**Response Letter**

**Redirecting the Route: Monocyte-Mediated Delivery of oHSV-1 Across a Human BBB-on-chip Model**

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**REVIEWER 1:**

The author used monocytes as vector cells to deliver the neuroattenuated strain of oncolytic herpes simplex virus type 1 (oHSV-1) to GBM and proved that monocytes, as vectors, could effectively deliver oncolytic viruses to glioma cells through static and dynamic microfluidic BBB-tumor in vitro models. This research has a certain degree of innovation, please consider modifying the following problems:

1. The key findings summarized in the article mentioned that 'monocytes preferentially migrate across the BBB in the presence of GBM spheroids, consistent with evidence that monocytes and tumor-associated macrophages (TAMs) are actively recruited to the glioma microenvironment through chemotactic cues (e.g., CD62L, CCL2, CXCL1, VEGF-A) and their corresponding receptors (e.g. CD62R, CCR2, CX3CR1, and VEGFR1)', 'infected monocytes do not release the virus en route nor infect BBB-resident cells. However, upon reaching GBM spheroids, viral replication is initiated, leading to robust infection and tumor cell death—comparable to direct free-virus treatment', there is a lack of key data support for monocytes responding to the chemokines and migration to tumor cells, monocytes not releasing viruses and not infecting BBB-related cells during the process, and tumor growth being strongly inhibited. Please consider supplementing experimental proofs.

We thank the reviewer for raising this important point. We agree that a clearer rationale and supporting evidence are needed to better support the proposed sequence of events regarding monocyte-mediated delivery of oHSV-1 to GBM spheroids. We previously demonstrated (Reale et al. 2023. REF #16 of the revised version of the manuscript) that human monocytes (both THP-1 and primary from healthy donors) are susceptible to infection with an oHSV-1 construct equivalent to T-VEC, carrying a reporter gene (EGFP). Key findings include:

*1.* **Infection profile**: *i.* THP-1 cells supported productive infection; *ii.* primary monocytes showed susceptibility but were poorly permissive, rapidly losing EGFP signal and releasing limited infectious particles, consistent with published literature (e.g., Morahan et al., 1989; Lee & Ghiasi, 2017; Bruun et al., 1998); *iii.* no cytotoxicity was observed in infected primary monocytes, confirming their viability post-infection.

2. **Influence of tumor signals on viral replication**: *i.* EGFP-oHSV-1–infected monocytes expressed ICP4 only after exposure to tumor-conditioned media (from MDA-MB-231 and UM-SCC-11B), indicating that viral replication is activated by tumor-derived cues; *ii.* viral titers significantly increased in the same settings, in parallel with a rise in EGFP-positive cells; *iii.* co-culture with cancer cells further increased virus production compared to infected monocytes alone.

3. **Monocyte tumor tropism**: *i.* infected and uninfected monocytes equally migrated toward tumor-conditioned media in standard transwell assays; *ii.* EGFP-oHSV-1-infected monocytes transmitted infection to tumor cells, with viral titers increasing by several orders of magnitude.

4. **In vivo validation in a chorioallantoic membrane (CAM) model**: *i.* THP-1-mediated delivery of oHSV-1 to UM-SCC-11B tumors was validated; *ii.* tumors showed robust expression of ICP4 and EGFP, with minimal off-target spread (e.g. low detection in liver/kidney); *iii.* immunofluorescence confirmed localization of infected monocytes (CD14+) within tumor tissue.

In this study, other than developing the BBB-on-chip and prior to applying this strategy to a 3D GBM model, we verified that primary monocytes infected with oHSV1-mCherry could successfully migrate toward GBM-conditioned media (U87-MG and LN229). We also assessed whether viral replication increased post-migration (data not shown in the main manuscript, but **Figure** provided in this Response Letter to the Reviewers). We believe this contributes to a validation of our proposed concept in glioma-relevant settings.

A graph of a patient's reaction

AI-generated content may be incorrect.***Figure Rev1.*** **Primary human monocytes loaded with oHSV1-mCherry migrate towards human GBM cells with a boost of viral replication**. **A.** Primary human monocytes infected with oHSV1-mCherry (MOI=5 PFU/cell) migrate towards serum-free medium conditioned by human GBM cells (LN229-c.m. and U87-MG-c.m., respectively) through a 5.0 µm-pore filter for 3 h. Cells were counted from at least 3 independent fields of view in the lower chamber. **B.** Infected monocytes were cultured at a 1:1 ratio with confluent U87-MG or LN229 cells, as indicated.

Regarding functional efficacy, we demonstrated that both THP-1 and primary monocyte-loaded oHSV-1 impaired tumor spheroid viability in static co-culture (MTT assays; **Fig. 2D**, **S3B**). Although direct quantification was technically challenging in the BBB-on-chip setup, qualitative evaluation of infected spheroids in Fig. 4B (day 5) and Fig. 5D (day 7) is consistent with a proposed cytopathic effect, showing that red (infected) cells are detaching, have altered shapes and reduced compactness. We also softened the language used while mentioning the lack of infection in BBB-resident cells and now cite a recent publication showing that THP-1 monocytes migrate across endothelial barriers only in the presence of GBM spheroids (now ref #38). We also further discussed the choice of monocytes as carriers, rooting the motivation in their physiological relevance: monocytes are precursors to tumor-associated macrophages (TAMs) and actively recruited to tumor microenvironments, including GBM, via multiple chemotactic axes. We finally added that ongoing experiments will further clarify the molecular signals guiding this recruitment and the behavior of monocytes, studies that go beyond the main scope of this manuscript.

These aspects and results are now better highlighted in the modified version of the manuscript.

1. In Figure 1E, the fluorescence staining of ZO-1 lacked typical membrane localization characteristics, and the cytoplasm staining of CD31 was very deep. There were two images of DAPI and GFAP placed under the blood site part, and the fluorescence intensity clarity was inconsistent. Please explain the reasons and check if there are any errors in the annotations in the figure. It looks like the bright field had been superimposed in the merge image. The entire image is very unclear and does not reflect effective information such as co-location. Please consider modifying it. The red fluorescence in Figure 3D seemed to have a very low resolution, and it was not focused and clear enough. Please consider modifying it. It is not easy to understand the image in Figure 4A and please check whether there was any mistake in marking the group. Please consider modifying and marking the structural instructions of the captured content.

We thank the reviewer for the comments and acknowledge that hat the original **Figure 1E** lacked clarity and contained some issues in both resolution and panel consistency. The suboptimal appearance of ZO-1 membrane localization and deep cytoplasmic CD31 signal is attributable to imaging through the porous membrane of the microfluidic BBB chip. The membrane's thickness, limited working distance, and light scattering significantly reduce resolution compared to standard 2D imaging setups. Nonetheless, the observed staining patterns are consistent with previous reports using similar microfluidic platforms. To address the Reviewer's concerns, we have replaced Figure 1E with improved, higher-quality images that enhance clarity and marker localization.

We similarly thank the Reviewer for pointing out the duplicated DAPI and GFAP panels. We apologize for the mistake, now corrected in the revised version. Regarding the merged image, no brightfield channel was intentionally included. However, contrast and overlay settings have now been optimized to improve signal-to-noise ratio and eliminate the appearance of any artifacts. Similarly, we fully agree that the red channel in the original version of **Figure 3D** lacked sufficient resolution and focus. A new, higher-quality image has now replaced the original in the revised version, providing improved clarity and signal definition. The Reviewer also noted issues with **Figure 4A**: we have now carefully reviewed it, verifying the group assignments, and made all necessary corrections. To improve interpretability, we also added structural annotations to better indicate the key features and contextualize the observed phenomena. We believe that the revised figure now facilitates a more intuitive and accurate reading of the data. Finally, with regards to our validation for the correct BBB formation beyond immunofluorescence, we underline how both the provided TEER measurements and permeability assays are considered functional evaluations supporting the successful establishment of a selective and robust barrier, strengthening the conclusions drawn from morphological data. We sincerely hope the Reviewer agrees with us.

1. In Figure 2C, the red oncolytic virus were released from the green THP-1 cells. Please explain the reason why there was no co-localization shown in the merge image. In Figure 2D, the tumor activity of the group intervened by THP-1 cells alone seemed to have significantly increased. Please explain the reason. The green fluorescence of image 72 hr in Figure 3C was quite different from that of 72hr-Control below. Please explain the reason. The image on the right side in Figure 4B Day 5, there was a distinct red fluorescence around the boundary of the tumor sphere. Please explain the reason. In the Day 1 No Ab group shown in Figure 5A and 5B, both the red fluorescence and the green fluorescence exhibited an unnatural, patchy, pasty localization and expression pattern. Please explain the reason.

We thank the Reviewer for these detailed observations. Each point is addressed below:

**Figure 2.C**: we agree that co-localization of green-labeled THP-1 cells and red oHSV-1 signal (mCherry) is not immediately obvious in the merged image. However, yellow cells, indicative of overlap between green (THP-1 vital dye) and red (virus-derived mCherry), are present and have now been more clearly highlighted with arrows in the revised figure. The relatively low number of visible yellow cells may be due to strong green fluorescence partially masking the red signal. We now removed the brightfield overlay in the merged panel and included a zoomed-in inset showing representative yellow cells. These changes are now incorporated into the revised Figure.

**Figure 2.D**: the observed increase in MTT signal in the THP-1-only group compared to the cancer cell control is expected. The co-culture includes metabolically active THP-1 cells which are not present in the control group. These cells contribute to the overall viability readout, explaining the elevated MTT signal. Conversely, in the oHSV-1-treated condition (THP-1 + GBM + virus), cell viability decreases due to viral cytopathic effects on both cell populations. We appreciate the Reviewer drawing attention to this and have added a clarifying comment in the Results section.

**Figure 3.C**: the Reviewer correctly noted differences between the 72 hr image and control. This reflects the fact that uninfected THP-1 cells proliferate over time, leading to increased green fluorescence. In contrast, infected THP-1 cells, particularly under conditions that promote viral replication, experience cytopathic effects, resulting in cell death and reduced green fluorescence. This distinction is consistent with the MTT results in Figure 2D and has been explicitly discussed in the revised manuscript.

**Figure 4.B**: the red fluorescence surrounding the spheroid in the Day 5 image refers to GBM cells infected by oHSV-1. These cells likely detached from the spheroid due to viral lysis and disrupted cell adhesion, a hallmark of viral replication and cytotoxicity. We now added this explanation to the revised Results section, highlighting this as additional evidence of oncolytic virus activity within the BBB-on-chip model.

**Figure 5.A and 5.B**: We agree with the Reviewer in observing that Day 1 panels in Figures 5A and 5B appear inconsistent and less informative, with uneven fluorescence likely due to cell adhesion artifacts shortly after seeding. To address this, we have removed the Day 1 panels from the revised figures, thereby improving clarity and focus on the more relevant and representative time points. We believe this enhances figure quality without impacting the scientific message.

1. In part 2.3 mentioned that 'no infection of the BBB cells is observed, confirmed by the negligible red fluorescent signals', the flickering fluorescence could still be seen in the image. The use of 'no' here is not quite precise. Please consider modifying it.

We apologize for the imprecision (which we now corrected in the revised manuscript). The Reviewer is right in pointing out that a “full no” is an overstatement. We also mitigated this statement in the Abstract and the Conclusion of the revised version of the manuscript.

**REVIEWER 2:**

This article explores the use of monocytes as vehicles for delivery of oncolytic viruses across a blood-brain barrier (BBB)-on-chip model to infect glioblastoma (GBM) spheroids. The authors established and validated a human microfluidic BBB-on-chip model, incorporating endothelial, astrocytic, and pericytic layers. They showed that monocytes loaded with oncolytic herpes simplex virus type 1 (oHSV-1) can deliver oHSV-1 across the BBB to infect GBM spheroids, shield oHSV-1 from neutralization by antibodies and avoid off-target infection of BBB. While the concept and results are interesting, the following comments need to be addressed:

1. Conceptual novelty. Does the novelty lie in the advancement of the microfluidic blood brain barrier (BBB) / blood-brain tumor barrier (BBTB) model design over chips? Or is the novelty on the advancement of oncolytic viruses for crossing BBB via the monocytes? What is the major research question? For example, does this model show any novelty in the microfluidic BBB / BBTB models over the other studies such as Li et al., OOC, 5, 100027 (2023), Hajal et al., Nat. Protoc., 17, 95-128 (2022).

We thank the Reviewer for this chance of better highlighting where we believe our advancements of the state of the art mostly reside. Our study contributes to both fields mentioned, BBB/BBTB microfluidic modeling and oncolytic virotherapy, yet we believe the most impactful advancement lies in the integration of both these aspects into a functional and translational proof-of-concept system.

With regards to the on-chip platforms, we agree with the reviewer in acknowledging the sophistication of recent BBB/BBTB-on-chip model, including those developed by Li et al. and Hajal et al, greatly advancing the field through high-fidelity multicellular constructs, extracellular matrices, and real-time permeability assessments. However, our model introduces complementary strengths that we believe make it novel and more readily translatable such as *i.* simplicity and accessibility, *ii.* modularity in the tumor compartment, *iii.* physiologically relevant flows and shear, and *iv.* rapid maturation and adaptability.

Our platform is designed to simplify operation without sacrificing biological fidelity. Unlike some complex models that require extensive optimization and/or proprietary components, our system enables fast barrier formation, reduced experimental setup time, and reproducibility. In addition, the integrated and customizable tumor compartment allows for direct seeding of 3D spheroids in proximity to and in communication with the endothelial barrier. This is not a common feature in many cited models, where tumor compartments are either remote or absent. Our controlled flow profiles, unlike gravity-driven perfusion systems, better mimic physiological shear stresses, critical for proper endothelial cell behavior and barrier integrity. Finally, our platform allows barrier formation in a streamlined process, differently from other models which rely on complex matrix gel composition and lengthy cell co-culture times. To reflect these points, we have added a brief comparative statement in the Conclusions section of the revised manuscript.

Regarding the potential of oncolytic virotherapy, we believe that our most significant advancement is demonstrating for the first time that human monocytes can be used to deliver oHSV-1 across an in vitro BBB to GBM spheroids, while retaining their cargo and shielding it from neutralizing antibodies. While monocyte migration to tumors is well described, our work is the first to show that monocytes infected with oHSV-1 can cross a biologically relevant BBB model and deliver functional oncolytic agents to GBM spheroids on the far side of the barrier. At the same time, we provide evidence that monocytes protect oHSV-1 from pre-existing immunity during transit, addressing one of the key translational barriers in oncolytic virotherapy. Finally, out data show that monocytes-mediated virus delivery results in tumor cell infection comparable to free virus, without infecting the BBB-resident cells en route, highlighting both safety and specificity.

Overall, the novelty of our manuscript is represented by this dual innovation in both model and application. We believe that our approach of course builds upon previous literature, but simultaneously proposes a streamlined and translatable tool for studying therapeutic delivery across the BBB in a multiplicity of disease models.

1. Mechanism of delivery and therapeutic action. The mechanism of monocyte passage through BBB and into the spheroid tumours, and subsequent release of viral particles is unclear. How are the oHSV-1 particles released from monocytes to the tumor cells? How do monocytes and viruses enter and exit the BBB? How do they enter tumour spheroid cells, and cross all other cell types of the model? Finally, please discuss the advantage of using monocytes as the delivery strategies over other delivery strategies across the blood-brain barrier/advantage of viral delivery over other gene therapies in the field. (Please cite and discuss these papers: Howard et al., Small, 18, 13, 2104763 (2022), Luo et al., Adv. Sci., 9, 26, 2201740 (2022), Lee et al, Mol. Pharmaceutics, 18, 2, 610-626 (2021)).

We thank the Reviewer for the constructive comment, which allowed us to further clarify both the biological mechanisms and the translational rationale behind our approach.

Monocytes are physiologically capable of crossing the BBB through both paracellular and transcellular routes, responding to chemokines and inflammatory cues, particularly in pathological conditions such as glioblastoma (Amann et al., Nat. Immunol., 2023; Séguin et al., J Neuropathol Exp Neurol., 2003). This innate tropism is especially relevant in GBM, where tumor-associated macrophages (TAMs) are known to originate at least in part from circulating monocytes (Xu et al., Front. Immunol., 2022). Our BBB-on-chip reproduces this behavior by enabling oHSV-1-loaded monocytes to migrate across a functional endothelial barrier and deliver their viral cargo directly to 3D GBM spheroids. The release of viral particles is induced once infected monocytes enter the tumor compartment and are exposed to the tumor microenvironment, likely due to metabolic and cytokine-mediated stimulation, as we have previously shown (Reale et al., IJMS, 2023). These stimuli boost viral replication, leading to release of newly formed virus particles capable of infecting adjacent tumor cells. Another important aspect is the fact that the monocytes cargo does not infect the endothelial cells lining the BBB, and viral replication is triggered only after arrival in the tumor zone, minimizing off-target infection. This is supported by our results and our previous in vitro and CAM-model studies. We have clarified these mechanisms on the revised manuscript.

As correctly highlighted, several strategies have been proposed for overcoming the BBB in glioma therapy, including those by Howard et al. (Small, 2022) who used magnetic nanoparticles derived from bacteria to deliver oHSV-1. While elegant and relevant, this approach requires extensive detoxification and external magnetic guidance for targeting, limiting broad applicability and scalability. Similarly, Luo et al. (Adv. Sci., 2022) and Lee et al. (Mol. Pharmaceutics, 2021) described nanocarriers for gene or mRNA delivery. These typically require intracranial or intrathecal injection due to limited BBB permeability, underlining the major translational bottleneck in GBM therapy that we aimed to tackle.

Overall, our proposed use of monocytes as cellular carriers offers multiple advantages: *i.* monocytes display a natural tropism towards tumor cells, and as such are actively recruited to GBM via chemotactic signals such as CCL2 and CX3CL1 (Qian et al., Nature, 2011), including toward early and small metastatic lesions; *ii.* monocytes shield the virus from neutralizing antibodies during circulation and are readily obtainable from peripheral blood with minimal manipulation; *iii.* future off-the-shelf carriers could be derived from hypoimmunogenic iPSC-derived monocytes (Trionfini et al., IJMS, 2023; Park et al., STAR Protoc., 2024), reducing the need for patient-specific processing.

We believe that OVs bring several advantages compared to traditional gene therapy vectors. Among those we cite: *i.* the self-amplification within tumor cells, increasing their own therapeutic payload; *ii.* the tumor-specific cytotoxicity, minimizing harm to healthy tissue; *iii.* their possible immunomodulatory effect pushing the tumor immune microenvironment towards an antitumor profile. This combination of features makes the monocyte-OVs pairing particularly powerful for brain tumors, as shown in our proof-of-concept platform.

We have incorporated this discussion and the recommended citations into the revised version of the manuscript.

1. Material characterization: The claims are heavily based on the assumption of successful ex vivo loading of oHSV-1 to THP-1 and primary human monocytes, but confirmation and characterization are missing (e.g., how much virus per cell?). Please confirm that both cell types are susceptible to oHSV-1 infection and whether they support viral replication.

We thank the Reviewer for highlighting this important point. We confirm that both THP-1 cells and primary human monocytes are susceptible to oHSV-1 infection, and that viral loading and replication dynamics have been previously characterized in detail in our earlier study (Reale et al., Int. J. Mol. Sci., 2023; REF #16 in the revised manuscript). Briefly, we demonstrated that THP-1 cells, due to their malignant origin, are fully permissive to oHSV-1 infection and support productive viral replication, with release of infectious particles and robust expression of viral proteins (e.g., ICP4, EGFP). Similarly, primary human monocytes, while susceptible to infection, are poorly permissive, displaying transient viral protein expression and limited viral release under basal conditions. However, when exposed to tumor-conditioned medium, they exhibit a significant boost in viral replication and infectivity, suggesting that the tumor microenvironment activates latent viral production.

Regarding loading quantification, in our previous study we estimated the infectious viral dose per cell, monitored viral replication kinetics via plaque assays, and quantified ICP4 expression using different techniques. These metrics determined the functional loading efficiency, which was in line with values reported in the literature for monocyte-based viral delivery.

Agreeing with the need of additional info, in this revised version we now expanded the Introduction to summarize key findings from Reale et al., 2023, including permissivity, viral titer, and tumor-triggered replication enhancement. These data are also referenced in the Conclusion to reinforce the rationale for selecting monocytes as delivery vehicles in our BBB-on-chip model. We trust these additions provide the necessary support for our experimental assumptions and clarify the robustness of our delivery system.

1. Data interpretation: In Fig. 3C, a small portion of THP-1 cells enters the tumour spheroid over 72 hours, but the overall number of THP-1 cell signals is much lower compared to the control group. Please explain why the targeted migration of THP-1 cells towards the tumour spheroids is less observable in THP-1/oHSV-1 group. Also, the authors claimed that infected monocytes initiate viral replication upon reaching GBM spheroids. In Fig. 3D, in the free oHSV-1 group, viral signals are observed throughout the spheroid, while in Fig. 3C, in THP-1/oHSV-1 group, the signals are located at the edges of the spheroid and appear much less. Similarly, it is difficult to see the difference in Fig. 5. Is viral replication capability of oHSV-1 hindered after delivery with monocytes?

We thank the Reviewer for raising these insightful comments, allowing us to better contextualize our findings regarding THP-1 cell dynamics and oHSV-1 delivery.

As correctly observed also by Reviewer #1, the THP-1/oHSV-1 group (**Figure 3.C**) shows less visible, green-labeled cells compared to the uninfected THP-1 control. This can be attributed to the fact that uninfected THP-1 cells, being of malignant origin, are metabolically active and proliferative, leading to a sustained and increasing signal over time. In contrast, THP-1 cells infected with oHSV-1 experience viral cytopathic effects that begin to compromise their viability, particularly once viral replication is reactivated upon reaching tumor spheroids. As a result, the infected THP-1 population declines, leading to reduced fluorescence signal. We have now clarified this interpretation in the Results section of the revised manuscript. In **Figure 3.D**, the different infection patterns in the experimental groups are a consequence of their distinct delivery mechanisms. Free oHSV-1 are directly introduced (perfused) into the circuit at a defined PFU and can immediately diffuse into the spheroid, leading to widespread infection throughout the tumor mass. In the THP-1/oHSV-1 condition, the same viral dose is first incubated with monocytes off-chip, then the carrier cells are perfused into the flow system and must: *i*. cross the BBB; *ii.* migrate toward the tumor spheroids; *iii.* reactivate viral replication upon reaching the spheroids (triggered by tumor-derived signals); *iv.* release progeny virions that initiate secondary infection of GBM cells.

This multi-step delivery process inherently introduces a temporal delay in infection onset compared to direct virus injection. Furthermore, initial viral spread is spatially constrained to the periphery of the spheroid where THP-1 cells first make contact, explaining why red fluorescence is primarily observed at the edges. The revised Discussion section has now been modified to include these details and ensure a better understanding of the kinetics and spatial distribution of infection in the two delivery models.

Our know-how also leads us to believe that the virus does not loose replicative capacity when delivered by monocytes. Rather, the kinetics and localization of replication are influenced by the re-initiation of viral replication in monocytes, the reduced number of viable carrier cells over time due to viral cytopathic effects, and to the sequential viral transfer from monocytes to GBM cell. Nevertheless, the THP-1/oHSV-1 system effectively delivers the virus across the BBB and initiates oncolytic infection at the tumor site. As also shown in our data, infection is maintained even in the presence of neutralizing antibodies, demonstrating a key functional advantage of cell-based delivery. This supports our claim that monocytes are promising carriers for stealth viral delivery in CNS malignancies.

1. BBB model. Did the authors actually model BBB or BBTB? While some previous studies would claim the use of HUVEC cell for BBB modeling, HUVEC is more acceptable for BBTB modeling. If the authors want to model human BBB in the microfluidic device, why would they choose HUVEC as the cell line? As this study is intended for glioblastoma treatment, it is better for the authors to change the wording from BBB to BBTB.

We thank the Reviewer for raising this insightful and important observation. We fully acknowledge the limitations associated with using HUVECs for modeling the human BBB, as they do not inherently replicate all the defining features of brain microvascular endothelial cells (BMECs) such as high TEER values, robust expression of tight junction proteins (e.g., claudin-5, occludin), and selective transport mechanisms. Here, the primary aim was to develop a versatile and modular microfluidic platform to study the cell-mediated transport of OVs across an endothelial barrier and into a GBM tumor microenvironment. Our platform was designed as a proof-of-concept system, with an emphasis on functionality, reproducibility, and adaptability. For this reason, we decided to use HUVECs, a cell model we have extensive know-how on, and that offers consistent growth behavior, ease of handling, and reliable barrier formation in microfluidic formats. Multiple recent studies successfully used HUVECs to model aspects of the BBB or tumor-associated endothelial barriers *in vitro*, particularly where the focus is on relative permeability, immune-endothelial interactions, or drug delivery. Examples include Singh et al., Cells (2022) presenting a BBB-on-chip for neurovascular modeling; Bolden et al., Sci. Rep. (2023), an endothelial barrier model of traumatic brain injury; Vakilian et al., Biotechnol. J. (2021) with drug transport in tumor-associated vasculature; and Margari et al., Cells (2025) building stromal-endothelial interactions in microfluidic settings. For our studies, we decided to first form a “healthy” barrier, prior to the introduction of tumor spheroids, recapitulating an intact physiological barrier before simulating the early stages of glioma progression and transmigration of carrier cells. For these reasons and given the platform’s flexibility to potentially also model tumor-altered vasculature (BBTB), we used the term BBB-on-chip to emphasize the preserved barrier integrity and immune-transmigration features central to our investigation.

Once again, we truly appreciate the Reviewer’s comments given it both allowed us to expand the discussion in the revised manuscript to explicitly address the distinction between BBB and BBTB models, and it suggested interesting future direction for our research. Provided our preference would be to maintain the “BBB” designation for consistency and to reflect the barrier’s physiological properties prior to tumor remodeling, should the Reviewer strongly prefer it, we are open to adopting more specific terminology (e.g., BBTB-on-chip).