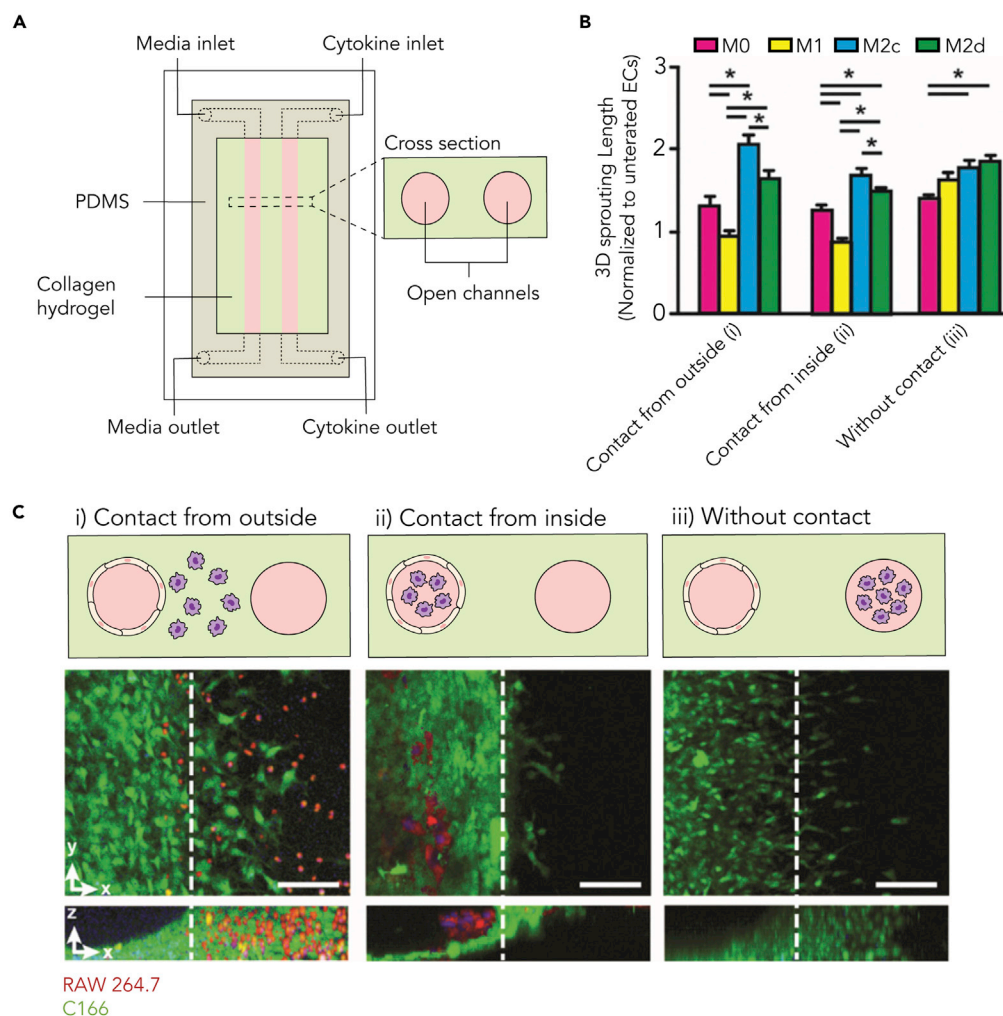
(B) MDA-MB-231 breast cancer cell invasion speed through collagenous ECM in the absence or presence of pre-invaded macrophages measured every 2 h. Data represent mean  $\pm$  S.E.M.

(C) Time-lapse images of GFP-tagged cancer cells in the absence (i, top) or presence (ii, bottom) of pre-invaded macrophages (scale bar, 150  $\mu$ m).

(D) Confocal fluorescence images of collagen fibers (green), F-actin (red), and nuclei (blue) showing cancer cells (white dotted line) in the presence of pre-invaded macrophages (yellow line). Yellow arrowheads indicate the microtracks generated by macrophages (scale bar, 10  $\mu$ m). Reprinted with permission from Hyunho Kim et al., 2019b.

in each category and assessing their gene and protein expression using standard molecular biology tools such as quantitative reverse transcription polymerase chain reaction (RT-qPCR), immunocytochemistry, or enzyme-linked immunosorbent assays (ELISAs). While this approach has historically worked well for assessing M1/M2 polarization *in vitro* after traditional cytokine-induced activation (LPS + IFN- $\gamma$ , IL-4), macrophage polarization in engineered TME models and *in vivo* may require more extensive characterization. As a result, future studies should utilize novel high-throughput analysis techniques, including gene set analysis, RNA-seq, and CyTOF to assess macrophage phenotype. These techniques can also be harnessed to identify potential ligand-receptor interactions that occur in the TME, which serve as potential therapeutic targets (Kumar et al., 2018). Retrieving and isolating individual cell populations from 3D scaffold-based models for downstream analysis is possible but not straightforward. We anticipate that combining sophisticated 3D experimental models with advanced transcriptomic analysis will prove instrumental in elucidating TAM polarization and heterotypic cellular interactions within the TME.

Validation is a critical step in the development of 3D TME models and ensures that *in vitro* discoveries generate useful predictions of clinical relevance. Although not yet fully standardized, validation methods for cell-embedded hydrogel platforms generally include verifying that the matrix properties (stiffness, composition, porosity) closely mimic that of native tissue, and correlating cell behavior (morphology, migration, gene/protein expression) with analogous measurements made in murine or human tumors. Ideally, validation should be performed at multiple time points throughout model development to systematically test mechanistic and phenotypic predictions against the *in vivo* response. Close collaboration



**Figure 5. Microfluidic Device to Study TAM-Mediated Angiogenesis**

(A) Schematic of microfluidic device consisting of two parallel open channels within a collagen hydrogel.

(B) Quantified 3D sprouting length (normalized to untreated ECs) under different EC-macrophage interactions with the presence of different macrophage subsets. \* $p < 0.05$ ; Data represent mean  $\pm$  S.E.M.

(C) Representative 3D fluorescent projections of confocal image stack showing distinct 3D EC-macrophage interactions. Raw 264.7 murine macrophages labeled red and C166 endothelial cells labeled green (scale bar, 200  $\mu$ m). Reprinted with permission from Cui et al. (2018).

between academic researchers, clinicians, and pathologists will prove fruitful in the creation of engineered models to greatly improve drug discovery and validation.

Scaffold-based platforms provide the opportunity to study cancer and stromal cells within a 3D biomaterial that partially resembles the dimensionality and mechanics of human tissue; however, these models offer limited control over the spatial distribution of heterogeneous cell populations at the single-cell level. The microscale dimensions and compartmentalization of microfluidic devices are better suited for multicellular cultures, but some complex microfluidic designs are not well suited for scaffold-based 3D cell culture. 3D bioprinting represents an especially promising and increasingly accessible technology for addressing these challenges. 3D bioprinting innovates upon 3D printing used in additive manufacturing by employing living cells, ECM components, biomaterials, and biochemical factors as “inks” to fabricate living, 3D structures with complex architectures and multiple cell types (Zhang et al., 2016). Although the use of 3D bioprinting in tumor models has tended to focus on breast cancer (Belgodere et al., 2018; Grolman et al., 2015), a recent study printed a mini-brain with spatially controlled populations of glioma cells and macrophages (Heinrich et al., 2019). The gelatin-based 3D model recapitulated features of TAM recruitment,

### Box 1. M1/M2 Polarization Model

Macrophage polarization refers to the process by which macrophages adopt distinct functional phenotypes in response to microenvironmental stimuli (Murray, 2017; Sica and Mantovani, 2012). The M1/M2 polarization model was first introduced to systematically describe macrophage activation in response to a particular stimulus (Mills et al., 2000). Since then, the model has evolved to include multiple macrophage subtypes that are distinguished by assessing the gene and protein expression of a relatively standardized list of phenotypic markers (Guilliams and van de Laar, 2015). In brief, classically activated macrophages, termed M1, arise from stimulation with toll-like receptor ligands, such as lipopolysaccharide (LPS), and interferon- $\gamma$  (IFN- $\gamma$ ). M1 macrophages secrete pro-inflammatory mediators (TNF- $\alpha$ , IL-1 $\beta$ , and IL-12), have antigen-presenting and co-stimulatory abilities, and play an instrumental role in host response to pathogens. M2 macrophages, also known as alternatively activated macrophages, occur after stimulation with IL-4, IL-10, or IL-13. Alternatively activated macrophages secrete immunosuppressive cytokines (IL-6, IL-10, TGF- $\beta$ 1) and mediate ECM remodeling and angiogenesis. M2 macrophages are further subdivided into M2a (type II inflammation, allergy), M2b (immunoregulation), and M2c (immunoregulation, matrix deposition, tissue remodeling). With regard to tumor immunity, it is generally thought that M1 macrophages are anti-tumorigenic, whereas M2 macrophages are pro-tumorigenic (Mantovani, 2014). The M1/M2 paradigm is recognized as a simplification of macrophage polarization *in vivo* and its relevance toward understanding macrophage phenotype and function in homeostasis and disease is controversial (Guilliams and van de Laar, 2015; Nahrendorf and Swirski, 2016; Sankowski et al., 2019).

polarization, and induction of GBM invasion, and it demonstrated the potential of harnessing macrophage-glioma interactions to enhance therapeutic response. We anticipate that this family of advanced fabrication techniques will become increasingly useful for recapitulating the 3D TME architecture while accommodating its rich diversity of cell types.

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### AUTHOR CONTRIBUTIONS

Conceptualization, E.A.A. and S.K.; Writing – Original Draft, E.A.A.; Writing – Reviewing & Editing, E.A.A., M.K.A., and S.K.

### REFERENCES

- Adlerz, K.M., Aranda-Espinoza, H., and Hayenga, H.N. (2016). Substrate elasticity regulates the behavior of human monocyte-derived macrophages. *Eur. Biophys. J.* 45, 301–309.
- Arandjelovic, S., and Ravichandran, K.S. (2015). Phagocytosis of apoptotic cells in homeostasis. *Nat. Immunol.* 16, 907–917.
- Bahlmann, L.C., Smith, L.J., and Shoichet, M.S. (2020). Designer biomaterials to model cancer cell invasion *in vitro*: predictive tools or just pretty pictures? *Adv. Funct. Mater.* 1909032, 1–11.
- Baker, K. (2018). Organoids provide an important window on inflammation in cancer. *Cancers (Basel)* 10, 151, <https://doi.org/10.3390/cancers10050151>.
- Belgodere, J.A., King, C.T., Bursavich, J.B., Burow, M.E., Martin, E.C., and Jung, J.P. (2018). Engineering breast cancer microenvironments and 3D bioprinting. *Front. Bioeng. Biotechnol.* 6, 66.
- Benner, B., Scarberry, L., Suarez-Kelly, L.P., Duggan, M.C., Campbell, A.R., Smith, E., Lapurga, G., Jiang, K., Butchar, J.P., Tridandapani, S., et al. (2019). Generation of monocyte-derived tumor-associated macrophages using tumor-conditioned media provides a novel method to study tumor-associated macrophages *in vitro*. *J. Immunother. Cancer* 7, 1–14.
- Bian, S., Repic, M., Guo, Z., Kavirayani, A., Burkard, T., Bagley, J.A., Krauditsch, C., and Knoblich, J.A. (2018). Genetically engineered cerebral organoids model brain tumor formation. *Nat. Methods* 15, 631–639.
- Blakney, A.K., Swartzlander, M.D., and Bryant, S.J. (2012). The effects of substrate stiffness on the *in vitro* activation of macrophages and *in vivo* host response to poly(ethylene glycol)-based hydrogels. *J. Biomed. Mater. Res. A* 100, 1375–1386.
- Bonaventura, P., Shekarian, T., Alcazer, V., Valladeau-Guilemond, J., Valsesia-Wittmann, S., Amigorena, S., Caux, C., and Depil, S. (2019). Cold tumors: a therapeutic challenge for immunotherapy. *Front. Immunol.* 10, 1–10.
- Boussommier-Calleja, A., Atiyas, Y., Haase, K., Headley, M., Lewis, C., and Kamm, R.D. (2019). The effects of monocytes on tumor cell extravasation in a 3D vascularized microfluidic model. *Biomaterials* 198, 180–193.
- Boussommier-Calleja, A., Li, R., Chen, M.B., Wong, S.C., and Kamm, R.D. (2016). Microfluidics: a new tool for modeling cancer-immune interactions. *Trends Cancer* 2, 6–19.
- Bowman, R.L., Klemm, F., Akkari, L., Pyonteck, S.M., Sevenich, L., Quail, D.F., Dhara, S., Simpson, K., Gardner, E.E., Iacobuzio-Donahue, C.A., et al. (2016). Macrophage ontogeny underlies differences in tumor-specific education in brain malignancies. *Cell Rep.* 17, 2445–2459.
- Brown, J.A., Pensabene, V., Markov, D.A., Allwardt, V., Diana Neely, M., Shi, M., Britt, C.M., Hoilett, O.S., Yang, Q., Brewer, B.M., et al. (2015). Recreating blood-brain barrier physiology and structure on chip: a novel neurovascular microfluidic bioreactor. *Biomicrofluidics* 9, 054124.
- Butowski, N., Colman, H., De Groot, J.F., Omuro, A.M., Nayak, L., Wen, P.Y., Cloughesy, T.F., Marimuthu, A., Haidar, S., Perry, A., et al. (2016). Orally administered colony stimulating factor 1 receptor inhibitor PLX3397 in recurrent glioblastoma: an Ivy Foundation Early Phase

- Clinical Trials Consortium phase II study. *Neuro. Oncol.* 18, 557–564.
- Cannarile, M.A., Weisser, M., Jacob, W., Jegg, A.M., Ries, C.H., and Rüttinger, D. (2017). Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *J. Immunother. Cancer* 5, 1–13.
- Castro, B.A., Flanigan, P., Jahangiri, A., Hoffman, D., Chen, W., Kuang, R., De Lay, M., Yagnik, G., Wagner, J.R., Mascharak, S., et al. (2017). Macrophage migration inhibitory factor downregulation: a novel mechanism of resistance to anti-angiogenic therapy. *Oncogene* 36, 3749–3759.
- Cha, J., and Kim, P. (2019). Time series assessment of the effects of hypoxic stress on glioma tumorsphere development within engineered microscale niches. *Biomaterials* 194, 171–182.
- Charles, N.A., Holland, E.C., Gilbertson, R., Glass, R., and Kettenmann, H. (2011). The brain tumor microenvironment. *Glia* 59, 1169–1180.
- Chen, J.W.E., Pedron, S., Shyu, P., Hu, Y., Sarkaria, J.N., and Harley, B.A.C. (2018). Influence of hyaluronic acid transitions in tumor microenvironment on glioblastoma malignancy and invasive behavior. *Front. Mater.* 5, 1–21.
- Chen, Z., Feng, X., Herting, C.J., Garcia, V.A., Nie, K., Pong, W.W., Rasmussen, R., Dwivedi, B., Seby, S., Wolf, S.A., et al. (2017). Cellular and molecular identity of tumor-associated macrophages in glioblastoma. *Cancer Res.* 77, 2266–2278.
- Chen, Z., Ross, J.L., and Hambardzumyan, D. (2019). Intravital 2-photon imaging reveals distinct morphology and infiltrative properties of glioblastoma-associated macrophages. *Proc. Natl. Acad. Sci. U S A* 116, 14254–14259.
- Choi, J., Mai, N., Jackson, C., Belcaid, Z., and Lim, M. (2018). It takes two: potential therapies and insights involving microglia and macrophages in glioblastoma. *Neuroinflammation* 5, 42.
- Cisneros Castillo, L.R., Oancea, A.D., Stüllein, C., and Régner-Vigouroux, A. (2016). A novel computer-assisted approach to evaluate multicellular tumor spheroid invasion assay. *Sci. Rep.* 6, 1–16.
- Civita, P., Leite, D.M., and Pilkington, G.J. (2019). Pre-clinical drug testing in 2d and 3d human in vitro models of glioblastoma incorporating non-neoplastic astrocytes: tunneling nano tubules and mitochondrial transfer modulates cell behavior and therapeutic respons. *Int. J. Mol. Sci.* 20, 6017.
- Coniglio, S.J., Eugenin, E., Dobrenis, K., Stanley, E.R., West, B.L., Symons, M.H., and Segall, J.E. (2012). Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. *Mol. Med.* 18, 519–527.
- Cui, X., Morales, R.T.T., Qian, W., Wang, H., Gagner, J.P., Dolgalev, I., Placantonakis, D., Zagzag, D., Cimmino, L., Snuderl, M., et al. (2018). Hacking macrophage-associated immunosuppression for regulating glioblastoma angiogenesis. *Biomaterials* 161, 164–178.
- da Silva, B., Mathew, R.K., Polson, E.S., Williams, J., and Wurdak, H. (2018). Spontaneous glioblastoma spheroid infiltration of early-stage cerebral organoids models brain tumor invasion. *SLAS Discov.* 23, 862–868.
- Darmanis, S., Sloan, S.A., Croote, D., Mignardi, M., Chernikova, S., Samghababi, P., Zhang, Y., Neff, N., Kowarsky, M., Caneda, C., et al. (2017). Single-cell RNA-seq analysis of infiltrating neoplastic cells at the migrating front of human glioblastoma. *Cell Rep.* 21, 1399–1410.
- De Palma, M., and Lewis, C.E. (2013). Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell* 23, 277–286.
- Delcassian, D., Malecka, A.A., Opoku, D., Cabeza, V.P., Merry, C., and Jackson, A.M. (2019). Primary human macrophages are polarized towards pro-inflammatory phenotypes in alginate hydrogels. *bioRxiv*. <https://doi.org/10.1101/824391>.
- Deshmane, S.L., Kremlev, S., Amini, S., and Sawaya, B.E. (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. *J. Interf. Cytokine Res.* 29, 313–325.
- Di Modugno, F., Colosi, C., Trono, P., Antonacci, G., Ruocco, G., and Nisticò, P. (2019). 3D models in the new era of immune oncology: focus on T cells, CAF and ECM. *J. Exp. Clin. Cancer Res.* 38, 1–14.
- Dijkstra, K.K., Cattaneo, C.M., Weeber, F., Chalabi, M., van de Haar, J., Fanchi, L.F., Slagter, M., van der Velden, D.L., Kaing, S., Kelderman, S., et al. (2018). Generation of tumor-reactive T cells by Co-culture of peripheral blood lymphocytes and tumor organoids. *Cell* 174, 1586–1598.e12.
- Flores-Toro, J.A., Luo, D., Gopinath, A., Sarkisian, M.R., Campbell, J.J., Charo, I.F., Singh, R., Schall, T.J., Datta, M., Jain, R.K., et al. (2020). CCR2 inhibition reduces tumor myeloid cells and unmasks a checkpoint inhibitor effect to slow progression of resistant murine gliomas. *Proc. Natl. Acad. Sci. U S A* 117, 1129–1138.
- Friebel, E., Kapolou, K., Unger, S., Núñez, N.G., Utz, S., Rushing, E.J., Regli, L., Weller, M., Greter, M., Tugues, S., et al. (2020). Single-cell mapping of human brain cancer reveals tumor-specific instruction of tissue-invading leukocytes. *Cell* 181, 1626–1642.e20.
- Gabrusiewicz, K., Rodriguez, B., Wei, J., Hashimoto, Y., Healy, L.M., Maiti, S.N., Thomas, G., Zhou, S., Wang, Q., Elakkad, A., et al. (2016). Glioblastoma-infiltrated innate immune cells resemble M0 macrophage phenotype. *JCI Insight* 1, e85841.
- Gieryng, A., Psczolkowska, D., Walentynowicz, K.A., Rajan, W.D., and Kaminska, B. (2017). Immune microenvironment of gliomas. *Lab. Invest.* 97, 498–518.
- Ginhoux, F., Lim, S., Hoeffel, G., Low, D., and Huber, T. (2013). Origin and differentiation of microglia. *Front. Cell. Neurosci.* 7, 1–14.
- Goodenberger, M.L., and Jenkins, R.B. (2012). Genetics of adult glioma. *Cancer Genet.* 205, 613–621.
- Grégoire, H., Roncali, L., Rousseau, A., Chérel, M., Delneste, Y., Jeannin, P., Hindré, F., and Garcion, E. (2020). Targeting tumor associated macrophages to overcome conventional treatment resistance in glioblastoma. *Front. Pharmacol.* 11, 1–17.
- Grolman, J.M., Zhang, D., Smith, A.M., Moore, J.S., and Kilian, K.A. (2015). Rapid 3D extrusion of synthetic tumor microenvironments. *Adv. Mater.* 27, 5512–5517.
- Guilliams, M., and van de Laar, L. (2015). A hitchhiker's guide to myeloid cell subsets: practical implementation of a novel mononuclear phagocyte classification system. *Front. Immunol.* 6, 1–12.
- Gutbier, S., Wanke, F., Dahm, N., Rummelin, A., Zimmermann, S., Christensen, K., Köchl, F., Rautanen, A., Hatje, K., Geering, B., et al. (2020). Large-scale production of human IPSC-derived macrophages for drug screening. *Int. J. Mol. Sci.* 21, 1–23.
- Gutmann, D.H., and Kettenmann, H. (2019). Microglia/brain macrophages as central drivers of brain tumor pathobiology. *Neuron* 104, 442–449.
- Hambardzumyan, D., Gutmann, D.H., and Kettenmann, H. (2015). The role of microglia and macrophages in glioma maintenance and progression. *Nat. Neurosci.* 19, 20–27.
- Heinrich, M.A., Bansal, R., Lammers, T., Zhang, Y.S., Michel Schiffelers, R., and Prakash, J. (2019). 3D-Bioprinted mini-brain: a glioblastoma model to study cellular interactions and therapeutics. *Adv. Mater.* 31, 1–9.
- Herrera-Perez, R.M., Voytik-Harbin, S.L., Sarkaria, J.N., Pollok, K.E., Fishel, M.L., and Rickus, J.L. (2018). Presence of stromal cells in a bioengineered tumor microenvironment alters glioblastoma migration and response to STAT3 inhibition. *PLoS One* 13, 1–20.
- Herter, S., Morra, L., Schlenker, R., Sulcova, J., Fahrni, L., Waldhauer, I., Lehmann, S., Reisländer, T., Agarkova, I., Kelm, J.M., et al. (2017). A novel three-dimensional heterotypic spheroid model for the assessment of the activity of cancer immunotherapy agents. *Cancer Immunol. Immunother.* 66, 129–140.
- Hoppstädter, J., Seif, M., Dembek, A., Cavelius, C., Huwer, H., Kraegeloh, A., and Kiemer, A.K. (2015). M2 polarization enhances silica nanoparticle uptake by macrophages. *Front. Pharmacol.* 6, 1–12.
- Hsieh, J.Y., Keating, M.T., Smith, T.D., Meli, V.S., Botvinick, E.L., and Liu, W.F. (2019). Matrix crosslinking enhances macrophage adhesion, migration, and inflammatory activation. *APL Bioeng.* 3, 016103.
- Hubert, C.G., Rivera, M., Spangler, L.C., Wu, Q., Mack, S.C., Prager, B.C., Couce, M., McLendon, R.E., Sloan, A.E., and Rich, J.N. (2016). A three-dimensional organoid culture system derived from human glioblastomas recapitulates the hypoxic gradients and cancer stem cell heterogeneity of tumors found in vivo. *Cancer Res.* 76, 2465–2477.
- Hussain, S.F., Kong, L.Y., Jordan, J., Conrad, C., Madden, T., Fokt, I., Priebe, W., and Heimberger, A.B. (2007). A novel small molecule inhibitor of signal transducers and activators of transcription

3 reverses immune tolerance in malignant glioma patients. *Cancer Res.* 67, 9630–9636.

Huszthy, P.C., Daphu, I., Niclou, S.P., Stieber, D., Nigro, J.M., Sakariassen, P.Ø., Miletic, H., Thorsen, F., and Bjerkvig, R. (2012). In vivo models of primary brain tumors: pitfalls and perspectives. *Neuro. Oncol.* 14, 979–993.

Hutter, G., Theruvath, J., Graef, C.M., Zhang, M., Schoen, M.K., Manz, E.M., Bennett, M.L., Olson, A., Azad, T.D., Sinha, R., et al. (2019). Microglia are effector cells of CD47-SIRP $\alpha$  antiphagocytic axis disruption against glioblastoma. *Proc. Natl. Acad. Sci. U S A* 116, 997–1006.

Insua-Rodríguez, J., and Oskarsson, T. (2016). The extracellular matrix in breast cancer. *Adv. Drug Deliv. Rev.* 97, 41–55.

Jacob, F., Salinas, R.D., Zhang, D.Y., Nguyen, P.T.T., Schnoll, J.G., Wong, S.Z.H., Thokala, R., Sheikh, S., Saxena, D., Prokop, S., et al. (2020). A patient-derived glioblastoma organoid model and biobank recapitulates inter- and intratumoral heterogeneity. *Cell* 180, 188–204.e22.

Jain, N., Moeller, J., and Vogel, V. (2019). Mechanobiology of macrophages: how physical factors coregulate macrophage plasticity and phagocytosis. *Annu. Rev. Biomed. Eng.* 21, 267–297.

Katt, M.E., Placone, A.L., Wong, A.D., Xu, Z.S., and Searson, P.C. (2016). In vitro tumor models: advantages, disadvantages, variables, and selecting the right platform. *Front. Bioeng. Biotechnol.* 4, 12.

Kim, H., Cha, J., Jang, M., and Kim, P. (2019a). Hyaluronic acid-based extracellular matrix triggers spontaneous M2-like polarity of monocyte/macrophage. *Biomater. Sci.* 7, 2264–2271.

Kim, H., Chung, H., Kim, J., Choi, D.H., Shin, Y., Kang, Y.G., Kim, B.M., Seo, S.U., Chung, S., and Seok, S.H. (2019b). Macrophages-triggered sequential remodeling of endothelium-interstitial matrix to form pre-metastatic niche in microfluidic tumor microenvironment. *Adv. Sci.* 6, 1900195.

Klemm, F., Maas, R.R., Bowman, R.L., Kornete, M., Soukup, K., Nassiri, S., Brouland, J.P., Iacobuzio-Donahue, C.A., Brennan, C., Tabar, V., et al. (2020). Interrogation of the microenvironmental landscape in brain tumors reveals disease-specific alterations of immune cells. *Cell* 181, 1643–1660.e17.

Kowal, J., Kornete, M., and Joyce, J.A. (2019). Re-education of macrophages as a therapeutic strategy in cancer. *Immunotherapy* 11, 677–689.

Kumar, M.P., Du, J., Lagoudas, G., Jiao, Y., Sawyer, A., Drummond, D.C., Lauffenburger, D.A., and Raue, A. (2018). Analysis of single-cell RNA-seq identifies cell-cell communication associated with tumor characteristics. *Cell Rep.* 25, 1458–1468.e4.

Larsen, A.M.H., Kuczek, D.E., Kalvisa, A., Siersbæk, M.S., Thorseth, M.-L., Johansen, A.Z., Carretta, M., Grøntved, L., Vang, O., and Madsen, D.H. (2020). Collagen density modulates the immunosuppressive functions of macrophages. *J. Immunol.* 205, 1461–1472.

Laviron, M., and Boissonnas, A. (2019). Ontogeny of tumor-associated macrophages. *Front. Immunol.* 10, 1799.

Lee, H., Pal, S.K., Reckamp, K., Figlin, R.A., and Yu, H. (2011). STAT3: a target to enhance antitumor immune response. *Curr. Top Microbiol. Immunol.* 344, 41–59.

Leidi, M., Gotti, E., Bologna, L., Miranda, E., Rimoldi, M., Sica, A., Roncalli, M., Palumbo, G.A., Introna, M., and Golay, J. (2009). M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than M1 cells in vitro. *J. Immunol.* 182, 4415–4422.

Lewis, D.M., and Gerecht, S. (2016). Microfluidics and biomaterials to study angiogenesis. *Curr. Opin. Chem. Eng.* 11, 114–122.

Li, R., Hebert, J.D., Lee, T.A., Xing, H., Boussommier-Calleja, A., Hynes, R.O., Lauffenburger, D.A., and Kamm, R.D. (2017). Macrophage-secreted TNF $\alpha$  and TGF $\beta$ 1 influence migration speed and persistence of cancer cells in 3D tissue culture via independent pathways. *Cancer Res.* 77, 279–290.

Lim, S.Y., Yuzhalin, A.E., Gordon-Weeks, A.N., and Muschel, R.J. (2016). Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget* 7, 28697–28710.

Lin, J.M.G., Kang, C.C., Zhou, Y., Huang, H., Herr, A.E., and Kumar, S. (2018). Linking invasive motility to protein expression in single tumor cells. *Lab Chip* 18, 371–384.

Linkous, A., Balamatsias, D., Snuderl, M., Edwards, L., Miyaguchi, K., Milner, T., Reich, B., Cohen-Gould, L., Storka, A., Nakayama, Y., et al. (2019). Modeling patient-derived glioblastoma with cerebral organoids. *Cell Rep.* 26, 3203–3211.e5.

Linville, R.M., Boland, N.F., Covarrubias, G., Price, G.M., and Tien, J. (2016). Physical and chemical signals that promote vascularization of capillary-scale channels. *Cell. Mol. Bioeng.* 9, 73–84.

Liu, M., Tolg, C., and Turley, E. (2019). Dissecting the dual nature of hyaluronan in the tumor microenvironment. *Front. Immunol.* 10, 1–9.

Lu-emerson, C., Snuderl, M., Kirkpatrick, N.D., Goveia, J., Davidson, C., Huang, Y., Riedemann, L., Taylor, J., Ivy, P., Duda, G., et al. (2013). Increase in tumor-associated macrophages after antiangiogenic therapy is associated with poor survival among patients with recurrent glioblastoma. *Neuro Oncol* 15, 1079–1087.

Lu, J., Xu, Z., Duan, H., Ji, H., Zhen, Z., Li, B., Wang, H., Tang, H., Zhou, J., Guo, T., et al. (2020). Tumor-associated macrophage interleukin- $\beta$  promotes glycerol-3-phosphate dehydrogenase activation, glycolysis and tumorigenesis in glioma cells. *Cancer Sci.* 111, 1979–1990.

Lugano, R., Ramachandran, M., and Dimberg, A. (2020). Tumor angiogenesis: causes, consequences, challenges and opportunities. *Cell. Mol. Life Sci.* 77, 1745–1770.

Lv, D., Hu, Z., Lu, L., Lu, H., and Xu, X. (2017). Three-dimensional cell culture: a powerful tool in tumor research and drug discovery. *Oncol. Lett.* 14, 6999–7010.

Ma, Y.-H.V., Middleton, K., You, L., and Sun, Y. (2018). A review of microfluidic approaches for investigating cancer extravasation during metastasis. *Microsystems Nanoeng* 4, 1–13.

Maas, S.L.N., Abels, E.R., Van De Haar, L.L., Zhang, X., Morsett, L., Sil, S., Guedes, J., Sen, P., Prabhakar, S., Hickman, S.E., et al. (2020). Glioblastoma hijacks microglial gene expression to support tumor growth. *J. Neuroinflammation* 17, 1–18.

Mantovani, A. (2014). Macrophages, neutrophils, and cancer: a double edged sword. *New J. Sci.* 2014, 1–14.

Mantovani, A., and Allavena, P. (2015). The interaction of anticancer therapies with tumor-associated macrophages. *J. Exp. Med.* 212, 435–445.

Martinez, F.O., Helming, L., Milde, R., Varin, A., Melgert, B.N., Draijer, C., Thomas, B., Fabbri, M., Crawshaw, A., Ho, L.P., et al. (2013). Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. *Blood* 121, 57–69.

McGranahan, T., Therkelsen, K.E., Ahmad, S., and Nagpal, S. (2019). Current state of immunotherapy for treatment of glioblastoma. *Curr. Treat. Options Oncol.* 20, 24.

Mestas, J., and Hughes, C.C.W. (2004). Of mice and not men: differences between mouse and human immunology. *J. Immunol.* 172, 2731–2738.

Mills, C.D., Kincaid, K., Alt, J.M., Heilman, M.J., and Hill, A.M. (2000). M-1/M-2 macrophages and the Th1/Th2 paradigm. *J. Immunol.* 164, 6166–6173.

Miyai, M., Tomita, H., Soeda, A., Yano, H., Iwama, T., and Hara, A. (2017). Current trends in mouse models of glioblastoma. *J. Neurooncol.* 135, 423–432.

Mora, R., Abschuetz, A., Kees, T., Dokić, I., Joschko, N., Kleber, S., Geibig, R., Mosconi, E., Zentgraf, H., Martin-Villalba, A., and Régnier-Vigouroux, A. (2009). TNF- $\alpha$  and TRAIL-resistant glioma cells undergo autophagy-dependent cell death induced by activated microglia. *Glia* 57, 561–581.

Mrdjen, D., Pavlovic, A., Hartmann, F.J., Schreiner, B., Utz, S.G., Leung, B.P., Lelios, I., Heppner, F.L., Kipnis, J., Merkler, D., et al. (2018). High-dimensional single-cell mapping of central nervous system immune cells reveals distinct myeloid subsets in Health, aging, and disease. *Immunity* 48, 380–395.e6.

Müller, S., Kohanbash, G., Liu, S.J., Alvarado, B., Carrera, D., Bhaduri, A., Watchmaker, P.B., Yagnik, G., Di Lullo, E., Malatesta, M., et al. (2017). Single-cell profiling of human gliomas reveals macrophage ontogeny as a basis for regional differences in macrophage activation in the tumor microenvironment. *Genome Biol.* 18, 1–14.

Murray, P.J. (2017). Macrophage polarization. *Annu. Rev. Physiol.* 79, 541–566.

Nahrendorf, M., and Swirski, F.K. (2016). Abandoning M1/M2 for a network model of macrophage function. *Circ. Res.* 119, 414–417.



- Nakod, P.S., Kim, Y., and Rao, S.S. (2020). Three-dimensional biomimetic hyaluronic acid hydrogels to investigate glioblastoma stem cell behaviors. *Biotechnol. Bioeng.* *117*, 511–522.
- Neal, J.T., Li, X., Zhu, J., Giangarra, V., Grzeskowiak, C.L., Ju, J., Liu, I.H., Chiou, S.H., Salahudeen, A.A., Smith, A.R., et al. (2018). Organoid modeling of the tumor immune microenvironment. *Cell* *175*, 1972–1988.e16.
- Nguyen, D.H.T., Stapleton, S.C., Yang, M.T., Cha, S.S., Choi, C.K., Galie, P.A., and Chen, C.S. (2013). Biomimetic model to reconstitute angiogenic sprouting morphogenesis in vitro. *Proc. Natl. Acad. Sci. U S A* *110*, 6712–6717.
- Noorani, I. (2019). Genetically engineered mouse models of gliomas: technological developments for translational discoveries. *Cancers (Basel)* *11*, 1335.
- Novak, U., and Kaye, A.H. (2000). Extracellular matrix and the brain: components and function. *J. Clin. Neurosci.* *7*, 280–290.
- Nunes, A.S., Barros, A.S., Costa, E.C., Moreira, A.F., and Correia, I.J. (2019). 3D tumor spheroids as in vitro models to mimic in vivo human solid tumors resistance to therapeutic drugs. *Biotechnol. Bioeng.* *116*, 206–226.
- Nywening, T.M., Wang-Gillam, A., Sanford, D.E., Belt, B.A., Panni, R.Z., Cusworth, B.M., Toriola, A.T., Nieman, R.K., Worley, L.A., Yano, M., et al. (2016). Phase 1b study targeting tumour associated macrophages with CCR2 inhibition plus FOLFIRINOX in locally advanced and borderline resectable pancreatic cancer HHS Public Access. *Lancet Oncol.* *17*, 651–662.
- Ogawa, J., Pao, G.M., Shokhirev, M.N., and Verma, I.M. (2018). Glioblastoma model using human cerebral organoids. *Cell Rep.* *23*, 1220–1229.
- Oh, T., Fakurnejad, S., Sayegh, E.T., Clark, A.J., Ivan, M.E., Sun, M.Z., Safaei, M., Bloch, O., James, C.D., and Parsa, A.T. (2014). Immunocompetent murine models for the study of glioblastoma immunotherapy. *J. Transl. Med.* *12*, 1–10.
- Ott, M., Kassab, C., Marisetty, A., Hashimoto, Y., Wei, J., Zamler, D.B., Leu, J.-S., Tomaszowski, K.-H., Sabbagh, A., Fang, D., et al. (2020). Radiation with STAT3 blockade triggers dendritic cell-T cell interactions in the glioma microenvironment and therapeutic efficacy. *Clin. Cancer Res.* *26*, 4983–4994.
- Pang, L., Qin, J., Han, L., Zhao, W., Liang, J., Xie, Z., Yang, P., and Wang, J. (2016). Exploiting macrophages as targeted carrier to guide nanoparticles into glioma. *Oncotarget* *7*, 37081–37091.
- Piao, Y., Liang, J., Holmes, L., Zurita, A.J., Henry, V., Heymach, J.V., and De Groot, J.F. (2012). Glioblastoma resistance to anti-VEGF therapy is associated with myeloid cell infiltration, stem cell accumulation, and a mesenchymal phenotype. *Neuro. Oncol.* *14*, 1379–1392.
- Pires-Afonso, Y., Niclou, S.P., and Michelucci, A. (2020). Revealing and harnessing tumour-associated microglia/macrophage heterogeneity in glioblastoma. *Int. J. Mol. Sci.* *21*, 689.
- Poh, A.R., and Ernst, M. (2018). Targeting macrophages in cancer: from bench to bedside. *Front. Oncol.* *8*, 1–16.
- Polacheck, W.J., Kutys, M.L., Tefft, J.B., and Chen, C.S. (2019). Microfabricated Blood Vessels for Modeling the Vascular Transport Barrier. *Nature Protocols (Springer US)*. <https://doi.org/10.1038/s41596-019-0144-8>.
- Porter, K., Lin, Y., and Liton, P.B. (2013). Cathepsin B is up-regulated and mediates extracellular matrix degradation in trabecular meshwork cells following phagocytic challenge. *PLoS One* *8*, 1–18.
- Pyonteck, S.M., Akkari, L., Schuhmacher, A.J., Bowman, R.L., Sevenich, L., Quail, D.F., Olson, O.C., Quick, M.L., Huse, J.T., Teijeiro, V., et al. (2013). CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat. Med.* *19*, 1264–1272.
- Qi, L., Yu, H., Zhang, Y., Zhao, D., Lv, P., Zhong, Y., and Xu, Y. (2016). IL-10 secreted by M2 macrophage promoted tumorigenesis through interaction with JAK2 in glioma. *Oncotarget* *7*, 71673–71685.
- Qian, X., Song, H., and Ming, G.L. (2019). Brain organoids: advances, applications and challenges. *Development* *146*, dev166074.
- Quail, D.F., and Joyce, J.A. (2017). The microenvironmental landscape of brain tumors. *Cancer Cell* *31*, 326–341.
- Rajab, N., Rutar, M., Laslett, A.L., and Wells, C.A. (2018). Designer macrophages: pitfalls and opportunities for modelling macrophage phenotypes from pluripotent stem cells. *Differentiation* *104*, 42–49.
- Rauch, U. (2004). Extracellular matrix components associated with remodeling processes in brain. *Cell. Mol. Life Sci.* *61*, 2031–2045.
- Rianna, C., Radmacher, M., and Kumar, S. (2020). Direct evidence that tumor cells soften when navigating confined spaces. *Mol. Biol. Cell* *31*, 1726–1734.
- Saavedra-López, E., Roig-Martínez, M., Cribaro, G.P., Casanova, P.V., Gallego, J.M., Pérez-Vallés, A., and Barcia, C. (2020). Phagocytic glioblastoma-associated microglia and macrophages populate invading pseudopalisades. *Brain Commun.* *2*, 1–20.
- Sankowski, R., Böttcher, C., Masuda, T., Geirsdottir, L., Sagar, Sindram, E., Seredenina, T., Muhs, A., Scheiwe, C., Shah, M.J., et al. (2019). Mapping microglia states in the human brain through the integration of high-dimensional techniques. *Nat. Neurosci.* *22*, 2098–2110.
- Schneemann, M., and Schoeden, G. (2007). Macrophage biology and immunology: man is not a mouse. *J. Leukoc. Biol.* *81*, 579.
- Schroder, K., Irvine, K.M., Taylor, M.S., Bokil, N.J., Le Cao, K.A., Masterman, K.A., Labzin, L.I., Semple, C.A., Kapetanovic, R., Fairbairn, L., et al. (2012). Conservation and divergence in Toll-like receptor 4-regulated gene expression in primary human versus mouse macrophages. *Proc. Natl. Acad. Sci. U S A* *109*, E944–53.
- Sedgwick, J.D., Riminton, D.S., Cyster, J.G., and Körner, H. (2000). Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol. Today* *21*, 110–113.
- Sevenich, L. (2018). Brain-resident microglia and blood-borne macrophages orchestrate central nervous system inflammation in neurodegenerative disorders and brain cancer. *Front. Immunol.* *9*, 1–16.
- Shi, C., and Pamer, E.G. (2011). Monocyte recruitment during infection and inflammation. *Nat. Rev. Immunol.* *11*, 762–774.
- Shih, J., Yuan, A., Chen, J.J., and Yang, P. (2006). Tumor-associated macrophage: its role in cancer invasion and metastasis. *J. Cancer Mol.* *2*, 101–106.
- Sica, A., and Mantovani, A. (2012). Plasticity and polarization. *J. Clin. Invest.* *122*, 787–795.
- Sørensen, M.D., Dahlrot, R.H., Boldt, H.B., Hansen, S., and Kristensen, B.W. (2018). Tumour-associated microglia/macrophages predict poor prognosis in high-grade gliomas and correlate with an aggressive tumour subtype. *Neuropathol. Appl. Neurobiol.* *44*, 185–206.
- Spiller, K.L., Anfang, R.R., Spiller, K.J., Ng, J., Nakazawa, K.R., Daulton, J.W., and Vunjak-Novakovic, G. (2014). The role of macrophage phenotype in vascularization of tissue engineering scaffolds. *Biomaterials* *35*, 4477–4488.
- Spiller, K.L., Wrona, E.A., Romero-Torres, S., Pallotta, I., Graney, P.L., Witherell, C.E., Panicker, L.M., Feldman, R.A., Urbanska, A.M., Santambrogio, L., et al. (2016). Differential gene expression in human, murine, and cell line-derived macrophages upon polarization. *Exp. Cell Res.* *347*, 1–13.
- Sridharan, R., Cameron, A.R., Kelly, D.J., Kearney, C.J., and O'Brien, F.J. (2015). Biomaterial based modulation of macrophage polarization: a review and suggested design principles. *Mater. Today* *18*, 313–325.
- Stanley, E.R., and Chitu, V. (2014). CSF-1 receptor signaling in myeloid cells. *Cold Spring Harb. Perspect. Biol.* *6*, 1–22.
- Stupp, R., Hegi, M.E., Gilbert, M.R., and Chakravarti, A. (2007). Chemoradiotherapy in malignant glioma: standard of care and future directions. *J. Clin. Oncol.* *25*, 4127–4136.
- Sung, K.E., and Beebe, D.J. (2014). Microfluidic 3D models of cancer. *Adv. Drug Deliv. Rev.* *79*, 68–78.
- Szulzewsky, F., Pelz, A., Feng, X., Synowitz, M., Markovic, D., Langmann, T., Holtman, I.R., Wang, X., Eggen, B.J.L., Boddeke, H.W.G.M., et al. (2015). Glioma-associated microglia/macrophages display an expression profile different from M1 and M2 polarization and highly express Gpnmb and Spp1. *PLoS One* *10*, 1–27.
- Tao, L., and Reese, T.A. (2017). Making mouse models that reflect human immune responses. *Trends Immunol.* *38*, 181–193.
- Tevis, K.M., Cecchi, R.J., Colson, Y.L., and Grinstaff, M.W. (2017a). Mimicking the tumor microenvironment to regulate macrophage

- phenotype and assessing chemotherapeutic efficacy in embedded cancer cell/macrophage spheroid models. *Acta Biomater.* 50, 271–279.
- Tevis, K.M., Colson, Y.L., Grinstaff, M.W., and Colson, Y. (2017b). Embedded spheroids as models of the cancer microenvironment corresponding authors HHS public access author manuscript. *Adv. Biosyst.* 1, 1–33.
- Thorsson, V., Gibbs, D.L., Brown, S.D., Wolf, D., Bortone, D.S., Ou Yang, T.-H., Porta-Pardo, E., Gao, G.F., Plaisier, C.L., and Eddy, J.A. (2018). The immune landscape of cancer. *Immunity* 48, 812–830.e14.
- Tibbitt, M.W., and Anseth, K.S. (2009). Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol. Bioeng.* 103, 655–663.
- Tomaszewski, W., Sanchez-Perez, L., Gajewski, T.F., and Sampson, J.H. (2019). Brain tumor microenvironment and host state: implications for immunotherapy. *Clin. Cancer Res.* 25, 4202–4210.
- Truong, D., Fiorelli, R., Barrientos, E.S., Melendez, E.L., Mehta, S., Nikkhah, M., and Brain, I. (2020). A three-dimensional (3D) organotypic microfluidic model for glioma stem cells – Vasc. Interactions. *Biomaterials* 198, 63–77.
- Tugal, D., Liao, X., and Jain, M.K. (2013). Transcriptional control of macrophage polarization. *Arterioscler. Thromb. Vasc. Biol.* 33, 1135–1144.
- van der Helm, M.W., van der Meer, A.D., Eijkel, J.C.T., van den Berg, A., and Segerink, L.I. (2016). Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue Barriers* 4, e1142493.
- Venteicher, A.S., Tirosh, I., Hebert, C., Yizhak, K., Neftel, C., Filbin, M.G., Hovestadt, V., Escalante, L.E., Shaw, M.L., Rodman, C., et al. (2017). Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science* 355, 355.
- Viallard, C., and Larrivé, B. (2017). Tumor angiogenesis and vascular normalization: alternative therapeutic targets. *Angiogenesis* 20, 409–426.
- Wakimoto, H., Mohapatra, G., Kanai, R., Curry, W.T., Yip, S., Nitta, M., Patel, A.P., Barnard, Z.R., Stemmer-Rachamimov, A.O., Louis, D.N., et al. (2012). Maintenance of primary tumor phenotype and genotype in glioblastoma stem cells. *Neuro. Oncol.* 14, 132–144.
- Walentynowicz, K.A., Ochocka, N., Pasierbinska, M., Wojnicki, K., Stepniak, K., Mieczkowski, J., Ciechomska, I.A., and Kaminska, B. (2018). In search for reliable markers of glioma-induced polarization of microglia. *Front. Immunol.* 9, 1–13.
- Wang, Q., Hu, B., Hu, X., Kim, H., Squatrito, M., Scarpace, L., deCarvalho, A.C., Lyu, S., Li, P., Li, Y., et al. (2017). Tumor evolution of glioma-intrinsic gene expression subtypes associates with immunological changes in the microenvironment. *Cancer Cell* 32, 42–56.e6.
- Wang, S.C., Yu, C.F., Hong, J.H., Tsai, C.S., and Chiang, C.S. (2013). Radiation therapy-induced tumor invasiveness is associated with SDF-1-regulated macrophage mobilization and vasculogenesis. *PLoS One* 8, e69182.
- Wesolowski, R., Sharma, N., Reebel, L., Rodal, M.B., Peck, A., West, B.L., Marimuthu, A., Severson, P., Karlin, D.A., Dowlati, A., et al. (2019). Phase Ib study of the combination of pexidartinib (PLX3397), a CSF-1R inhibitor, and paclitaxel in patients with advanced solid tumors. *Ther. Adv. Med. Oncol.* 11, 1–13.
- Whitesides, G.M. (2006). The origins and the future of microfluidics. *Nature* 442, 368–373.
- Wolf, K.J., Chen, J., Coombes, J.D., Aghi, M.K., and Kumar, S. (2019). Dissecting and rebuilding the glioblastoma microenvironment with engineered materials. *Nat. Rev. Mater.* 4, 651–668.
- Wolf, K.J., Lee, S., and Kumar, S. (2018). A 3D topographical model of parenchymal infiltration and perivascular invasion in glioblastoma. *APL Bioeng.* 2, 031903.
- Wu, A., Wei, J., Kong, L.Y., Wang, Y., Priebe, W., Qiao, W., Sawaya, R., and Heimbeger, A.B. (2010). Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro. Oncol.* 12, 1113–1125.
- Xiao, W., Sohrabi, A., and Seidlits, S.K. (2017). Integrating the glioblastoma microenvironment into engineered experimental models. *Future Sci. OA* 3, FSO189.
- Xiao, W., Wang, S., Zhang, R., Sohrabi, A., Yu, Q., Liu, S., Ehsanipour, A., Liang, J., Bierman, R.D., Nathanson, D.A., and Seidlits, S.K. (2020). Bioengineered scaffolds for 3D culture demonstrate extracellular matrix-mediated mechanisms of chemotherapy resistance in glioblastoma. *Matrix Biol.* 85, 128–146.
- Xu, H., Li, Z., Yu, Y., Sizdahkhani, S., Ho, W.S., Yin, F., Wang, L., Zhu, G., Zhang, M., Jiang, L., et al. (2016). A dynamic in vivo-like organotypic blood-brain barrier model to probe metastatic brain tumors. *Sci. Rep.* 6, 1–12.
- Xu, Y., Liao, C., Liu, R., Liu, J., Chen, Z., Zhao, H., Li, Z., Chen, L., Wu, C., Tan, H., et al. (2019). IRGM promotes glioma M2 macrophage polarization through p62/TRAF6/NF- $\kappa$ B pathway mediated IL-8 production. *Cell Biol. Int.* 43, 125–135.
- Yan, D., Kowal, J., Akkari, L., Schuhmacher, A.J., Huse, J.T., West, B.L., and Joyce, J.A. (2017). Inhibition of colony stimulating factor-1 receptor abrogates microenvironment-mediated therapeutic resistance in gliomas. *Oncogene* 36, 6049–6058.
- Yang, M., McKay, D., Pollard, J.W., and Lewis, C.E. (2018). Diverse functions of macrophages in different tumor microenvironments. *Cancer Res.* 78, 5492–5503.
- Ye, X., Xu, S., Xin, Y., Yu, S., Ping, Y., Chen, L., Xiao, H., Wang, B., Yi, L., Wang, Q., et al. (2012). Tumor-associated microglia/macrophages enhance the invasion of glioma stem-like cells via TGF- $\beta$ 1 signaling pathway. *J. Immunol.* 189, 444–453.
- Zeiner, P.S., Preusse, C., Golebiewska, A., Zinke, J., Iriondo, A., Muller, A., Kaoma, T., Filipski, K., Müller-Eschner, M., Bernatz, S., et al. (2019). Distribution and prognostic impact of microglia/macrophage subpopulations in gliomas. *Brain Pathol.* 29, 513–529.
- Zhang, B., Peticone, C., Murthy, S.K., and Radisic, M. (2013). A standalone perfusion platform for drug testing and target validation in micro-vessel networks. *Biomicrofluidics* 7, 44125.
- Zhang, H., Xue, C., Shah, R., Bermingham, K., Hinkle, C.C., Li, W., Rodrigues, A., Tabita-Martinez, J., Millar, J.S., Cuchel, M., et al. (2015). Functional analysis and transcriptomic profiling of iPSC-derived macrophages and their application in modeling mendelian disease. *Circ. Res.* 117, 17–28.
- Zhang, Y.S., Duchamp, M., Oklu, R., Ellisen, L.W., Langer, R., and Khademhosseini, A. (2016). Bioprinting the cancer microenvironment. *ACS Biomater. Sci. Eng.* 2, 1710–1721.
- Zhou, W., Ke, S.Q., Huang, Z., Flavahan, W., Fang, X., Paul, J., Wu, L., Sloan, A.E., McLendon, R.E., Li, X., et al. (2015). Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. *Nat. Cell Biol.* 17, 170–182.