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## Engineering blood–brain barrier microphysiological systems to model Alzheimer’s disease monocyte penetration and infiltration†

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Alzheimer’s disease (AD) is a progressive and neurodegenerative disease, predominantly causing dementia. Despite increasing clinical evidence suggesting the involvement of peripheral immune cells such as monocytes in AD pathology, the dynamic penetration and infiltration of monocytes crossing blood–brain barrier (BBB) and inducing neuroinflammation is largely understudied in an AD brain. Herein, we engineer BBB-like microphysiological system (BBB-MPS) models for recapitulating the dynamic penetration and infiltration of monocytes in an AD patient’s brain. Each BBB-MPS model can be engineered by integrating a functional BBB-like structure on a human cortical organoid using a 3D-printed device within a well of a plate. By coculturing these BBB-MPS models with monocytes from AD patients and age-matched healthy donors, we found that AD monocytes exhibit significantly greater BBB penetration and brain infiltration compared to age-matched control monocytes. Moreover, we also tested the interventions including Minocycline and Bindarit, and found they can effectively inhibit AD monocyte infiltration, subsequently reducing neuroinflammation and neuronal apoptosis. We believe these scalable and user-friendly BBB-MPS models may hold promising potential in modeling and advancing therapeutics for neurodegenerative and neuroinflammatory diseases.

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### Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder that primarily impairs memory, thinking, social skills, and behavior.<sup>1,2</sup> It is the most common cause of dementia among older adults and is quickly becoming one of the most expensive, lethal, and burdening diseases of this century.<sup>3,4</sup> Although current therapeutic strategies for AD effectively aim to improve symptoms or slow disease progression, a definitive cure has yet to be discovered. One promising perspective is to innovate therapeutics targeting the neuroinflam-

mation in AD. The immune system plays a vital role in the development and progression of AD.<sup>5,6</sup> Research on brain-resident immune cells in the progression of AD is extensive, yet studies on the peripheral immune system remain limited.<sup>7–10</sup> As one of the most important peripheral innate immune cells in the blood, monocytes can be recruited into the CNS due to the increasing permeability of blood–brain barrier (BBB) in AD and participate in AD development.<sup>11–14</sup> Specifically, the CC-chemokine ligand 2 (CCL2) generated by activated microglia and astrocytes binds to the C–C motif chemokine receptor (CCR2) on monocytes and mediates the recruitment of these monocytes. Once recruited, monocytes are directed to the BBB, where they undergo diapedesis, a process that enables them to penetrate through the BBB and infiltrate into the brain parenchyma.<sup>15–19</sup> The activation of infiltrated monocytes also exacerbates neuroinflammation, leading to further neuronal damage and contributing to the progression of AD.<sup>20–22</sup> However, to date, the dynamics of monocytes penetrating the BBB and infiltrating the brain parenchyma remain poorly understood in AD, partly due to the absence of suitable human models with functional BBB structures.

Current knowledge of monocytes’ participation in AD progression primarily derives from animal models, particularly transgenic mouse models.<sup>23–25</sup> However, transgenic mouse

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models cannot adequately mimic the complex pathological features of AD.<sup>26</sup> On the one hand, transgenic mouse models mainly simulate symptoms of familial AD, which accounts for only 2–5% of AD cases. On the other hand, there are significant neurophysiological differences between human and mouse brains across multiple bio-hierarchical levels, such as the morphogenesis and cytoarchitecture of the cortex, BBB tight junction and efflux expression and efflux transporter<sup>27,28</sup> as well as the neuron polarization of neuroepithelium. Superior to animal models, emerging human brain organoid (hBO) provides a more human-relevant model to investigate neurological diseases.<sup>29,30</sup> Human brain organoids derived from human pluripotent stem cells (hPSCs) can recapitulate some critical physiological features of the human brain, including cellular diversity, cytoarchitecture, and neural activities.<sup>31–34</sup> More importantly, hBOs can be derived from patient-specific induced PSCs, thereby accurately recapitulating the individual's unique genetic background and associated mutations.<sup>35,36</sup> However, brain organoids encounter several significant challenges, including the absence of BBB structures and immune cells, as well as the limitation of oxygen and nutrient perfusion.

So far, efforts have been made to advance hBOs for neurological studies and disease modeling. By lowering the concentrations of the neuroectodermal stimulant heparin and delaying Matrigel embedding in the cerebral organoids, microglia naturally developed within these organoids, exhibiting characteristic ramified morphology and the capacity to mediate inflammatory responses.<sup>29</sup> In addition, we have previously presented a tubular organoid-on-a-chip device by employing a three-dimensional (3D) printed hollow mesh scaffold to improve oxygen and nutrient perfusion and further reduce the hypoxia and necrosis of organoids.<sup>30</sup> Microglia incorporated into the device were then exposed to an opioid receptor agonist to stimulate microglia-mediated neuroinflammation. Despite progress in investigating neuroimmune interactions with existing models, a major limitation persists in the absence of a model that can monitor the dynamic process by which peripheral innate immune cells, such as monocytes, cross the BBB and infiltrate brain tissue. The lack of capability to observe these dynamics hinders a deeper understanding of how monocytes contribute to neuroinflammation and disease progression.

Herein, we engineered novel BBB-MPS models that incorporate human cortical organoids with an integrated BBB for recapitulating the dynamic penetration and infiltration of monocytes in an AD brain. Our BBB-MPS models have several advantages: (1) The functional BBB-like structures are easily created by culturing endothelial cells and human brain organoids containing astrocytes on 3D-printed devices. (2) Our previously reported 3D-printed devices incorporate *in situ* air-liquid interface culture, promoting efficient nutrient and oxygen exchange. (3) Our devices are designed to be compatible with commercially available 24-well microtiter plates, allowing for the simultaneous investigation of multiple experimental conditions which include drug screening to inhibit the

penetration of AD patient-derived monocytes and subsequently mitigate neuroinflammation. Applying novel BBB-MPS models, we monitor the dynamic infiltration of monocytes cross through BBB and demonstrate that reducing the penetration of AD monocytes through the BBB can effectively decrease neuroinflammation and neuronal apoptosis. Consequently, the BBB-MPS models hold significant potential for investigating the role of peripheral innate immune cells in neurodegenerative diseases.

## Materials and methods

### Design and fabrication of 3D-printed holder devices

Following the protocol that we developed previously,<sup>37–39</sup> the holder devices were designed using AutoCAD software (Autodesk) and fabricated using a stereolithography 3D printer (Form 3B, Formlabs) and FormLabs Clear Resin V4. Design details of the holder device are shown in Fig. S1.† The polyester porous membranes (Sterlitech) were cut by employing a CO<sub>2</sub> laser engraver and bonded to the holder devices using double-sided adhesive (3M).

### Human stem cell culture

The human embryonic stem cell (hESC) line (WA09) was purchased from WiCell institute. The cells were handled under the guidelines of both WiCell Institute and Indiana University Biosafety Committee. WA09 cells were cultured with mTESR plus medium (Stemcell Technologies) on Growth Factor Reduced Matrigel (Corning) coated 6-well plates in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. The medium was changed every other day. WA09 cells were passed every 5 days using ReLeSR (Stemcell Technologies).

### Generation of human cortical organoids

Human cortical organoids were fabricated from the hESC line (WA09). The cortical organoids were cultured according to a previously reported protocol.<sup>40,41</sup> The detailed composition of cortical organoid differentiation medium is described in Table S1.† Specifically, embryonic bodies (EBs) were generated using a 96-well U bottom microplate (Sarstedt) by aggregation of ~9000 hESCs per well and then cultured in 100 µL cortical organoid medium 1 (COM I). After EB formation (Day 1), the EBs were switched to COM I without Y-27632. After 3 days of culture, the cultures were transferred to cortical organoid medium 2 (COM II) for the next 7 days. On day 10, the cultures were maintained in cortical organoid medium 3 (COM III) for 7 days, followed by 7 additional days in cortical organoid medium 4 (COM IV). Next, the cultures were transferred to cortical organoid medium 5 (COM V) to facilitate maturation, glycolysis, and enhance cellular activity. After 22 days, the cortical organoids were maintained in cortical organoid medium 6 (COM VI). Medium change was performed every other day during this process except when specified. From day 30 on, the organoid cultures were transferred to a 6-well ultralow adhesion plate (Corning) and kept on an orbital shaker at a

speed of 90 rpm. The immunofluorescence characterization of the cortical organoids is shown in Fig. S2.†

### Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell growth medium (Lonza) in 6-well microtiter plates. After confluence, HUVECs were digested with 0.25% trypsin ethylenediaminetetraacetic acid (EDTA) (Gibco) and used for the following experiments. To minimize experimental variability, only the primary cells in passages 6–8 were used for experiments.

### Permeability assay

The permeability of the BBB-MPS was tested by using 70 kDa dextran (Invitrogen) using our well-developed protocol.<sup>42</sup> Briefly, the BBB-MPS was cultured in 1 mL culture medium. The 50  $\mu$ L of 500  $\mu$ g mL<sup>-1</sup> dextran were prepared in the culture medium and replaced the medium in the holder devices from the BBB side. The holder devices were placed in an incubator for 30 min. Then, the medium in the bottom well was collected, and the fluorescence was quantified at 555/580 nm (excitation/emission, gain: 65) using a microplate reader. A standard curve was made of 500  $\mu$ g mL<sup>-1</sup> dextran, and the apparent permeability coefficient ( $P_{app}$ ) was calculated according to the equation below:

$$P_{app} (\text{cm s}^{-1}) = \frac{V_t \times C_t}{A \times C_0 \times t}$$

where  $V_t$  is the measured volume of the bottom well,  $C_t$  is the measured dextran concentration at time  $t$ ,  $A$  is the effective permeability area of the membrane, and  $C_0$  is the original dextran concentration.

### Isolation of monocytes from human PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples obtained from different healthy donors and AD patients. Monocytes were isolated from PBMCs using the Classical Monocyte Isolation Kit according to the manufacturer's protocol (Miltenyi Biotec GmbH) with MS Column (Miltenyi Biotec GmbH). Typically,  $5 \times 10^4$  monocytes per milliliter of whole blood were obtained. All experiments were performed in accordance with the guidelines of Indiana University biological sample procurement and biobanking protocol, and experiments were approved by the ethics committee at Indiana university. Informed consents were obtained from human participants of this study.

### Imaging monocyte infiltration

Monocyte infiltration was characterized by adapting our previously developed protocols.<sup>43–46</sup> The monocytes were labeled with Vybrant DiL (red) (Invitrogen) at 1:1000 dilutions at 37 °C for 30 min before being introduced into BBB-MPS models. Simultaneously, the BBB-MPS models were labeled with Vybrant DiO (green) (Invitrogen) at 1:1000 dilutions at 37 °C for 30 min. A total of  $1 \times 10^5$  labeled monocytes were added to each BBB-MPS. After co-culturing in a 37 °C incuba-

tor for 24 h, the BBB-MPS models were imaged using an Olympus OSR spinning disk confocal microscopy with a 10 $\times$  objective inside a Tokai Hit on-stage incubator set at 37 °C and 5% CO<sub>2</sub>. The acquired Z-stack images were analyzed using Imaris (version 9.0.1). A 3D reconstruction of the BBB-MPS structure was generated based on the DiO signal to define the boundary of the organoid. Monocytes (DiI signal) were detected and segmented using the software's spot detection function. Only monocytes located within the reconstructed 3D boundary were considered infiltrated. The infiltrated rate was defined as the percentage of infiltrated monocytes out of the total number of monocytes added to the system.

### Drug treatment

Minocycline (STEMCELL Technologies) 20 to or 100  $\mu$ M Bindarit (Cayman Chemical) were used for BBB-MPS model treatment with the addition of  $1 \times 10^5$  labeled monocytes. The co-culture was then incubated at 37 °C for 24 h before confocal imaging.

### Cryo-sectioning of BBB-MPS

Cryo-sectioning of the BBB-MPS was conducted using our well-developed protocol.<sup>47</sup> Samples were initially rinsed twice with 1 $\times$  PBS and fixed in 4% paraformaldehyde (Thermo Fisher) in 1 $\times$  PBS at 4 °C overnight. Post-fixation, samples were cryoprotected by immersion in 30% sucrose (Sigma) in 1 $\times$  PBS (w/v) at 4 °C overnight. The cryoprotected samples were then embedded in the O.C.T. compound (Fisher Scientific) using cryomolds (Sakura Finetek USA) and frozen on dry ice. After that, the frozen samples were sectioned to a thickness of 30  $\mu$ m using a cryostat (Eprelia) and collected on Superfrost™ Plus microscope slides (VWR).

### Immunofluorescence staining

Immunofluorescence staining was conducted directly on cryo-sectioned samples collected on slides. The sections were washed twice with 1 $\times$  PBS to remove residual O.C.T. compound. Antigen retrieval was performed by treating the sections with sodium citrate buffer (Sigma) for 20 minutes. After rinsing twice with 1 $\times$  PBS, the sections were permeabilized and blocked using 0.3% Triton X-100/5% normal donkey serum (Jackson ImmunoResearch) in 1 $\times$  PBS for 1 hour. Primary antibodies were then applied in a diluted antibody solution and incubated overnight at 4 °C, humidified chamber. The slides were subsequently rinsed three times with 1 $\times$  PBS and incubated with secondary antibodies at room temperature for 2 hours. Finally, the sections were washed three times with 1 $\times$  PBS, and coverslips were mounted using ProLong Gold Antifade Mountant with DAPI (Invitrogen). Detailed antibody information and dilution factors are shown in Table S2.†

### Quantification of astrocyte activation and neuronal apoptosis

Astrocyte activation quantification was conducted based on immunofluorescence slides stained with an anti-GFAP antibody. Neuronal apoptosis was quantified with immunofluores-

cence slides stained with anti-NeuN and anti-cleaved caspase-3 antibodies. One staining slide per cortical organoid was selected for quantification. The visualization was conducted using a laser scanning confocal microscopy (Stellaris 8, Leica). The quantification of fluorescence intensity and cell ratio was performed with ImageJ (v 1.54f). The value obtained from one slide per organoid was presented as one data point in the results.

### Statistical analysis

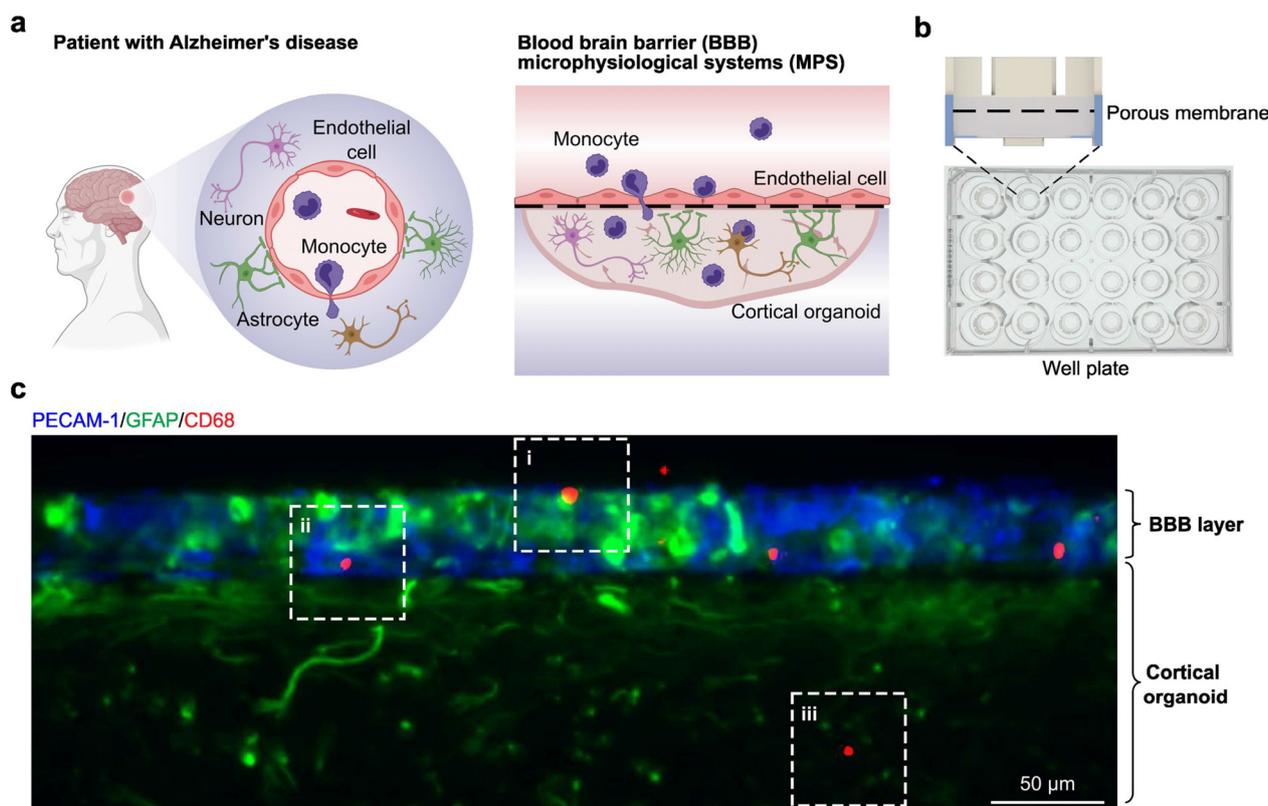
The statistical comparison of each group was performed *via t*-test with GraphPad Prism 8 (GraphPad Software). All data are presented as mean  $\pm$  SEM values. The statistical significance of differences in values is denoted as following \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.005$ .

## Results

### Engineering BBB-MPS models to study dynamic penetration and infiltration of AD monocytes

In AD, monocytes migrate from the bloodstream into the brain by penetrating the BBB, resulting in infiltration in the brain parenchyma.<sup>48,49</sup> Within the AD brain, these infiltrating monocytes become activated in response to A $\beta$  plaques and other

pathological stimuli, releasing pro-inflammatory cytokines such as necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), and interleukin-6 (IL-6). This inflammatory cascade exacerbates neuronal damage, amplifies neuroinflammation, and contributes to the neurodegenerative processes that are hallmark features of AD pathology (Fig. 1a).<sup>10,20,48</sup> We engineered BBB-MPS models to precisely observe the penetration and infiltration process of the monocytes from BBB to the cortical brain (Fig. 1b and Fig. S3 $\dagger$ ). In the microphysiological system, the human umbilical vein endothelial cell (HUVEC) monolayer (upside) and cortical organoids (downside) were located on both sides of the polyester porous membrane of the 3D-printed holder device. Based on the previous instruction, cortical organoids were cultured on the air-liquid interface to improve oxygen and nutrient exchanges and to mediate the formation of organoids with flattened shapes.<sup>50-53</sup> It is well mentioned that the BBB-MPS models are compatible with the current commercial microtiter plates and thus are applied for high-throughput and user-friendly drug screening aiming at mitigating neuroinflammation induced by AD patient-derived monocytes. Furthermore, the robustness and simplicity of the model allow users to easily “switch-out” cell types to incorporate various BBB-like cell types and disease-specific organoids. Immunofluorescent staining showed that after co-culture with BBB-MPS models for 48 h,



**Fig. 1** Engineering BBB-MPS models for recapitulating dynamic penetration and infiltration of AD monocytes. (a) Schematics showing the penetration and infiltration of monocytes and the subsequent neuroimmune interactions in AD patients (left) and the BBB-MPS model for recapitulating the same process. (b) A 24-well plate with 24 BBB-MPS models. (c) Immunofluorescent image of the BBB-MPS models after 48 h treatment with AD patient-derived monocytes.

CD68<sup>+</sup> monocytes derived from AD patients migrated through the endothelial monolayer into healthy cortical organoids (Fig. 1c). Using the BBB-MPS model, we can dynamically monitor and assess monocyte penetration across the BBB and subsequent infiltration into the brain parenchyma.

### Characterization of BBB-MPS models

The brain is regarded as an immune-privileged organ due to the protective role of the BBB. In BBB, endothelial cells are tightly aligned to form a continuous monolayer, while astrocytes enwrap the outer surface of these endothelial cells with their end-feet, enhancing the selective permeability and structural integrity of the barrier.<sup>45–49</sup> In AD, the BBB becomes increasingly susceptible to immune cell infiltration. This vulnerability arises from the upregulation of adhesion molecules, the breakdown of BBB tight junctions, and other processes exacerbated by both aging and disease pathology.<sup>50–53</sup> In the BBB-MPS models, HUVECs (upside) formed an integrated monolayer that positively expressed platelet endothelial cell adhesion molecule-1 (PECAM-1) as an endothelial cell marker, along with well-developed tight junction protein zonula occludens-1 (ZO-1) and adherent junction protein vascular endothelial cadherin (VE-cadherin) (Fig. 2a). On the cortical organoid side, astrocytes and mature neurons were identified by staining for glial fibrillary acidic protein (GFAP) and microtubule-associated protein 2 (MAP2), respectively (Fig. 2b and Fig. S4†). The astrocytes in the BBB-MPS models extended their end-feet to wrap around the endothelium (Fig. 2c and d). The paracellular permeability assay, a standard assessment of epithelial and endothelial barrier integrity, demonstrated that

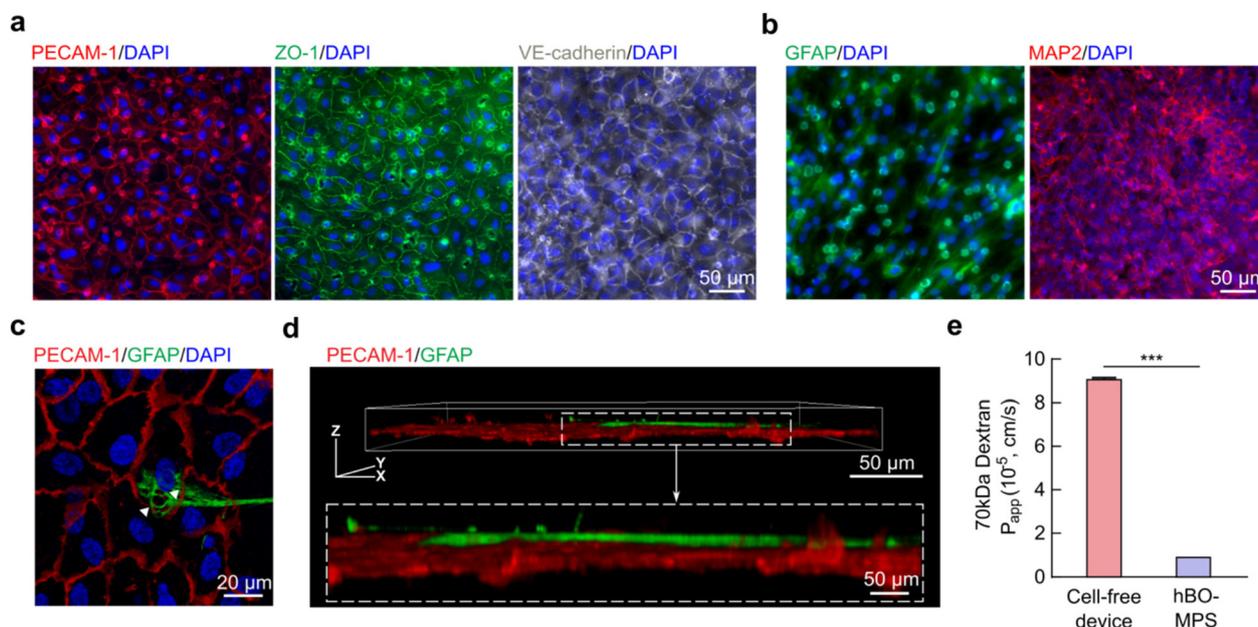
the permeability of the tracer marker, 70 kDa dextran, was lower in the BBB-MPS models, indicating that this microphysiological system possesses an integrated BBB (Fig. 2e). Taken together, the BBB-MPS effectively recapitulates the essential features of the BBB.

### Investigation of monocyte infiltration using BBB-MPS models

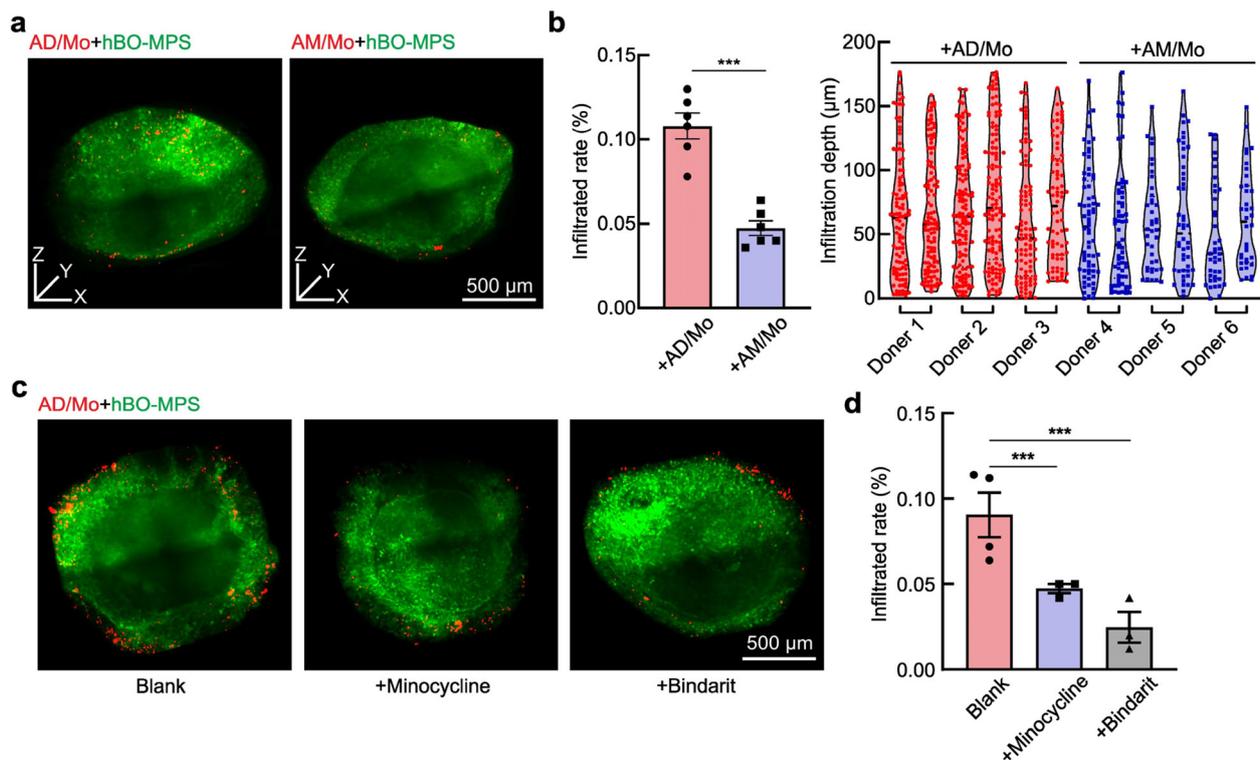
As mentioned previously, monocytes exhibit an enhanced capacity to penetrate the BBB and infiltrate the brain parenchyma. To investigate the differential infiltration capacity of monocytes derived from AD patients compared to age-matched control individuals, we introduced primary monocytes isolated from PBMCs into BBB-MPS models. We collected the AD monocytes (AD/Mo) from the PBMCs of patients aged over 70 years and obtained age-matched control monocytes (AM/Mo) from donors of the same age group. After adding AD/Mo or AM/Mo to BBB-MPS models, they gradually penetrated the BBB and infiltrated the cortical organoids (Fig. S5†). To quantify the infiltration of monocytes into cortical organoids, we analyzed the recorded images (Fig. 3a). We found that the infiltrated rate of AD/Mo was approximately twice that of age-matched AM/Mo (left in Fig. 3b). Furthermore, AD/Mo exhibited a greater infiltration depth (right in Fig. 3b), indicating that they possess a higher infiltration capacity than AM/Mo.

### Minocycline and Bindarit treatments reduce neuroinflammation and neuronal apoptosis

Next, we investigate the effects of two promising therapeutic agents, Minocycline and Bindarit, on inhibiting the infiltration



**Fig. 2** Characterization of engineered BBB-MPS models. (a) Immunofluorescent images of the BBB labeled with PECAM-1, ZO-1, and VE-cadherin. (b) Immunofluorescent images of the cortical organoids labeled with GFAP and MAP2. Top view (c) and side view (d) showing GFAP-labeled astrocytes extending end-feet to wrap around PECAM-1 labeled endothelium. (e) The permeability of dextran (70 kDa) was compared in cell-free devices and BBB-MPSs. Data are presented as mean  $\pm$  SEM.



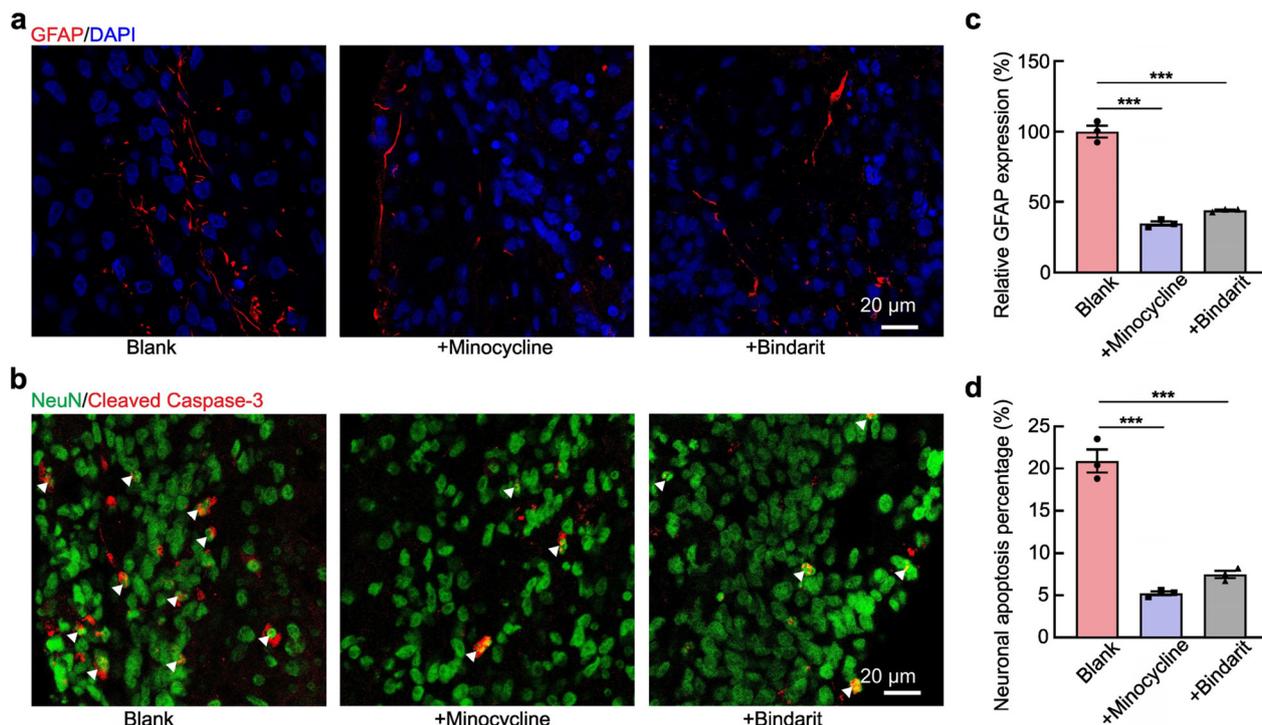
**Fig. 3** Characterization and intervention of AD monocyte infiltration of BBB-MPS models. (a) Comparison of infiltration capacity of monocytes isolated from AD patients (>70-year-old, as AD/Mo) with monocytes isolated from age-matched control individuals (as AM/Mo). Scale bar: 500  $\mu\text{m}$ . (b) Quantification of infiltrated rate (left) and infiltration depth (right) between AD/Mo and AM/Mo. (c) Comparison of infiltration capacity of AD monocytes after the Minocycline (Minocycline+) and Bindarit (Bindarit+) treatments. Scale bar: 500  $\mu\text{m}$ . (d) Quantification of infiltrated rate. Data are presented as mean  $\pm$  SEM.

of monocytes into cortical organoids. Both Minocycline and Bindarit have anti-inflammatory and neuroprotective properties.<sup>54–57</sup> Notably, both Minocycline (50  $\mu\text{M}$ ) and Bindarit (25  $\mu\text{M}$ ) significantly suppressed the infiltrated rate and depth of monocytes derived from AD patients, indicating that these agents reduce monocyte infiltration in AD (Fig. 3c, d and Fig. S6†). Additionally, it has been reported that infiltrating monocytes can amplify inflammatory responses in microglia and astrocytes within the CNS and further exacerbate neuronal damage.<sup>58–60</sup> We next explored the regulatory mechanism of Minocycline and Bindarit. GFAP staining was used to assess astrocyte activation, revealing that GFAP levels were significantly suppressed in both the Minocycline and Bindarit treatment groups compared to the AD/Mo group (Fig. 4a and b). We also evaluated the neuroprotective effects of Minocycline and Bindarit on neuronal apoptosis, potentially associated with neuroinflammation induced by AD monocytes. Neuron nucleus were labeled with NeuN, while apoptotic cells were labeled with cleaved caspase-3. Co-staining for NeuN and cleaved caspase-3 indicated neuronal apoptosis in the AD/Mo group. The percentage of cleaved caspase-3-positive neurons in brain organoids was significantly reduced in the Minocycline and Bindarit treatment groups, suggesting that these two drugs might reverse AD monocyte-induced neuronal apoptosis by inhibiting monocytes penetration through BBB (Fig. 4c and d).

## Discussion and conclusion

In this study, we engineered novel BBB-MPS models to investigate monocyte crossing BBB and the related neuroimmune interactions in AD. Our findings indicate that monocytes from AD patients have a significantly higher capacity to penetrate the BBB and infiltrate brain tissue than those from age-matched control individuals, underscoring their potential role in exacerbating neuroinflammation in AD. Furthermore, the therapeutic agents Minocycline and Bindarit markedly reduced monocyte infiltration, subsequently decreasing neuroinflammation and neuronal apoptosis. The BBB-MPS models thus provide a valuable platform for examining immune cell dynamics in neurodegenerative diseases and evaluating novel therapeutic approaches.

Dysregulation of the immune system is a key feature in AD, with immune cells undergoing dynamic changes throughout disease progression and fulfilling diverse roles. Monocytes, a type of white blood cell produced in bone marrow, are critical participants in AD pathology. Studies in amyloid precursor protein/presenilin 1 (APP/PS1) mouse models have shown that amyloid deposits in blood vessel walls interact with monocytes, which aid in clearing these deposits.<sup>61</sup> Once monocytes infiltrate the CNS *via* the CCL2/CCR2 axis,<sup>62–64</sup> they modulate neuroinflammation and protect neuron synapses from the



**Fig. 4** Investigation and intervention of AD monocyte-mediated neuroinflammation and neuronal apoptosis. (a) The expression of GFAP was identified by immunofluorescence staining and the quantifications. (b) The expression of NeuN and cleaved Caspase 3 was identified by immunofluorescence staining, and the quantifications for (c) relative GFAP fluorescence intensity statistics, and (d) neuronal apoptosis percentage. Scale bars: 20  $\mu$ m. Data are presented as mean  $\pm$  SEM.

neurotoxic effects of A $\beta$ 42 oligomers.<sup>65</sup> Additionally, monocytes can transport A $\beta$  to the bloodstream, reducing its accumulation in the brain.<sup>66</sup> However, these models predominantly capture monocyte activity through endpoint analyses, such as post-mortem tissue sampling, which only provides static representations of monocyte presence.<sup>67</sup> Consequently, the lack of dynamic observation limits our understanding of monocyte migration and interactions within the CNS, highlighting the need for human-based models capable of real-time observation. Our BBB-MPS models allow the real-time observation of monocyte penetration through the BBB and infiltration into brain organoids. The system consists of a 3D-printed holder device with a polyester porous membrane, compatible with standard 24-well microtiter plates and suitable for imaging techniques like fluorescence and confocal microscopy. HUVECs form a monolayer on one side of the membrane, mimicking the BBB, while cortical organoids are cultured on the opposite side to represent brain tissue. AD and age-matched monocytes are introduced on the BBB side, where they migrate towards the cortical organoids, enabling dynamic observation of this process. Our results demonstrate that AD-derived monocytes infiltrate brain tissue more effectively than those from healthy controls, emphasizing their role in AD pathology. This increased infiltration can be attributed to the factors, including the enhanced migratory capacity of AD monocytes, driven by elevated expression of receptors such as CCR1 and migration-related genes (*e.g.*,

SEMA6B, FGR, and FOXO3), which facilitate their penetrate through the BBB.<sup>68</sup>

Blocking the infiltration of peripheral immune cells into the CNS presents a promising strategy for treating AD and other neurodegenerative disorders.<sup>69</sup> In this study, we explored the effects of two therapeutic agents, Minocycline and Bindarit, on reducing monocyte migration into the CNS. Minocycline has been demonstrated to be a potential AD therapeutic due to its anti-inflammatory and neuroprotective properties, including its ability to suppress the production of pro-inflammatory chemokines such as CCL2.<sup>54,70</sup> Moreover, Minocycline can downregulate the expression of adhesion molecules on endothelial cells, which may help to limit monocyte infiltration into brain tissue. Bindarit, an anti-inflammatory small molecule, inhibits the synthesis of monocyte chemoattractant proteins (MCPs) by modulating the NF- $\kappa$ B pathway.<sup>55,56</sup> By targeting these chemokines, Bindarit reduces monocyte recruitment to sites of inflammation, thereby helping to regulate the inflammatory response. Our findings indicate that treatment with Minocycline and Bindarit significantly reduced both the number and depth of monocyte infiltration in cortical organoids. Furthermore, these agents alleviated AD-related neuropathology induced by monocyte infiltration, including astrocyte activation and neuronal apoptosis. These findings position the engineered BBB-MPS as a promising model for testing therapies aimed at regulating immune cell infiltration in AD and other neurodegenerative diseases.

While the current BBB-MPS model effectively simulates the BBB, it has some limitations. One limitation of current BBB-MPS models is that HUVECs cannot fully replicate the unique properties of brain endothelial cells such as specialized tight junctions and transport mechanisms. Moreover, the absence of pericytes limits the physiological relevance of the model, as pericytes are known to regulate BBB stability and immune cell trafficking.<sup>68</sup> Pericytes have been shown to secrete chemokines that facilitate monocyte recruitment and transmigration. Their interaction with infiltrating monocytes plays a key role in modulating neuroinflammation and BBB integrity under disease conditions.<sup>71,72</sup> Furthermore, the current model lacks fluidic stimulation, which is crucial for mimicking blood flow and shear stress *in vivo*. Fluid shear stress plays a significant role in endothelial cell function, including the regulation of tight junctions and monocyte adhesion. To address these limitations, further development of the model could incorporate brain endothelial cells, pericytes, and fluidic stimulation to better replicate the *in vivo* conditions of the BBB.<sup>73,74</sup> Additionally, our study focused on dynamically monitoring and evaluating the penetration of monocytes across the BBB and their subsequent infiltration into the brain parenchyma. While in AD, the BBB becomes compromised, leading to increased permeability and dysfunction. This disruption allows the entry of potentially harmful substances and immune cells into the brain. Conversely, the infiltrated immune cells further exacerbate BBB breakdown by releasing inflammatory mediators such as IL-1 $\beta$  and TNF- $\alpha$ , which impair endothelial integrity, exacerbate neuroinflammation, and contribute to neuronal damage.<sup>75,76</sup> Further study can investigate how monocytes interact with and contribute to BBB breakdown in the context of AD. To obtain more disease-relevant insights, it will be critical to utilize models that incorporate patient-specific pathological features of AD, as the current models lack disease specificity and may not fully capture the complexity of the AD-associated BBB. To conclude, our BBB-MPS devices are simple, user-friendly, and compatible with microtiter plates and lab settings. We believe that the BBB-MPS holds considerable promise for exploring the involvement of peripheral innate immune cells in neurodegenerative diseases.

## Author contributions

Longjun Gu: data curation, investigation, methodology, validation, visualization, writing – original draft, writing – review & editing. Xiangdi Mao: data curation, writing – review & editing. Chunhui Tian: methodology, writing – review & editing. Yang Yang: data curation, writing – review & editing. Kaiyuan Yang: visualization, writing – review & editing. Scott G. Canfield: data curation, writing – review & editing. Donghui Zhu: data curation, writing – review & editing. Mingxia Gu: data curation, writing – review & editing. Feng Guo: conceptualization, data curation, funding acquisition, project administration, resources, supervision, writing – review & editing.

## Data availability

The data supporting this article have been included as part of the ESI.†

## Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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