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In vitro microglia models: the era of engineered cell microenvironments

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Background: The most widely employed approach by cell biologists to performing *in vitro* cell culture assays is the one using 2D plastic culture ware systems, which allows reproducibility and ease of use. Moreover, this method is cost-effective. However, in most cases, these flat surfaces lead to the formation of unrealistic 2D cell monolayers, which do not reproduce the complex configuration characteristics of native tissues in terms of dimensionality, rigidity, and topography. For this reason, a new generation of interdisciplinary scientists, working across microengineering and cell biology has started to develop engineered cell microenvironments (Huang et al., 2017) by employing advanced materials and fabrication approaches (Fan et al., 2019) over the last two decades. Depending on the level of resolution of the adopted manufacturing technique, the geometrical features of these structures can reach micrometric or even sub-micrometric dimensions comparable to the ones of cellular somas or cellular filopodia, therefore fostering cell-biomaterial interactions. The developed structures are pivotal for a better investigation of fundamental mechanobiology (Lemma et al., 2019), the optimization of *in vitro* disease modeling, drug/treatment screening (Gao et al., 2021), and tissue engineering (Mani et al., 2022).

Among all tissues in our body, brain tissue is one of the most complex. Several efforts have been made (Accardo et al., 2019) to create physiologically relevant and biomimetic artificial niches that are able to efficiently host different neuronal and glial cell lineages. Among these, microglia are the resident macrophages of the central nervous system and contribute to maintaining the brain's homeostasis. In addition, they have a vital role in tissue repair. Activation and dysfunction of microglial cells are associated with neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. In the human brain, four main phenotypes of microglia are reported, namely ramified, primed, reactive and amoeboid. The ramified or "resting" phenotype is the most abundant in the healthy brain and features a cellular morphology with multiple extensively ramified processes that probe chemical or physical cues that indicate the presence of a foreign body or cellular debris. Microglia that display an amoeboid or "activated" phenotype secrete pro-inflammatory mediators and neurotoxins, initiating a pro-inflammatory signal cascade that results in phagocytosis, among other things. This amoeboid phenotype is characterized by a flat cellular morphology, a wide cell body, and few (i.e., less than three) or no branched primary processes. One of the main issues when primary microglia are isolated from the brain and are seeded in 2D plastic culture ware systems is that they display either round, globular-like, or flat, amoeboid-like, morphologies (**Figure 1A**; Jeong et al., 2013). Although progress has been made in the optimization of microglia *in vitro* culture conditions, e.g. the elimination of serum from the culture medium, *in vitro* microglia are still characterized by an activated phenotype (Bohlen et al., 2017; Timmerman et al., 2022). This limits the potential of performing fundamental neuromechanobiology investigations, and of developing *in vitro* disease models or *in vitro* drug-screening studies that involve microglia. In order to overcome this limitation, research has been emerging over the last decade showing that micro- and nano-engineering approaches represent a powerful tool to create controlled and reproducible microenvironments able to guide the fate of microglia and to optimize current *in vitro* microglia models.

Engineered microglia environments: The guidance of microglia morphology and phenotype via engineered microenvironments can take place by exploiting four different parameters: substrate geometry, topography (e.g., roughness), stiffness, and biochemical coating (**Figure 1B**). A decade ago, excimer laser processing of thin hydrogenated amorphous silicon films deposited by plasma-enhanced chemical vapor deposition on glass substrates already enabled the formation of silicon micro and nano-structures (between 2 nm and 2.3 µm) resulting in substrates with various roughness which influenced the morphology of a murine microglial cell line (BV-2) (Persheyev et al., 2011). On amorphous flat silicon, BV-2 cells tended to adopt a rounded morphology with flat protrusions whereas in the presence of laser-processed silicon micro- and nano-structures, these cells displayed a more elongated, bipolar shape. The manufactured features are sensed by microglia via lamellipodia and filopodia in a process known as mechanosensing. More recently, another widely employed technique for the fabrication of cell scaffolds called electrospinning (**Figure 1C-i**), able to generate polymeric nanofibers using electric fields, was employed to assess the response of primary microglia (which have a higher pre-clinical relevance compared to the BV-2 cell line), isolated from Wistar rat pups (Pires et al., 2015), in presence of poly(trimethylene carbonate-co-ε-caprolactone) (P(TMC-CL)) fibers. A comparison between flat P(TMC-CL) films and P(TMC-CL) fibers showed that cells cultured on P(TMC-CL) films were larger and featured an increased circularity compared to microglia cultured on fibers. This can be considered as an indication of a more pro-inflammatory profile. However, when media from the primary microglia culture was introduced to astrocytes, astrogliosis was not exacerbated, signifying that microglia were not activated. Thus, it is important to mention that the association between microglia activation states and the display of distinct morphologies are not always linked. In another recent work, the addition of conditioned medium of BV-2 microglia grown within 3D graphene foams obtained by chemical vapor deposition (**Figure 1C-ii**) promoted neurosphere formation, which was not observed when neurons were exposed to conditioned medium from 2D cultured microglia, suggesting possible applications in the field of regenerative medicine (Jiang et al., 2016).

All the above-mentioned studies employed microglia cell lines or primary microglia coming from rodent models. However, in order to have more clinically relevant models for prospective *in vitro* disease modeling, drug-screening assays, or even neural tissue engineering purposes, it is of paramount importance to evaluate the behavior of microglia that are closer to human microglia. A recent advancement, making use of primary microglia isolated from adult rhesus macaques, reported for the first time how micro- and nano-metric 2.5D and 3D structures, fabricated via two-photon polymerization, directly influenced primary microglia morphology and branching (**Figure 1C-iii** and **D**; Sharaf et al., 2022). Two-photon polymerization is a manufacturing

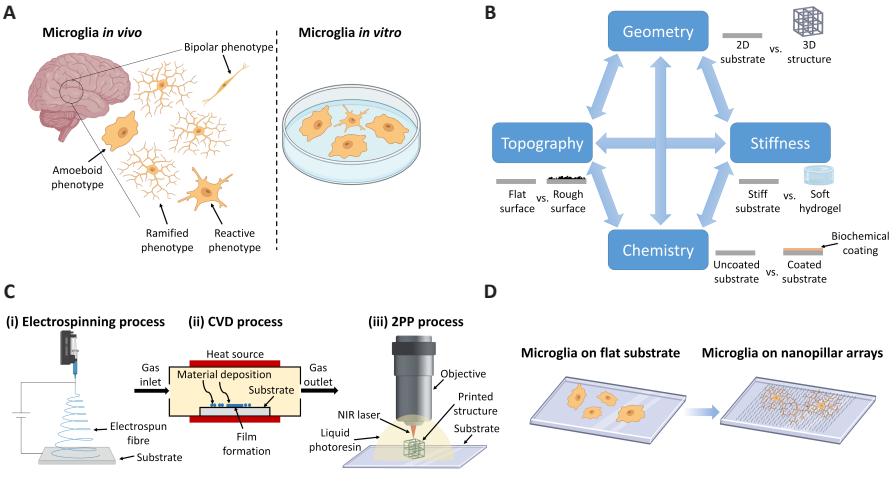

Figure 1 | Schematic representation of the various features of *in vitro* microenvironments, the processes used to fabricate them, and their effect on microglia.
(A) Microglia phenotypes observed *in vivo* and *in vitro*. (B) Overview of the parameters of *in vitro* microglia engineered microenvironments and their interplay therein. (C) Examples of processes employed to fabricate *in vitro* engineered microglia microenvironments (i) electrospinning, (ii) chemical vapor deposition (CVD), and (iii) two-photon polymerization (2PP). (D) An example of the effect of engineered microenvironments where nanopillar arrays, providing a topography and stiffness that resemble those of the native cell environment, fostered the expression of a ramified phenotype in primate-derived microglia compared to the amoeboid one on flat surfaces. Created with BioRender.com.

technique that exploits femtosecond near-infrared laser pulses to polymerize (i.e. solidify) photosensitive liquid materials within extremely confined regions, thus enabling sub-micrometric feature resolution. This technique has also been recently employed to realize glioblastoma-engineered microenvironments for both fundamental mechanobiology (Barin et al., 2022) and *in vitro* proton therapy treatment modeling (Akolawala et al., 2022). The aim of the developed structures was to approach the dimensionality, topography and stiffness of the extracellular matrix in the central nervous system (where the brain features protein fibers of tens to hundreds of nm diameter in a 3D spatial configuration and a Young's modulus E of 0.1–1 kPa, respectively), thus providing a biomimetic microenvironment. Although the intrinsic elastic modulus of the employed biomaterial, named IP-Dip, is in the GPa range, the presence of nanopillar arrays with a large aspect ratio resulted in a low effective shear modulus (i.e. the shear modulus experienced by a cell as it moves over the array) ranging from 250 kPa to 14.6 MPa. This is an added value as IP-Dip enables the creation of robust nanometric structures (therefore with dimensions in the range of filopodia processes) and does not suffer from the mechanical instability typical of other soft hydrogels. Compared to the cell culture on 2D glass substrates (E ≈ 70 GPa), where after 15 days primary microglia abundantly displayed an amoeboid/non-ramified phenotype, the developed nanostructures fostered a more complex ramified microglia morphology (which was characterized by scanning electron microscopy and immunofluorescence), an increase in the number of primary branches per single cell, and a cell-guidance effect. This demonstrates how the interplay of topography and effective shear modulus of the microfabricated architectures plays a fundamental role in the context of microglia-material interaction both at the micro- and the nano-scale.

Future perspectives: Engineered microglia cell culture models can help to overcome the limitations of conventional 2D plastic/glass culture ware approaches. These models have a pivotal role in better understanding fundamental neuromechanobiology processes but also for the development of more physiologically relevant *in vitro* drug screening platforms, which can be employed to test therapeutic targets when dealing with neurodegenerative diseases. By having culture models which better resemble the morphology and phenotype of cells in living tissues, it is theoretically possible to lower the amount of *in vivo* animal experiments and the time required to discover new drugs as well as related costs. The developed engineered microglia models are still very minimalistic, however, as they are usually composed of only one cell type while the brain niche features a highly heterogeneous population of cells (including neurons, brain endothelial cells, and glial cells). It will therefore be of paramount importance to assess the behavior of microglia cultured in the presence of other central nervous system cell types within such engineered microenvironments, especially for the development of *in vitro* disease models such as Alzheimer's disease and Parkinson's disease models. A second important point is to observe the behavior of microglia within even more complex tissue-like structures such as neural spheroids and organoids but in a "biomechanically controlled" configuration. Indeed, one of the main limitations of these widely employed 3D cell assemblies is that they rapidly develop a necrotic core which is detrimental for long-term studies. In the near future, we aim to merge scaffold-free and scaffold-based strategies in order to control the growth of pre-formed mini-neural-organoids seeded within meso-scale (mm or cm-sized) engineered porous microenvironments and facilitate the diffusion of nutrients to prevent early formation of necrotic cores. Another aspect, which needs to be further developed, is the compatibility between the current scaffolds technology and some relevant characterization techniques employed in cell biology, and particularly microglia, such as gene expression analysis, to confirm the homeostatic phenotype of these cells. To reach this goal, one solution is to integrate bio-inert coatings on the flat substrate (onto which the engineered structures are manufactured) enabling the cells to adhere only to the developed architectures and allowing therefore reliable functional and gene expression analyses (which would otherwise be polluted by inputs coming from the cells adhering to the flat substrate). The technical challenge, in this case, is to ensure that the bio-inert coating does not hinder the adhesion of the engineered structures to the substrate as this would lead to delamination issues. As soon as such needs can be addressed, it is foreseeable that these engineered microenvironments will become an indispensable actor in the neuroscientific landscape and that unexplored neuromechanobiological mechanisms will be unveiled, which will be beneficial for both diagnostic and therapeutic applications.

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