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Hacking macrophage-associated immunosuppression for regulating glioblastoma angiogenesis

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ABSTRACT

Glioblastoma (GBM) is the most lethal primary adult brain tumor and its pathology is hallmarked by distorted neovascularization, diffuse tumor-associated macrophage infiltration, and potent immunosuppression. Reconstituting organotypic tumor angiogenesis models with biomimetic cell heterogeneity and interactions, pro-/anti-inflammatory milieu and extracellular matrix (ECM) mechanics is critical for preclinical anti-angiogenic therapeutic screening. However, current *in vitro* systems do not accurately mirror *in vivo* human brain tumor microenvironment. Here, we engineered a three-dimensional (3D), microfluidic angiogenesis model with controllable and biomimetic immunosuppressive conditions, immune-vascular and cell-matrix interactions. We demonstrate *in vitro*, GL261 and CT-2A GBM-like tumors steer macrophage polarization towards a M2-like phenotype for fostering an immunosuppressive and proangiogenic niche, which is consistent with human brain tumors. We distinguished that GBM and M2-like immunosuppressive macrophages promote angiogenesis, while M1-like pro-inflammatory macrophages suppress angiogenesis, which we coin "inflammation-driven angiogenesis." We observed soluble immunosuppressive cytokines, predominantly TGF-β1, and surface integrin ($\alpha_v\beta_3$) endothelial-macrophage interactions are required in inflammation-driven angiogenesis. We demonstrated tuning cell-adhesion receptors using an integrin ($\alpha_v\beta_3$)-specific collagen hydrogel regulated inflammation-driven angiogenesis through Src-PI3K-YAP signaling, highlighting the importance of altered cell-ECM interactions in inflammation. To validate the preclinical applications of our 3D organoid model and mechanistic findings of inflammation-driven angiogenesis, we screened a novel dual integrin ($\alpha_v\beta_3$) and cytokine receptor (TGFβ-R1) blockade that suppresses GBM tumor neovascularization by simultaneously targeting macrophage-associated immunosuppression, endothelial-macrophage interactions, and altered ECM. Hence, we provide an interactive and controllable GBM tumor microenvironment and highlight the importance of macrophage-associated immunosuppression in GBM angiogenesis, paving a new direction of screening novel anti-angiogenic therapies.

1. Introduction

Malignant neovascularization and immunosuppression are two hallmarks of glioblastoma (GBM) [1], the most prevalent and aggressive primary adult brain tumor. Newly-diagnosed GBM patients survive less than 15 months despite combinatorial surgery, chemotherapy and radiation therapy [2], while recurrent GBM patients survive less than 6 months with conventional salvage therapies [3]. GBM has been traditionally defined by histological and genetic alterations, but its tumor microenvironment—aberrant neovascularization, infiltrating tumor-associated macrophage (TAMs), and dynamic extracellular matrix (ECM) alterations—is being increasingly recognized as a critical factor of relapse and

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therapeutic resistance. Consequently, considerable interest has been directed to engineering biomimetic preclinical models to validate therapeutic efficacy and dissect how tumor-stroma interactions confer a drug-resistant GBM subtype.

A diagnostic feature that distinguishes GBMs from low-grade gliomas is aberrant microvascular proliferation due to high expression levels of diverse proangiogenic factors, indicating angiogenic endothelial cells (ECs) are promising GBM therapeutic targets [4]. However, the effects of currently available anti-angiogenic therapies like vascular endothelial growth factor (VEGF) inhibition result in transient, meager clinical responses due to acquired resistance [5]. Increasing evidence suggests GBM immunity contributes to tumor angiogenesis and therapeutic resistance [1]. GBM casts a proangiogenic and immunosuppressive microenvironment by secreting VEGF and transforming growth factor beta (TGF-β1) [6], promoting tumor vascularization and suppress cytotoxic T cell proliferation and function [7] to impede anti-angiogenic therapy and immunotherapy [8]. GBM patients exhibit marked immunosuppression [9], where tumor-infiltrating TAMs can comprise up to 30% of bulk GBM tumor mass [10]. Our past studies prove increasing numbers of tumor-infiltrating, perivascular TAMs after antiangiogenic therapy correlate to poor survival among recurrent GBM patients [11,12]. These findings highlight TAMs are critical drivers of anti-angiogenic resistance, but the molecular and intercellular mechanisms remain poorly defined. Therefore, investigating the pro-tumorigenic roles of TAMs and the synergy between tumor immunosuppression and angiogenesis is highly relevant for identifying potential targets to optimize GBM anti-angiogenic therapy.

However, a physiologically-accurate, integrated analysis of GBM tumor angiogenesis requires incorporating tumor-associated immunity to understand their parallel evolution during tumor progression and therapeutic resistance [6]. The intrinsic limitations of conventional methods for studying tumor angiogenesis pose a preclinical challenge. Primary dissociated cultures permit functional *in vitro* assays, but studying an isolated, single cell population in a two-dimensional (2D) environment does not reflect the true intertumoral heterogeneity and interactions [13]. Three-dimensional (3D) *in vitro* angiogenesis assays are more physiologically-relevant to *in vivo* GBM tumors than conventional 2D *in vitro* capillary network formation assays. Recent studies have distinguished differences in pro-/anti-inflammatory signaling between 2D and 3D environments [14], suggesting distinct regulatory roles for pro-angiogenic factor secretion during 2D and 3D angiogenesis [15,16]. Furthermore, anti-angiogenic therapeutics are clinically delivered through 3D vasculature until it reaches and diffuses into the GBM tumor site, which can be only replicated in 3D vascularized tumor microenvironments. *In vivo* murine models are the current “gold standard” for cancer therapeutic screenings, but they exhibit several intrinsic limitations for longitudinal immune cell monitoring. Patient-derived xenografts involve suppressing host immune rejection for implanting and studying patient-specific tumors *in vivo*, but they lack physiologically-accurate human immune responses. Furthermore, genetically-engineered mice models lack the genetic heterogeneity of patient-specific GBM tumors [17]. Patient explant cultures are advantageous because they can preserve 3D tissue architecture and GBM tumor microenvironment interactions, but they lack ongoing, dynamic extracellular cues like altered cell-ECM interactions and controllable biochemical gradients [18].

Recent advances in microfluidics-based *in vitro* models provide promising solutions for assessing cancer therapeutics, but they are sufficient to only model pathophysiological features independently without complete multiparametric integration into a single chip. Our previous study established a 3D hydrogel microfluidics-based model and demonstrated the biophysical roles of flow shear stress in regulating angiogenesis, but it did not consider the proangiogenic role of immunity [19]. Furthermore, perivascular interactions have been recently linked to sprouting angiogenesis [20], but GBM studies have yet dissected the proangiogenic roles of TAMs and cell-ECM interactions for regulating tumor angiogenesis and therapeutic efficacy. However, there are no existing *in vitro* 3D physiologically-accurate systems that reconstruct the unique and complex GBM tumor microenvironment with immunosuppressive and angiogenic signatures to dissect the synergy between tumor immunity and angiogenesis.

This requires constructing a microenvironment that mirrors GBM tumor-stroma crosstalk, where communication is mostly mediated between membrane-bound receptors and soluble (cytokines) and nonsoluble (cell adhesion receptors) ligands. Previous studies demonstrate increasing numbers of TAMs being proximal or in physical contact with tumor vasculature, implicating perivascular macrophage-EC interactions may regulate tumor angiogenesis [21,22]. Furthermore, ECM adhesion is essential for regulating tumor angiogenesis and immunity [23]. In GBM, promoted integrin expression correlates with increasing vascular density [24] and cytokine production and suppressed tumor-cytotoxicity [25]. Mounting evidence has demonstrated elevated integrin $\alpha_v\beta_3$ expression on angiogenic ECs [26] and glioma cells [24], which may be upregulated due to perivascular macrophage-EC or cell-ECM interactions. In addition, biomaterial studies have demonstrated polysaccharide-based [27] and silicified collagen [16] hydrogels can activate macrophage/monocytes to secrete pro-angiogenic factors for promoting *in vitro* and *in vivo* 3D angiogenesis. Therefore, 3D cell-suspending hydrogels offer an opportunity to tune integrin activation and coupled cellular behaviors like macrophage-mediated inflammation and angiogenesis [21]. Probing the dynamic interactions among angiogenic ECs, GBM TAMs and GBM tumors in a tunable ECM can lead to the development of a promising GBM anti-angiogenic therapeutic strategy [18].

Here, we engineered a biomimetic, microfluidics-based brain tumor angiogenesis model by implanting GBM-like tumors (GL261 or CT-2A) in a 3D artificial vascularized hydrogel of tunable ECM properties and immunosuppression gradients to mimic the *in vivo* GBM tumor niche. This organotypic platform reconstitutes notable GBM hallmarks, including GBM tumor angiogenesis and macrophage-associated immunosuppression. We have demonstrated that our *in vitro* GBM tumor microenvironment more closely replicates *in vivo* pathology by fostering immunosuppressive conditions that steer macrophage polarization towards an alternatively-activated M2-like phenotype promoting proangiogenic activity. We show GBM-induced M2-like macrophages exhibit elevated secretion of anti-inflammatory cytokines TGF-β1 and IL-10 to promote EC capillary, proliferation, and angiogenic sprouting, while classically-activated M1-like macrophages suppress proangiogenic activity, which we termed “inflammation-driven angiogenesis.” We reveal soluble immunosuppressive cytokines, predominantly TGF-β1, and surface EC-macrophage interactions are involved in regulating GBM tumor angiogenesis. Our studies implicate perivascular macrophage-EC interactions regulate *in vitro* proangiogenic activity through integrin ($\alpha_v\beta_3$) receptors and Src-PI3K-YAP signaling, which can be facilitated either by cell-cell or cell-matrix interactions. Our biomimetic GBM model with macrophage-associated immunosuppression and GBM tumor angiogenesis enables a high throughput screening for testing novel therapeutic combinations such as dual integrin ($\alpha_v\beta_3$) and cytokine receptor (TGFβ-R1) blockade to improve GBM therapeutic efficacy and minimize anti-angiogenic resistance.