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# Meningeal lymphatic endothelial cells fulfill scavenger endothelial cell function and cooperate with microglia in waste removal from the brain

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## Abstract

Brain lymphatic endothelial cells (BLECs) constitute a group of loosely connected endothelial cells that reside within the meningeal layer of the zebrafish brain without forming a vascular tubular system. BLECs have been shown to readily endocytose extracellular cargo molecules from the brain parenchyma, however, their functional relevance in relation to microglia remains enigmatic. We here compare their functional uptake efficiency for several macromolecules and bacterial components with microglia in a qualitative and quantitative manner in 5-day-old zebrafish embryos. We find BLECs to be significantly more effective in the uptake of proteins, polysaccharides and virus particles as compared to microglia, while larger particles like bacteria are only ingested by microglia but not by BLECs, implying a clear distribution of tasks between the two cell types in the brain area. In addition, we compare BLECs to the recently discovered scavenger endothelial cells (SECs) of the cardinal vein and find them to accept an identical set of substrate molecules. Our data identifies BLECs as the first brain-associated SEC population in vertebrates, and demonstrates that BLECs cooperate with microglia to remove particle waste from the brain.

**KEYWORDS**
brain lymphatic endothelial cells, macromolecular uptake, meningeal lymphatic endothelial cells, microglia, scavenger endothelial cells, zebrafish

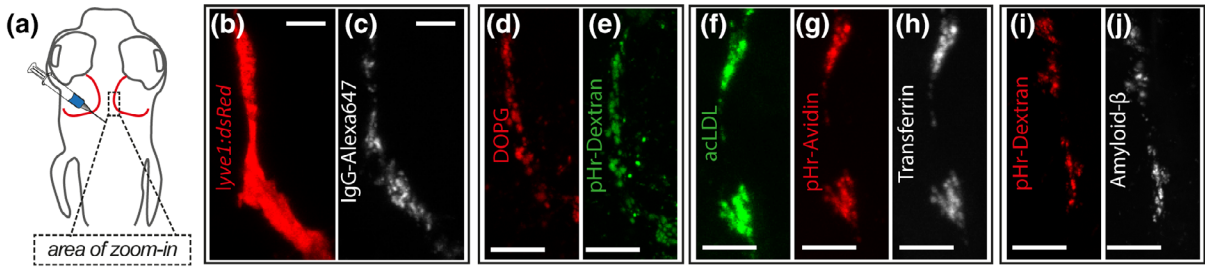
## 1 | INTRODUCTION

Microglia play a central role in the removal of waste products from the brain. Their high capacity to recognize, engulf and digest apoptotic neurons or to remove harmful degradation products, which are released from damaged and dying cells of the central nervous system (CNS), together with their function during synaptic pruning and neuronal circuit organization makes them a key cell type for the maintenance of brain homeostasis (Marquez-Ropero, Benito, Plaza-Zabala, & Sierra, 2020; Paolicelli & Ferretti, 2017; Tremblay et al., 2011). Besides microglia, also astrocytes have been reported to exert phagocytotic activity within the CNS, regulating synapse homeostasis through clearance of dead cells, neuronal debris, synapses and pathological protein aggregates (Damisah et al., 2020; Lee & Chung, 2021; Sloan & Barres, 2018). Whether the crucial function of waste product removal from the brain parenchyma is furthermore mediated by additional cell types associated with the brain, remains unclear.

In other tissues and organs, the lymphatic vascular system is essential for the uptake and drainage of interstitial fluid and macromolecules which are eventually transported back to the blood circulation (Petrova & Koh, 2020; Schulte-Merker, Sabine, & Petrova, 2011; Tammela & Alitalo, 2010). However, since the brain is considered to be devoid of lymphatic vessels, a possible contribution of the lymphatic system for waste removal in the brain has been neglected in the past. This is somewhat counter-intuitive, given the high metabolic activity of the brain, and the abundance of blood vessels (BVs) within it, which will invariably loose some of their contents into the interstitium, as is the case in all other vascularized tissues of the body. Recently, a lymphatic vascular network in the dura mater of the mouse meninges was (re-)discovered (Aspelund et al., 2015; Louveau et al., 2015; Mascagni & Bellini, 1816). These lymphatic vessels are located in the immediate proximity of the meningeal blood vasculature and absorb tracer from the brain interstitial fluid upon injection into the brain parenchyma (Aspelund et al., 2015; Louveau et al., 2015). The dural lymphatic vessels develop post-natally, originate around the foramina that form the entry and exit sites for the BVs and nerves, and migrate along BVs and the cranial and spinal nerves, eventually resulting in a fully developed lymphatic system at P28 (Antila et al., 2017). This meningeal lymphatic system appears to be conserved across mammals and has been described in humans and non-human primates (Absinta et al., 2017). Very recently, the presence of a similar lymphatic vessel network covering the inner lining of the skull has been reported in zebrafish, which drains substances and immune cells from the brain (Castranova et al., 2021). Whether and how meningeal lymphatics might contribute to waste removal from the brain has not been experimentally addressed but is a topic of significant interest, since accumulation of protein aggregates is one of the hallmark features of neurodegenerative diseases (Metcalf, Garcia-Arencibia, Hochfeld, & Rubinsztein, 2012). More generally, it is an outstanding question of significant importance how waste products are removed from the brain parenchyma in all vertebrates, and whether cells distinct from microglia serve key functions in the process.

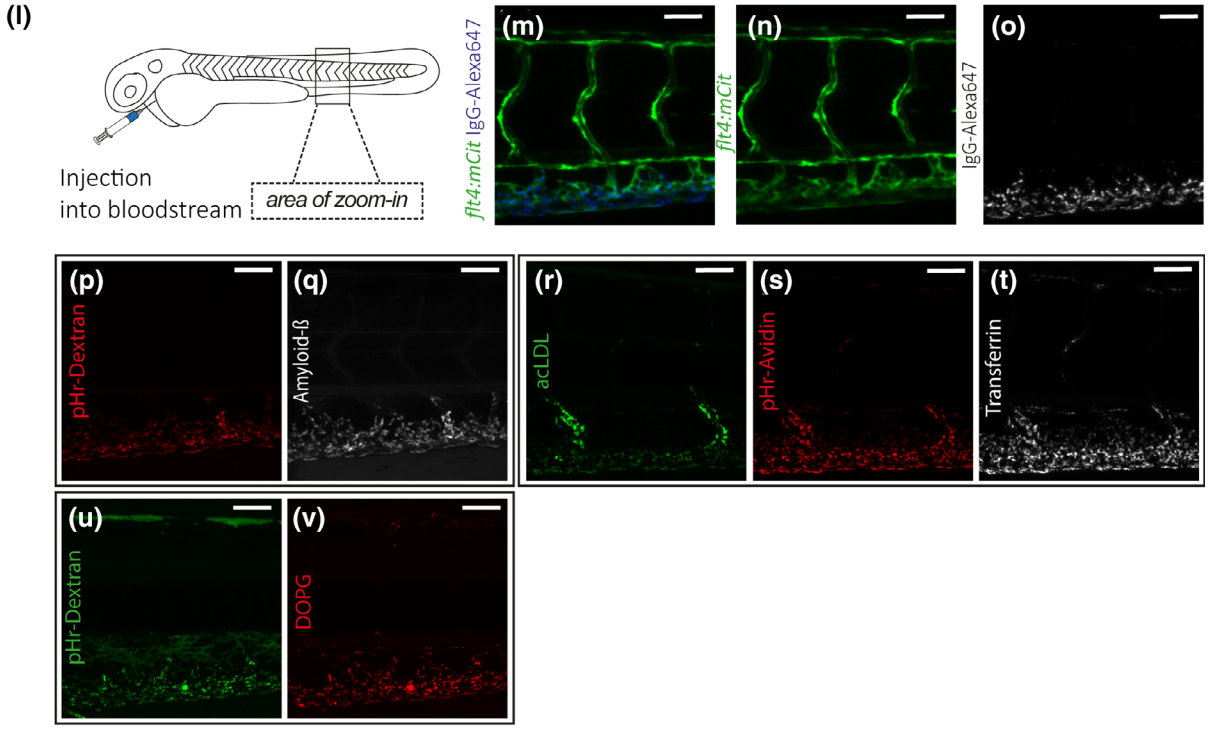
Recently, we and others have demonstrated the presence of lymphatic endothelial cells in the meningeal layer of the zebrafish brain (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017). Due to the simultaneous but independent discovery of these cells, they were termed either brain lymphatic endothelial cells (BLECs) (van Lessen et al., 2017), mural lymphatic endothelial cells (Bower et al., 2017a), or fluorescent granular epithelial cells (FGP cells) (Venero Galanternik et al., 2017), with all terms referring to the identical cell type. These cells express hallmark lymphatic marker genes such as the transcription factor prospero homeobox 1a (*prox1a*), the lymphatic vessel endothelial hyaluronan receptor *1* (*lyve-1)* as well as the vascular endothelial growth factor receptor 3 *(vegfr3*, also termed *flt4)*. Furthermore, and analogous to the lymphatic vasculature, they originate from the venous endothelium and their development is dependent on the activity of the conserved Vascular endothelial growth factor C (Vegfc)/Vegfr3 signaling pathway. Even though BLECs are in close proximity to the meningeal blood vasculature, they do not share—in contrast to pericytes—a common basement membrane with endothelial cells (van Lessen et al., 2017). Whole transcriptome profiling of sorted BLECs confirmed that BLECs are a distinct endothelial cell population, which show expression profiles different from macrophages and pericytes, while expressing lymphatic markers (Bower et al., 2017a; Venero Galanternik et al., 2017). It has been shown, that BLECs are not lineage-related with macrophages, and inhibition of myelopoesis by administrating a *pu.1* (*spi1b*) morpholino does not affect BLEC development. Remarkably, these cells do not form any vascular structure, but give rise to a network consisting of individual lymphatic endothelial cells expanding over the whole brain surface (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017). BLECs originate from the venous choroidal vascular plexus behind the eye and sprout around 56 hours post fertilization, at which point they downregulate blood vascular specific genes and upregulate lymphatic markers. Sprouting occurs bilaterally and cells migrate along the mesencephalic vein, resulting in symmetric loops of single cells that cover the optic tectum of the zebrafish embryo (Figure 1(a)). This network of individual cells continues to expand throughout the development of the fish and covers the whole surface of the brain at around 3 weeks of age. BLECs have been shown to play a role in regenerative processes within the brain (Chen et al., 2019) and have the ability to take up extracellular substances into subcellular vesicles in a process depending on receptor-mediated endocytosis. The capacity to endocytose macromolecules is acquired immediately upon sprouting from the choroidal vascular plexus (van Lessen et al., 2017).

The cellular morphology and the ability to take up macromolecules imply a functional similarity to the well-known microglia, which are the immune cells of the CNS that are considered to be the main constituent for waste removal within the brain. Microglia are able to protect the brain from invading microorganisms and can recognize and engulf dying neurons to prevent the diffusion of damaging degradation products (Lauber, Blumenthal, Waibel, & Wesselborg, 2004; Platt, da Silva, & Gordon, 1998). Here, we compare BLECs to the recently discovered scavenger endothelial cells of the cardinal vein, which are the zebrafish equivalent of sinusoidal endothelial cells within the mammalian liver and find them to act as scavenger




(k)

| substrates | | genotypes: wt | stab2-/- | mrc1a-/- | stab2-/-; mrc1a-/- |
|---|---|---|---|---|---|
| liposome | DOPG | + | + | + | + |
| lipoprotein | ocLDL | + | + | + | + |
| | acLDL | + | + | + | + |
| glycosaminoglycan | fluoHA | + | + | + | + |
| proteins | Avidin | + | + | – | – |
| | IgG | + | + | + | + |
| | Transferrin | + | + | + | + |
| | Amyloid-ß | + | + | + | + |
| polysaccharide | Dextran | + | + | + | + |




(w)

| substrates | | genotypes: wt | stab2-/- | mrc1a-/- | stab2-/-; mrc1a-/- |
|---|---|---|---|---|---|
| liposome | DOPG | + | – | + | – |
| lipoprotein | ocLDL | + | + | + | + |
| | acLDL | + | + | + | + |
| glycosaminoglycan | fluoHA | + | – | + | – |
| proteins | Avidin | + | + | + | + |
| | IgG | + | + | + | + |
| | Transferrin | + | + | + | + |
| | Amyloid-ß | + | + | + | + |
| polysaccharide | Dextran | + | + | + | + |

**FIGURE 1** Legend on next page.

endothelial cells of the brain. Furthermore, we compare microglia and BLECs in their capacity to take up different substrates in a qualitative and quantitative manner and report significant differences in their uptake efficiencies for several substances in zebrafish embryos at 5 days post fertilization (dpf). Our results therefore suggest a division of tasks between both cell types thereby assuring an efficient removal of extracellular particle waste from the interstitium of the brain.

## 2 | MATERIALS AND METHODS

### 2.1 | Zebrafish strains

Zebrafish strains were maintained under standard husbandry conditions according to FELASA guidelines (Aleström et al., 2020). Animal work followed guidelines of the animal ethics committees at the University of Münster, Germany. The following transgenic and mutant lines have been used in this study:

*Tg(kdr-l:HRAS-mCherry-CAAX)*$^{s916}$ referred to as *kdrl:mCherry* (Hogan et al., 2009); *Tg(lyve1:dsRed2)*$^{nz101}$ (Okuda et al., 2012), *Tg(flt4:mCitrine)*$^{hu7135}$ (van Impel et al., 2014), *Tg(flt1*$^{enh}$*:tdTomato)*$^{hu5333}$ (Bussmann et al., 2010), *Tg(mpeg1:EGFP)*$^{gl22}$ (Ellett, Pase, Hayman, Andrianopoulos, & Lieschke, 2011), *Tg(fli1a:nEGFP)*$^{y7}$ (Roman et al., 2002).

### 2.2 | CRISPR/Cas9

The guide RNA targeting *mrc1a* exon 4 (GGGGACAGTG ATCCAGTGAC) was designed using the chopchop algorithm (https://chopchop.cbu.uib.no/). The sgRNA was synthesized as described previously (Gagnon et al., 2014). A mixture containing 15 pg of gRNA with 300 pg of Cas9 mRNA was injected into the cytoplasm of one-cell stage zebrafish embryos. A resulting −7 bp deletion allele termed *mrc1a*$^{mu408}$ was maintained and used for the experiments.

*mrc1a* wt CTCTGGATGGGACAGTGATCCAGTGACTGGTGT ATTATATCAGAGGAATGTGCAG

*mrc1a*$^{mu408}$

CTCTGGATGGGACAGTGATC−−−−−TGGTGTATTATATCAGA GGAATGTGCAG

### 2.3 | Genotyping

*mrc1a*$^{-7bp}$ *and stab2*$^{-4bp}$ embryos were genotyped by KASP using the following primers: Mrc1aKASPAR_wt 5′-GAAGGTGACCAAGTT CATGCTCAGCTCTGGATGGGACAGTGATCC-3′, Mrc1aKASPAR_mut 5′-GAAGGTCGGAGTCAACGGATTCAGCTCTGGATGGGACAGTGAT CT-3′, Mrc1a_C2-R 5′-CCAAGTCAGTATTGACTGCACATTCCTCT-3′, Stab2KASPAR_wt 5′-GAAGGTGACCAAGTTCATGCTTTATGCAGC AATCAACCCGTGC, Stab2KASPAR_mut 5′-GAAGGTCGGAGTC AACGGATTTTATGCAGCAATCAACCCGTGA-3′, Stab2_C2-R 5′-CAC TGCATTCGCATGGCACAC-3′.

### 2.4 | Injection regimes

Injections were carried out with a pneumatic PicoPump. Embryos were embedded in 1.5% low melting point agarose (ThermoFischer, #16520100) containing 0.0168% tricaine (Sigma, #A5040) and injected with a total volume of 0.5–1 nl per injected bolus. For intratectal injection and injection into the cerebrospinal fluid, needles were inserted into the brain in a sloped angle. Care was taken not to penetrate deep into the brain tissue.

### 2.5 | Dyes

The following fluorescent dyes and concentrations were used for injection: 10 kDa dextran-conjugated Alexa Fluor 647 (2 mg/ml, ThermoFisher, #D22914), HiLyte Fluor 647-labeled Amyloid-β (1–40) (2 mg/ml, Anaspec, AS-60493), pHrodo Red Avidin (2 mg/ml, ThermoFisher, #P35362), pHrodo Red Dextran (2 mg/ml, ThermoFisher, P10361), pHrodo Green Dextran (2 mg/ml, ThermoFisher, P35368), acLDL (1 mg/ml, ThermoFisher, L23380), Donkey anti-Goat IgG (H + L) Alexa Fluor 647 Cross-Adsorbed Secondary Antibody (2 mg/ml, ThermoFisher, A21447), oxLDL (1 mg/ml, ThermoFisher L34357), Transferrin (2 mg/ml, ThermoFisher, T23366). Hyaluronic acid (fluoHA sodium salt, 100 kDa) was purchased from Lifecore Biomedical Inc. DOPG liposomes were prepared as previously described (Campbell et al., 2018). Of the HPV16 pseudoviruses a total of 36 pg were injected per embryo.

**FIGURE 1** BLECs in the meningeal layer and scavenger endothelial cells (SECs) in the cardinal vein show the same substrate specificity. (a) Cartoon depicting the zebrafish head region (dorsal view, anterior at the top) with the position of the two BLEC loop structures highlighted in red. The boxed area shows the position of the maximum projection of BLECs depicted in (b-j). Injection of the different fluorescently labeled substrates occurred either into the center of the optic tectum or into the ventricles of embryos at 5dpf. (b,c) Uptake of IgG-Alexa647 by BLECs. Dorsal confocal projections of a *lyve1:DsRed* positive BLEC (b), which has internalized fluorescent IgG-Alexa647 (c). (d-j) Confocal projections of BLECs that have internalized the indicated substrate combinations. (k) Overview about the uptake of the analyzed classes of substrate molecules by BLECs in either wild type, *stab2* $^{-/-}$ mutant, *mrc1a* $^{-/-}$ mutant or *stab2*$^{-/-}$;*mrc1a*$^{-/-}$ double mutant embryos. (l) Schematic overview of a zebrafish embryo, depicting injection of different fluorescent dyes into the bloodstream. Examples of substance uptake by SECs in the cardinal vein (boxed area) is shown in (m-v). (m-o) Maximum projection highlighting venous and lymphatic endothelial cells by *flt4:mCitrine* expression (n) and showing accumulation of the injected IgG-Alexa647 in the cardinal vein area (o). Uptake of different substrates by SECs in the cardinal vein (p-v). (w) Summary of the SEC uptake capacity for different classes of substrates which were tested in wild type, *stab2* $^{-/-}$ mutant, *mrc1a* $^{-/-}$ mutant and *stab2*$^{-/-}$;*mrc1a* $^{-/-}$ double mutants. (m-o) *mrc1a* $^{+/-}$, (p–t) wild type embryo; (u-v) *mrc1a* $^{+/-}$. Scale bars in (b-j) represent 12.5 μm and in (m-v) 50 μm

## 2.6 | Bacteria

*Escherichia coli* K-12 MG1655 λ*att*::P*frr-cfp-cat-yfp*-P*gadA* was a gift from Marco Chitto. Allelic replacement of the *Pfrr-cfp-cat*-yfp module by *cfp-aph(3′)-Ia* was accomplished by amplifying the cassette by PCR with the primers MC_163 and MBM_294 and pMB47 as template plasmid (Berger, Aijaz, Berger, Dobrindt, & Koudelka, 2019) according to the method described by (Datsenko & Wanner, 2000). The correctness of the transcriptional fusion of *cfp* to the *gadA* promoter in the *att* site of the *E. coli* K-12 MG1655 was verified by PCR over the 5′ and 3′ chromosome-cassette junctions with the primers UD 3343 and UD 598 and primers UD 3342 and 3344, respectively. The strain was thereafter named *E. coli* K-12 MG1655_blue*.

Oligonucleotides used for cloning were MC_163 5′-GGATA AATCCTACTTTTTTATTGCCTTCAAATAAATTTAAGGAGTTCGAAAT-GGTGTCTATCACTAAAGA-3′, MBM_294 5′-CACAGGTTGCTCCG GGCTATGAAATAGAAAAATGAATCCGTTGAAGCCTGAGGAAACAGC TATGACCATG-3′, UD 598 5′-GCTGAACTTGTGGCCGTTTA-3′, UD 3342 5′-GGTATTGATAATCCTGATATG-3′, UD 3343 5′-GGC GCAATGCCATCTGGTAT-3′ and UD 3344 5′-GACGGGAAACT GAAAATGTG-3′.

## 2.7 | Virus preparation

HPV16 pseudoviruses were prepared as previously described (Buck, Pastrana, Lowy, & Schiller, 2005). Briefly, 293TT cells were co-transfected with p16sheLL and the reporter plasmid pClneoEGFP. At 48 h post transfection, virus was harvested by incubation of the cell pellet with 0.35% Brij 58, 0.2% Plasmid Safe DNase and 0.2% benzonase for 24 h at 37°C. Pseudoviruses were purified using a linear 25%–39% OptiPrep density gradient and analyzed for virus content and purity by Coomassie staining of SDS-PAGE gels.

## 2.8 | Virus labelling

HPV16 pseudoviruses were labeled with pHrhodo as previously described (Samperio Ventayol & Schelhaas, 2015). Briefly, pseudoviruses were incubated with pHrhodo succinimidyl ester in virion buffer (635 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.1 mM KCl in PBS, pH 7.6) using a 1:8 molar ratio of the major capsid protein L1 to the dye for 1 h on an overhead rotator. Subsequently, virus was separated from free dye by ultracentrifugation using a 15–25–39% OptiPrep step gradient.

## 2.9 | Microscopy and image processing

Confocal imaging was performed on a Leica SP8 microscope employing Leica LAS X 3.5.6.21594 software and using a 20×/0.75 dry or a 40×/1.1 water immersion objective. For imaging, embryos were anesthetized with 0.0168% tricaine (Sigma, #A5040) and embedded in 1% low melting point agarose (ThermoFischer, #16520100). Confocal stacks were processed using Fiji-ImageJ version 1.51 g, and figures were assembled using Microsoft Power Point, Adobe Photoshop, and Adobe Illustrator. All data were processed using raw images with brightness, color and contrast adjusted for printing.

## 2.10 | Particle analysis of BLECs

Confocal maximum projections were analyzed as follows:

```
   roiManager("reset");
run("Duplicate...", " ");
run("Duplicate...", " ");
run("Median...", "radius=2");
run("Enhance Contrast...", "saturated=0.6
normalize");
run("Threshold...");
setThreshold(x, 255);
setOption("BlackBackground", true);
run("Convert to Mask");
run("Analyze Particles...", "show=Outlines add");
run("Tile");
waitForUser("select original");
roiManager("deselect");
roiManager("multi-measure measure_all");
roiManager("deselect");
```

## 2.11 | Particle analysis in *mpeg* positive macrophages

Threshold of the *mpeg:GFP* maximum projection was set in order to determine regions of interest, which were added to the ROI manager. The regions of interest were applied to the maximum projection of the injected molecules and the pixel intensity was measured. Regions containing a melanocyte were excluded from the analysis.

## 2.12 | Statistical analysis

Data sets were tested for normality (Shapiro–Wilk) and equal variance. *p*-values of data sets with normal distribution were determined by Welch's t-test. In case data values did not show normal distribution, a Mann–Whitney test was performed instead.

# 3 | RESULTS

## 3.1 | BLECs share substrate specificity with SECs in the cardinal vein

Endothelial cells usually line a lumenized space, which renders the rather loosely connected BLECs a unique population of cells.

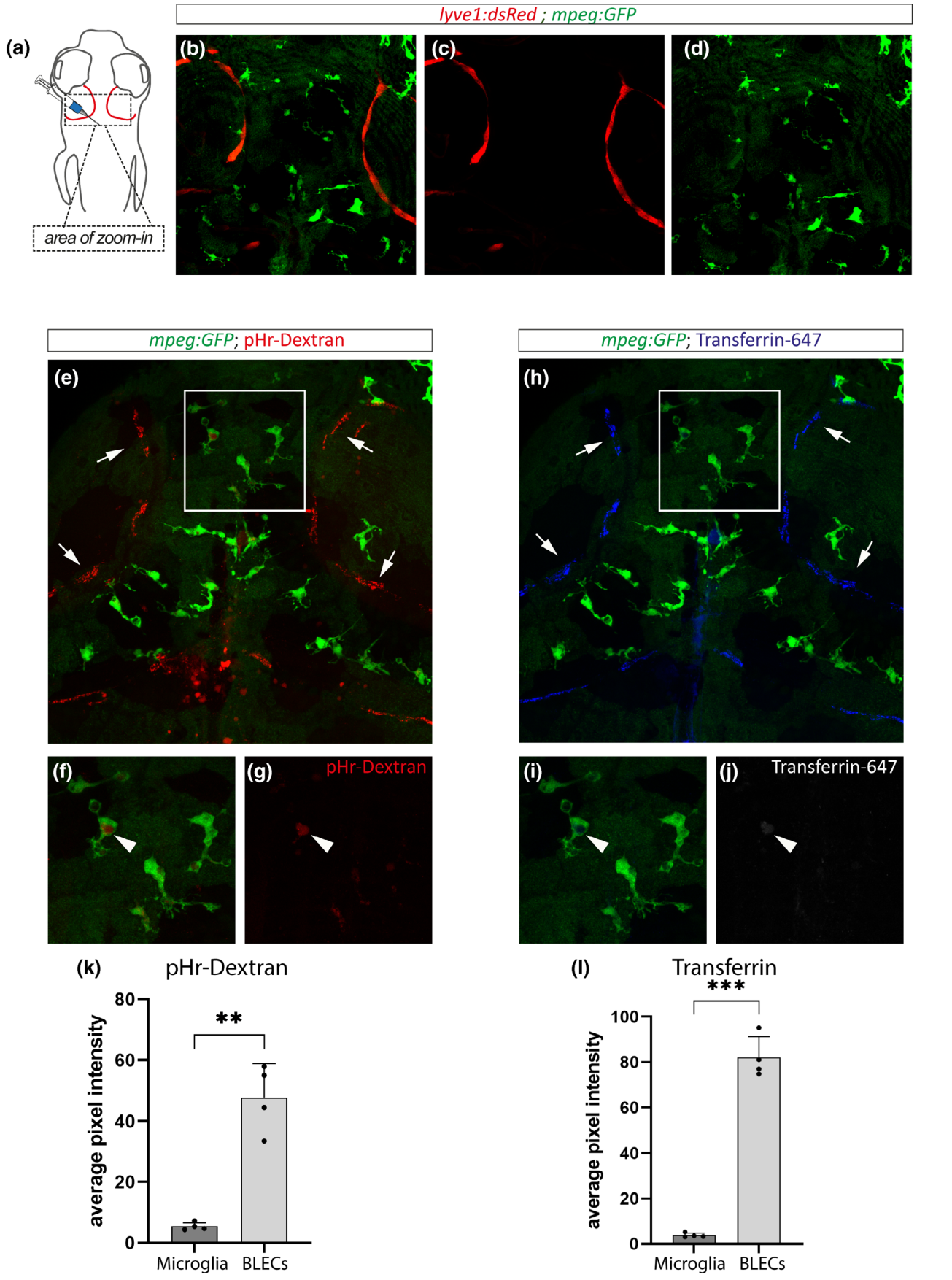



**FIGURE 2** Legend on next page.

Previously we have shown that BLECs form very characteristic loops covering the optic tectum of the zebrafish embryonic brain and that they efficiently endocytose exogenous protein in a dynamin-dependent manner after intracerebral injection (van Lessen et al., 2017). Based on these features, the closest similar group of ECs that could be compared to BLECs are scavenger endothelial cells, which are connected to each other, but do not necessarily form a conventional endothelial layer while exhibiting an extremely high endocytic capacity (e.g., in the mammalian liver) (Smedsrod, Pertoft, Gustafson, & Laurent, 1990). Very recently, a set of scavenger endothelial cells has been identified in the cardinal vein of the zebrafish embryo, with the capacity to take up various different substrate classes from the blood circulation (Campbell et al., 2018). We directly compared BLECs with the cardinal vein SECs in 5dpf embryos by injecting different classes of dye-conjugated substrate molecules into either the optic tectum (Figure 1(a–j)) or the bloodstream (Figure 1(l–v)) to analyze the similarity of both cell populations in terms of substrate specificity. To this end, we chose a wide variety of different substance classes, including liposomes (DOPG liposomes - Figure 1(d,v)), modified lipoproteins (acetylated-LDL, Figure 1(f,r); oxidated LDL, data not shown), glycosaminoglycans (fluorescent hyaluronic acid, fluoHA, data not shown), proteins including Avidin (Figure 1(g,s)), Transferrin (Figure 1(h,t)), Amyloid-β (1–40) (Figure 1(j,q)) and IgG-Alexa647 (Figure 1(c,o)) as well as the polysaccharide dextran (Figure 1(i,p)). In line with our initial experiments (van Lessen et al., 2017), we found that after injection of labeled proteins into the brain parenchyma, the substrates accumulated within BLECs in a punctate pattern indicating successful internalization of the molecules into endosomes (Figure 1(b–k) and Supporting Information 1). Likewise, also all other tested substrate molecules were rapidly internalized by BLECs indicating that BLECs can also endocytose various other substances. Injection of the same substrate molecules into the bloodstream resulted in an uptake and likewise dotted accumulation of the molecules within the cardinal vein SECs (Figure 1(m–w)). The finding that all tested substrate classes can efficiently be internalized by both cell populations strongly implies that BLECs and SECs of the cardinal vein have an identical substrate specificity, even though both cell types serve two completely different anatomical and physiological compartments. In conjunction with our previous dye injection experiments we therefore conclude, that BLECs exert a similar scavenger function in the brain meninges as SECs do in the blood vasculature.

In addition, we wanted to investigate which receptors are involved in the uptake of the different substrates, in order to determine whether BLECs and SECs are not only functionally similar and share the same substrate specificity, but also rely on the same cell surface receptors for cargo uptake. We therefore investigated the role of two different scavenger receptors, which are expressed both in BLECs (Bower et al., 2017a) as well as in SECs of the cardinal vein (Campbell et al., 2018): Mannose receptor 1a (Mrc1a) and Stabilin-2 (Stab2). We generated a knock-out allele for *mrc1a* harboring an out-of-frame deletion in exon 4 (Supporting Information 2) and analyzed the uptake of different injected substrate classes in stable *mrc1a*$^{-/-}$or *stab2*$^{-/-}$ single mutants, or in *mrc1a*$^{-/-}$; *stab2*$^{-/-}$ double mutant embryos. This analysis revealed that nearly all substances were still internalized by BLECs in the respective mutant scenarios (Figure 1(k)). However, in the case of *mrc1a*$^{-/-}$ single or *mrc1a*$^{-/-}$; *stab2*$^{-/-}$ double mutant BLECs, pHr-Avidin was not taken up anymore suggesting that the endocytosis of this particular substrate by BLECs is dependent on Mrc1a function (Figure 1(k)). When investigating the clearance capacity of SECs, we observed that the uptake of pHr-Dextran, Transferrin, Amyloid-β, IgG-Alexa647, oxLDL, acLDL, but also of pHr-Avidin, was not affected by the absence of Mrc1a, Stab2 or both receptors. Only the previously identified substrates of Stab2, hyaluronic acid and DOPG liposomes (Campbell et al., 2018), were no longer internalized by SECs in mutant scenarios lacking Stab2 while still being internalized by BLECs in the same mutant backgrounds (Figure 1(k,w)). Our results therefore demonstrate that although the overall range of accepted substrate molecules is identical for BLECs and cardinal vein SECs, both cell types show differences in the scavenger receptors employed for the respective cargo uptake. Furthermore, the fact that most of the substrates are still endocytosed by both cell types in the *mrc1a* and *stab2* mutant scenarios suggests that a set of additional receptors is involved in the uptake of cargo molecules.

Taken together, our data imply that BLECs represent a SEC-like cell population of the brain periphery that together with microglia ensure the removal of a wide spectrum of molecule classes from the brain parenchyma to maintain tissue homeostasis in the CNS.

## 3.2 | BLECs are more efficient in the uptake of macromolecules than microglia

After identifying this novel scavenger endothelial cell type in the meningeal layer of the brain, the question arises whether there is a division of labors with microglia, which are generally considered to constitute the main cell population that clears cellular or sub-cellular components from the brain (Marquez-Ropero et al., 2020; Platt et al., 1998). In order to explore the function of BLECs in direct

**FIGURE 2** Microglia are significantly less efficient in the uptake of dextran and Transferrin from the brain parenchyma compared to BLECs. (a) Schematic overview of the dorsal head region of a zebrafish embryo indicating the zoom-in area in (b-d, e, h). (b-d) Maximum projection showing the characteristic loops of *lyve1:DsRed* positive BLECs on top of the optical tecta and indicating the position of *mpeg:GFP* positive microglia at 5dpf. (e-j) *mpeg:GFP* positive zebrafish embryo in which pHr-dextran (e) and Transferrin-647 (h) were co-injected into the brain parenchyma. Arrows highlight an uptake of the respective substance by BLECs. (f, g) and (i, j) zoom-in of the boxed region in (e) and (h), respectively. Arrowheads indicate cargo uptake in a macrophage. (k) Quantification of the average pixel intensity of pHr-dextran shows a significantly higher uptake in BLECs compared to microglia (Welch's t-test, $p = .0044$). (l) The average pixel intensity for Transferrin-647 in BLECs is significantly higher compared to microglia (Welch's t-test, $p = .0004$). Number of analyzed embryos was n = 4. Values are presented as means ± SD

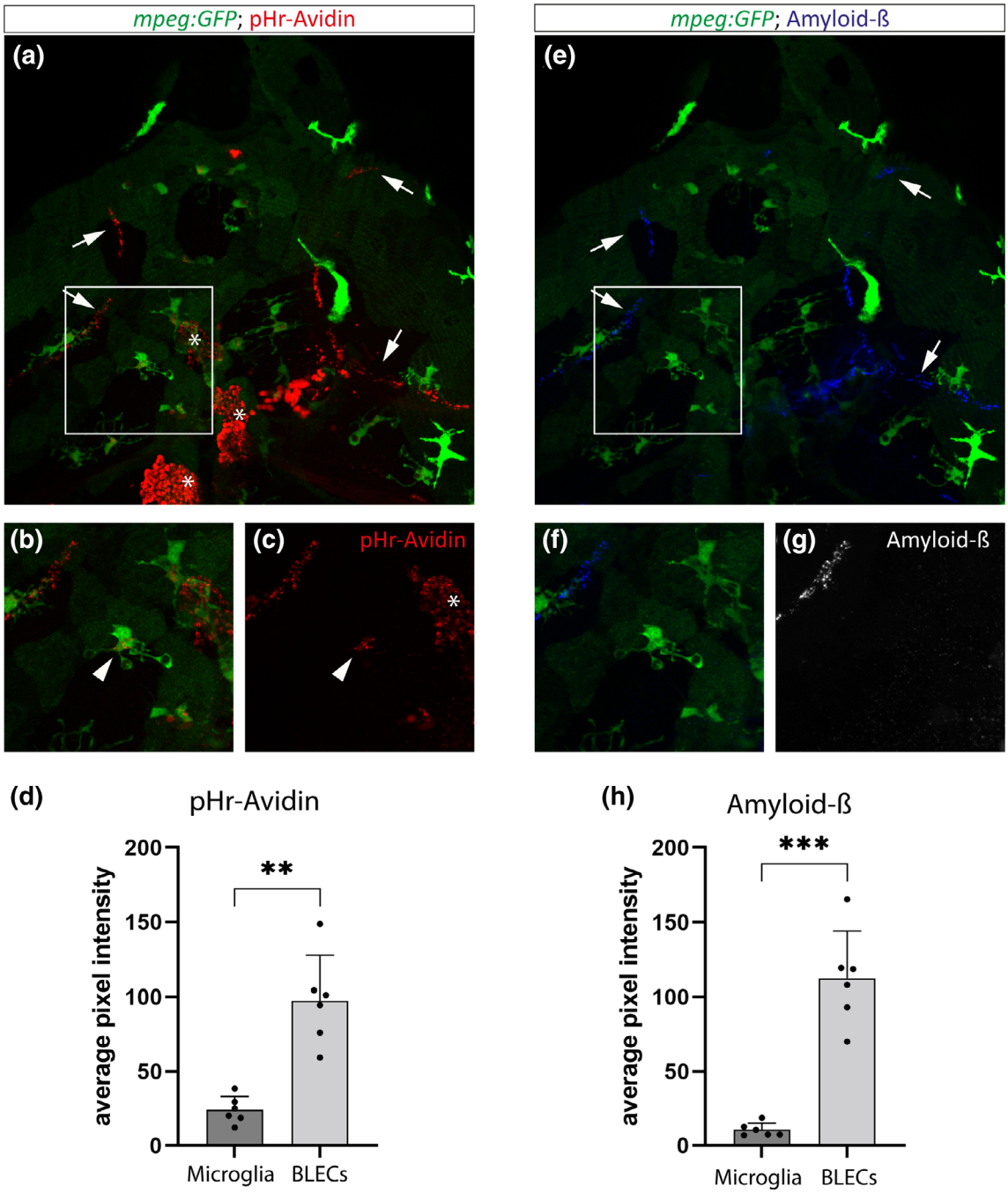



**FIGURE 3** Microglia take up Avidin and Amyloid-β less efficiently than BLECs. (a-c) Maximum projections of a 5dpf *mpeg:GFP* positive embryo in which pHr-Avidin was injected into the optic tectum. Note the auto-fluorescence in the red channel, marked by asterisks. (d) The average pixel intensity of the pHr-Avidin signal is significantly lower in microglia compared to BLECs (Welch's t-test, $p = .0014$). (e-g) Maximum projections of the same embryo, showing the distribution of HiLyte Fluor 647-labeled Amyloid-β (1–40) protein after parenchymal administration. (b, c and f, g) Zoom-in of the indicated regions in (a) and (e), respectively. Arrows highlight an uptake event in BLECs, arrowheads indicate an uptake by a *mpeg* + macrophage. (h) Quantification of the average pixel intensity of Amyloid-β accumulating in microglia and BLECs results in significantly higher values for BLECs as compared to microglia (Welch's t-test, $p = .0005$). Number of embryos analyzed was n = 4. Values are presented as means ± SD

comparison to microglia, we decided to investigate how the endocytic capacity of microglia compares to that of BLECs in a quantitative manner. Using *mpeg:GFP* transgenic embryos at 5 dpf that were injected with different fluorescent substrates into the brain parenchyma (Figure 2(a)), we were able to discriminate between the characteristic BLEC loop covering the optic tectum (Figure 2(b,c); arrows in (e,h)) and the *mpeg:GFP* positive microglia (Figure 2(b,d)). To assess the uptake efficiencies of both cell types in a quantitative manner, we measured the amount of fluorescently labeled substrate accumulating in BLECs and microglia by determining the average pixel intensity in

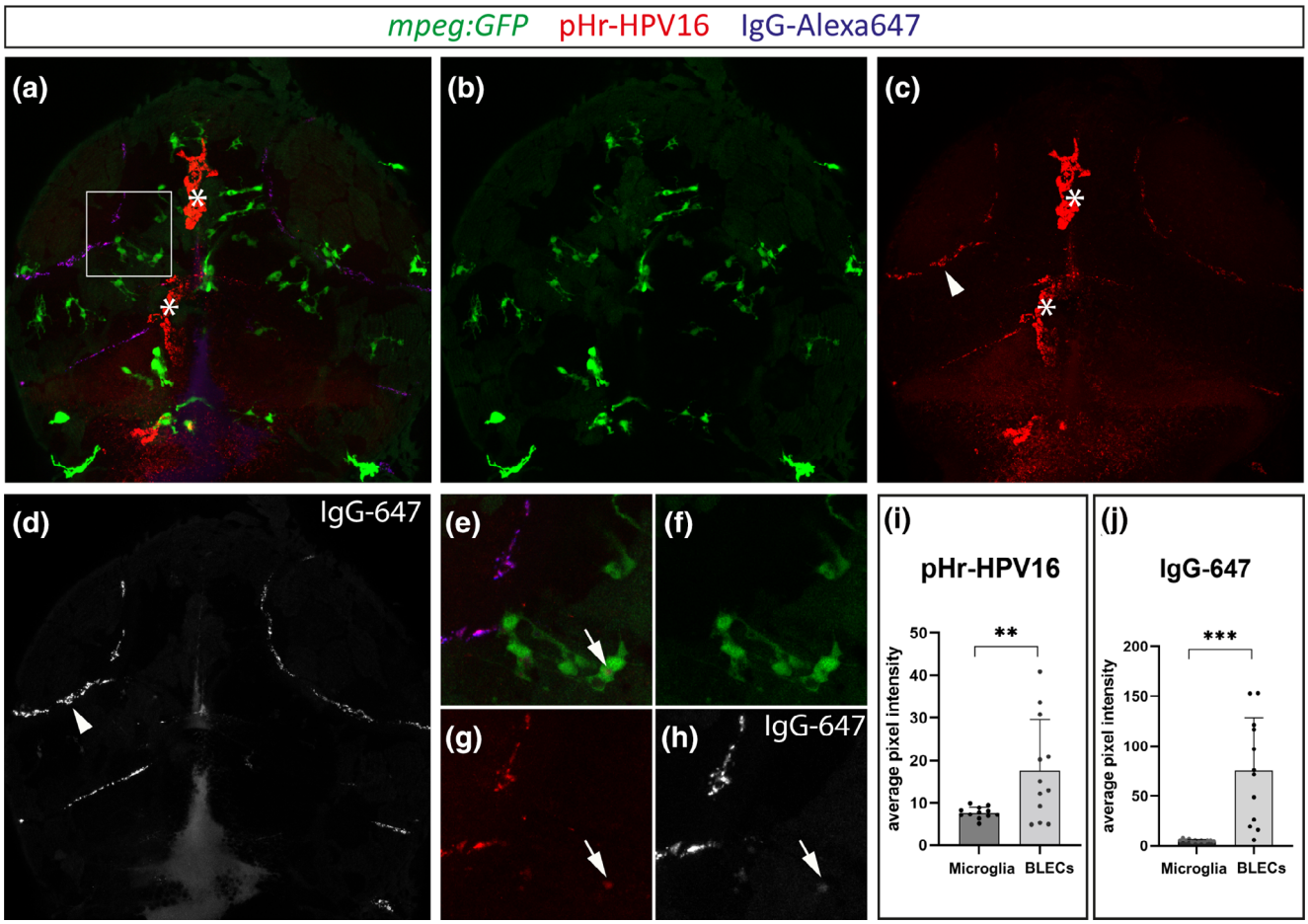



**FIGURE 4** BLECs ingest HPV-16 pseudoviruses more efficiently than microglia. (a-d) Maximum projection of a *mpeg:GFP* positive zebrafish embryo that was co-injected with pHr-HPV-16 and IgG-Alexa647 into the CSF at 5dpf. The white box indicates the zoom-in area in (e-h). Single channel of maximum projection in (a) for *mpeg:GFP* (b), pHr-HPV-16 (c) and IgG-Alexa647 (d). Arrowheads highlight uptake into BLECs while arrows mark endocytosis of the respective substrate in microglia. Note the auto-fluorescence of pigment cells in the red channel, marked by asterisks. (i, j) Quantification of the average pixel intensities within *mpeg:GFP*+ microglia and BLECs in n = 12 embryos shows a significantly higher uptake of pHr-HPV-16 (Welch's t-test, $p = .0097$) and IgG-Alexa647 (Welch's t-test, $p = .0001$) in BLECs when compared to microglia. Values are presented as means ± SD

the *mpeg:GFP*+ microglia and the characteristic BLEC loop within the same embryo at 1 h post-injection (hpi) (Supporting Information 3). In order to discriminate between an accumulation of substrates at the cell surface and the successful internalization of cargo molecules by the respective cell type, we co-injected pHrodo (pHr)-coupled dyes, which are almost non-fluorescent at neutral pH and only fluoresce upon internalization into acidic compartments of the cell.

When co-injecting pHr-Dextran and Transferrin, which mediates the transport of iron in the blood, into the brain parenchyma, we found both dyes to strongly accumulate within BLECs, leading to a bright signal of both substrates in this cell population (pHr-Dextran in BLECS: 47.68 average pixel intensity [api]; Transferrin-Alexa647 in BLECs 82.00 api) (Figure 2(e–g,k)). In *mpeg*+ microglia, however, the intensity of the dye accumulating in endosomes was considerably lower (pHr-Dextran in microglia 5.41 api; Transferrin in microglia 3.78 api), resulting in a significant difference in average pixel intensity between BLECs and microglia for both Transferrin (t-test; $p = .0004$) and pHr-Dextran (*t*-test; $p = .0044$) (Figure 2(h–j,l)).

Next, we co-injected pHr-Avidin and Amyloid-β. Amyloid-β is a protein that in case of mis-folding manifests itself into plaques that have been found in brains of Alzheimer Disease patients (Glenner & Wong, 2012; Haass & Selkoe, 1993; Selkoe & Hardy, 2016). After injecting pHr-Avidin and the Amyloid-β (1–40) C-terminal variant into the brain parenchyma we found that the average pixel intensity of both substrates was again significantly higher in BLECs compared to microglia (pHr-Avidin in BLECs 97.42 api vs microglia 23.89 api [t-test $p = .0014$] and Amyloid-β in BLECs 112.50 api vs. microglia 10.58 api [t-test $p = .0005$) (Figure 3(a–h)). This implies that BLECs are significantly more efficient in clearing those substances from the extracellular compartment than microglia.

To exclude the possibility, that parenchyma injections would favor an uptake of substances by BLECs and not by microglia, we furthermore injected both substrate combinations into the cerebrospinal fluid (CSF) of *mpeg:GFP*+ embryos. In line with the parenchyma injections, however, we found that in all cases BLECs exhibited a significantly higher uptake of cargo molecules when compared to microglia

(Supporting Information 4). These results therefore demonstrate that the site of injection (parenchyma versus CSF injection) is not causative for the overall higher uptake efficiency of BLECs compared to microglia.

Given the fact that the uptake of Avidin by BLECs is dependent on Mrc1a function (Figure 1(k)), we were wondering whether mircoglia would show an increased clearance activity for pHr-Avidin in *mrc1a* mutant embryos. We therefore co-injected pHr-Avidin and IgG-Alexa647 into the CSF of embryos from a *mrc1a*$^{+/-}$; *mpeg:GFP* incross. Although BLECs again failed to internalize pHr-Avidin in homozygous *mrc1a* mutants while still ingesting the IgG-Alexa647, microglia did not seem to functionally compensate in this scenario since the average pixel intensities within the *mpeg:GFP*-positive microglia were not significantly different between *mrc1a* mutants and sibling embryos (Supporting Information 5).

We also addressed the question whether microglia would be able to eventually accumulate similar amounts of test substances after an extended time span, and therefore analyzed the uptake of IgG-Alexa647 at 1hpi, 3hpi, and 6hpi. Although the IgG-Alexa647 signal intensity mildly increased in both cell types over time, the difference in the uptake efficiency between both populations persisted (Supporting Information 6). Considering the motility of microglia and the fact that microglia are in closer proximity to the actual injection sites in the brain parenchyma and the ventricles, it is remarkable that BLECs are an order of magnitude more efficient in the internalization of these soluble substrate molecules.

### 3.3 | CSF-injected virus particles can be ingested by BLECs and microglia

The liver has previously been established as a major site for clearance of circulating virus particles from the body. Importantly, more recent studies suggested a central role for sinusoidal endothelial cells, the scavenger endothelial cell population of the liver, in clearing virus or virus-like particles from the blood circulation more efficiently than the resident liver macrophages, called Kupffer cells (Ganesan et al., 2011; Mates et al., 2017; Oie et al., 2020; Simon-Santamaria et al., 2014). In order to investigate whether the BLECs as a novel scavenger endothelial cell population in the brain meninges could fulfill a similar virus-clearing function in the brain environment, we made use of fluorescently labeled HPV-16 pseudovirus particles and assessed the efficiency of internalization in microglia and BLECs. These spherical particles have a diameter of 55 nm and are coupled to pHr, allowing the visualization of their internalization into acidic compartments of cells. We co-injected the virus particles with IgG-Alexa647 into the CSF of 5dpf *mpeg:GFP*-positive embryos and subsequently quantified the average pixel intensity for both substrates in microglia and in the BLEC-loops on top of the optical tectum. Although the virus could be detected in both cell populations (Figure 4(a–h)), significantly higher pixel intensities could be detected within BLECs when compared to the uptake in microglia (pHr-HPV16 in microglia 7.69 api and in BLECs 17.59 api [t-test $p = .0097$]) (Figure 4(i)). As expected, the co-injected IgG-Alexa647 also showed a significantly higher uptake by

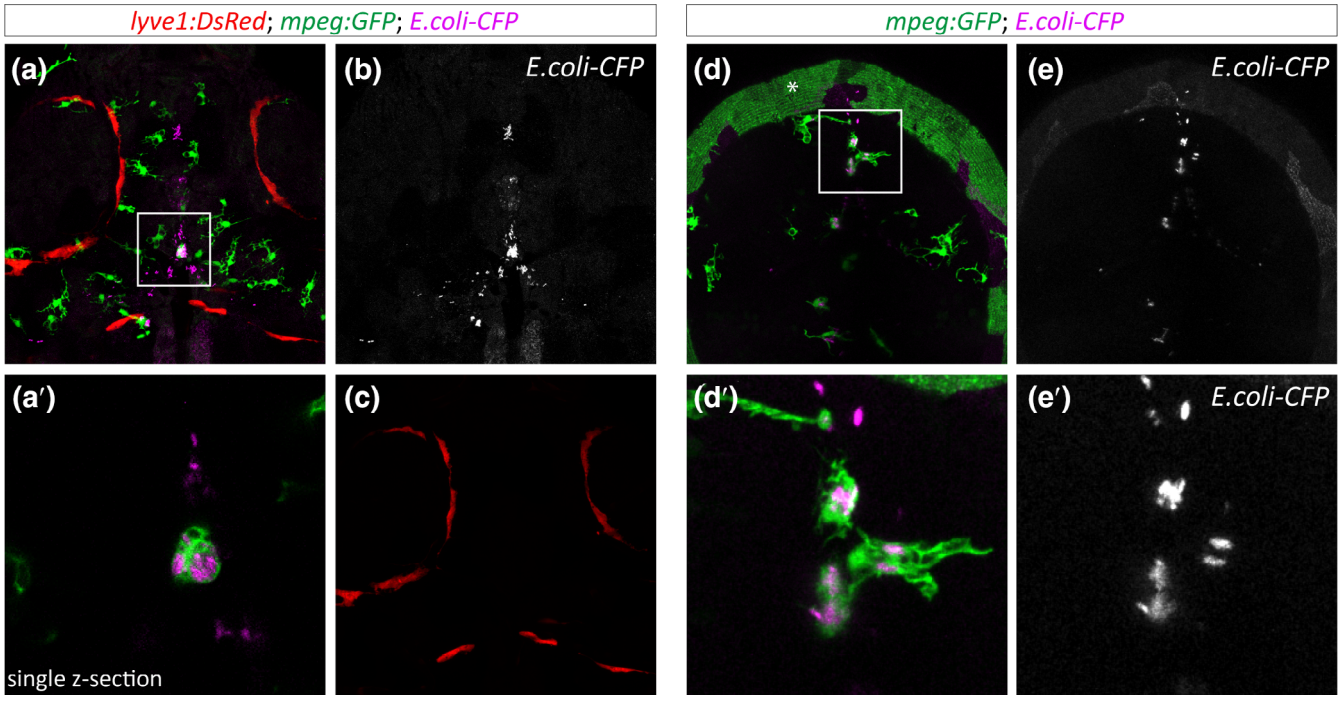



**FIGURE 5** Microglia but not BLECs internalize CFP-labeled *Escherichia coli* from the brain parenchyma. (a-c) Maximum projection of the head region (dorsal view) of a *lyve1:DsRed; mpeg:GFP* double transgenic embryo that was injected with CFP-labeled *E. coli* at 5dpf. (a') Single confocal slice of the boxed area in a, showing uptake of *E. coli* by a GFP-positive macrophage. (d-e') Partial projection of a *mpeg:GFP* positive embryo that was injected with CFP-labeled *E. coli*. The box indicates the area of zoom-in in (d′) and (e'). Note that the green signal marked by an asterisk in (d) represents auto-fluorescence of the embryo

BLECs (IgG-Alexa647 in microglia 5.32 api and in BLECs 75.30 api [*t*-test $p = .0001$]) (Figure 4(j)). Taken together, these results demonstrate that BLECs also show a higher endocytotic capacity for larger cargo such as the pHr-HPV16 and that they might therefore substantially contribute to the clearance of virus particles from the brain tissue.

### 3.4 | Bacteria are taken up by microglia but not by BLECs

The question then remains whether microglia serve a unique function, or whether microglia and BLECs constitute a fully redundant system. Since microglia have always been considered to provide a clearing mechanism which responds efficiently to tissue damage and infection by engulfing and processing pathogens (Platt et al., 1998), we intended to investigate whether BLECs can also contribute to the removal of bacteria. We therefore generated a fluorescently labeled *E. coli* K-12 strain expressing CFP, which we injected into 5dpf old embryos to evaluate the uptake in BLECs versus microglia. 22 embryos were injected either into the CSF (n = 10) or the parenchyma (n = 12) and the fluorescently labeled bacteria were visible in the brain directly after injection. At 1hpi, an obvious uptake of CFP-labeled *E. coli* by *mpeg*+ microglia could be observed (Figure 5(a,a')), whereas no uptake by BLECs was detectable (Figure 5(b,d)). Independent of the injection site (CSF or brain parenchyma), we never observed uptake of bacteria in BLECs even at 4hpi (data not shown). Microglia, however, sensed the presence of *E. coli* and extended processes in order to reach and engulf bacteria (Figure 5(d–e')). Our results therefore imply that BLECs are not involved in this aspect of the immune response in the zebrafish brain, and cannot phagocytose whole bacteria.

## 4 | DISCUSSION

Clearance of macromolecules from the brain parenchyma is a crucial process and has always been considered to be mainly carried out by microglia in vertebrates. The interplay and functional connection of microglia with meningeal lymphatic vessels on the one hand (Aspelund et al., 2015; Castranova et al., 2021; Louveau et al., 2015) and with BLECs in the meningeal layer of the teleost brain or with the so-called Leptomeningeal Lymphatic Endothelial Cells in the mammalian leptomeninges on the other hand (Shibata-Germanos et al., 2020), has only just begun to be explored, since these structures were only recently discovered (Suarez & Schulte-Merker, 2021). The experimental accessibility of the zebrafish allows us to address these questions, and we here show that BLECs and microglia constitute two distinct cell types that share the task of removing substances and pathogens from the larval brain. BLECs qualify as a scavenger endothelial cell population in the brain meninges, and are more efficient in uptake of exogenous substances than microglia. BLECs, however, are unable to take up living bacteria, which microglia readily engulf and phagocytose.

BLECs cover the optic tectum and other parts of the brain as an extensive network of cells in close proximity to meningeal BVs. They remain as single cells and never form lumenized structures, even after having expanded significantly during growth of the zebrafish. Gene expression profiling by RNA-seq demonstrated that this cell population is distinct from blood ECs, macrophages and pericytes, while expressing typical lymphatic marker genes and a range of endothelial growth factors and ligands (Bower et al., 2017b; Venero Galanternik et al., 2017). The question which function BLECs fulfill under normal physiological conditions, has not been sufficiently answered, even though a number of intriguing properties have been reported, such as an ability to respond to cerebrovascular damage and to fulfill a guidance function for re-growing BVs (Bower et al., 2017b; Chen et al., 2019). Furthermore, BLECs have the capacity to take up extracellular macromolecules from the brain parenchyma, which raises the question why the embryo establishes, next to microglia, a second cell population to perform what appears to be a very similar task. We compared BLECs with microglia in terms of uptake efficiency of several macromolecules, virus particles and bacteria, to investigate the function of BLECs in comparison to microglia in a qualitative and quantitative manner. We found that microglia and BLECs show a clear difference in the uptake efficiency of different substances, implying a task-division of the two cell types in the brain. Whereas microglia are involved in the immune response and removal of bacteria from the brain parenchyma, BLECs are more efficient in the uptake of smaller cargo such as proteins and polysaccharides.

The notion that BLECs constitute a pool of single endothelial cells that do not form vessels and that show a remarkable uptake capacity for exogenous substances, is reminiscent of scavenger endothelial cell properties. Recently, scavenger endothelial cells were discovered in embryonic zebrafish in several large veins, including the posterior cardinal vein and the common cardinal vein. They clear macromolecules, colloidal waste and viral particles from the blood circulation (Campbell et al., 2018). In terrestrial vertebrates, this specialized cell population is located in the liver sinusoids, termed liver sinusoidal endothelial cells. In adult teleost fish, sharks and lampreys these cells have been identified in various other organs (Seternes, Sorensen, & Smedsrod, 2002). By directly comparing BLECs to SECs in the cardinal vein, and by testing numerous different substrate classes such as proteins, liposomes, lipoproteins, polysaccharides and glycosaminoglycans (Figure 1), we were unable to find any difference in the substrate specificity of caudal vein SECs and BLECs in embryos at 5dpf. We therefore conclude that BLECs function as bona fide scavenger endothelial cells. To the best of our knowledge, this is the first demonstration of SECs associated with the brain in any vertebrate.

By analyzing the spectrum of possible scavenger receptor molecules, we assessed how similar BLECs are to cardinal vein SECs on a molecular level. We have focused on two genes that are highly expressed in BLECs (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017) and SECs (Campbell et al., 2018): *mrc1a* and *stab2*. We found that Mrc1a mediates the uptake of pHr-Avidin in BLECs. Importantly, Avidin, which is isolated from hen egg white, contains abundant high-mannose glycans (Bruch &

White, 1982; DeLange, 1970; Fiete, Beranek, & Baenziger, 1997; Green & Toms, 1970). Glycoproteins containing these glycans are known ligands for Mrc1a and such substances are rapidly cleared from the blood circulation via liver sinusoidal endothelial cells in mammals (Hubbard, Wilson, Ashwell, & Stukenbrok, 1979). In contrast, Transferrin normally does not contain abundant high-mannose glycans, providing a possible explanation for the selective requirement for Mrc1a in Avidin clearance. Surprisingly, Mrc1a does not mediate the uptake of Avidin in the SECs of the cardinal vein, indicating a difference in receptor preference within the two cellular compartments. Stab2 on the other hand is not required for the uptake of substrates in BLECs, even though Stab2 is the main receptor in liver sinusoidal endothelial cells for binding of hyaluronic acid in mice (Adachi & Tsujimoto, 2002; Schledzewski et al., 2011) and in the SECs of the zebrafish cardinal vein (Campbell et al., 2018). This indicates that additional receptors are involved in the endocytosis of different macromolecules. Since Stab1 has been considered a potential endocytosis receptor (Hansen et al., 2005), which is able to mediate the uptake of acetylated low-density protein and advanced glycation end products (Adachi & Tsujimoto, 2002), it is very likely that it plays at least a redundant role in macromolecular internalization of BLECs. Other potential receptors which would be interesting to consider are Lyve-1 and Cd44, which constitute hyaluronic acid receptors and are important for dendritic cell trafficking in mammals (Johnson et al., 2017). Since *lyve-1* is also expressed in BLECs (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017) it might also mediate endocytosis of hyaluronic acid in the BLECs and might play an essential role for macromolecule internalization. Future studies therefore need to investigate additional scavenger receptors, and double or even triple mutants might have to be employed in order to shed more light onto the molecular machinery involved in the endocytosis of proteins, liposomes, lipoproteins, polysaccharides and glycosaminoglycans.

Microglia are thought to represent the major cleaning system of the brain, quickly responding to tissue damage and infection by engulfing and degrading infiltrating pathogens (Marquez-Ropero et al., 2020; Platt et al., 1998). We therefore studied the functional capacity of BLECs and microglia in direct comparison. Apart from their cellular morphology and the expression of the mannose receptor, BLECs and microglia have little in common. First, microglia mediate waste removal mainly via phagocytosis (Barron, 1995; Kettenmann, 2007) whereas BLECs take up their cargos via receptor-mediated endocytosis (van Lessen et al., 2017). Second, BLECs are venous derived cells and sprout from the choroidal vascular plexus in the head (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017), whereas microglia stem from a hematopoietic precursor population (Goldmann et al., 2016). Third, BLECs and microglia are very different in motility. BLECs form a network of stationary cells and have not been observed to move towards exogenously delivered substrates, whereas microglia are distributed throughout the brain and use their long cellular extensions to remove dying neurons from the extracellular space (Mazaheri et al., 2014). The latter would intuitively suggest that microglia might be more efficient in clearing the brain from macromolecules, particularly since they reside in the brain parenchyma while BLECs are not in direct contact with neural tissue. We show, however, microglia to be less efficient in the uptake of pHr-Avidin, Amyloid-ß, pHr-Dextran, Transferrin, IgG-Alexa647 and fluorescently labeled virus particles than BLECs. This is a surprising finding and possibly challenges the long-thought paradigm of microglia being the main cell type responsible for waste removal in the brain. Apparently, BLECs play a dominant role in the clearance of macromolecules from the brain. Whether this is a particular feature of teleost embryos, and whether BLECs also serve this role during adult phases, remains to be seen. Importantly, the presence of a similar cell population in the murine leptominiges was recently reported. Analogous to BLECs in fish, these cells (termed leptomininges lymphatic endothelial cells) express lymphatic markers such as LYVE1, PROX1 and VEGFR3 together with MRC1 and show the capacity to internalize macromolecules from the CSF (Shibata-Germanos et al., 2020). It is therefore tempting to assume that the here described scavenger activity of BLECs associated with the zebrafish brain is evolutionary conserved (recently reviewed in (Suarez & Schulte-Merker, 2021)). More work is required to address the question whether the activity of BLECs and similar cells in the mammalian brain also declines with age, comparable to what has been reported for meningeal lymphatic vessels (Ahn et al., 2019; Da Mesquita et al., 2018), as this would have a major impact on the etiology of different neuropathologies that manifest themselves due to protein aggregation.

Our side by side comparison between microglia and BLECs concerning their capacity to ingest bacteria demonstrates that microglia readily take up whole *E. coli* while BLECs fail to do so, implying that the uptake of large substrates like bacteria or whole cells via phagocytosis is a specific feature of microglia, whereas BLECs are more efficient in the endocytosis of proteins (IgG-Alexa647, Amyloid-ß, Transferrin, Avidin), polysaccharides (Dextran) and other smaller substrates like the HP virus particles with a diameter of 55 nm. This leads to the assumption that microglia and BLECs execute a "division of labor", possibly accomplished by different substrate preferences and/or by their differential capacity for phagocytosis or clathrin-mediated endocytosis of substrate molecules. Theoretical and experimental evidence suggests that particles up to 100 nm in size are most efficiently taken up via clathrin-mediated endocytosis, while a particle size of about 3 μm is optimal for phagocytosis by cells of the immune system (Baranov, Kumar, Sacanna, Thutupalli, & van den Bogaart, 2020). Hence, it is plausible to assume that BLECs might be specialized in receptor-mediated endocytosis of soluble macromolecules and smaller particles from the brain parenchyma and CSF, while microglia as "professional phagocytes" of the brain are much more efficient when it comes to uptake of larger particles and pathogens like bacteria. Besides the actual size of the cargo, also differential expression of cell surface receptors on microglia compared to BLECs might enable physiologically distinct roles. Pattern recognition receptors such as TLR2, TLR4, and TLR9 which bind bacterial lipopeptides, lipopolysaccharides, and bacterial CpGDNA have been shown to be expressed by mouse microglia (Olson & Miller, 2004) but might actually not be expressed in BLECs, which could explain why injection of live *E. coli* into the optic tectum or the CSF resulted in an uptake of

fluorescently labeled bacteria in microglia but not in BLECs (Figure 5). It would therefore be interesting to compare the gene expression profile of BLECs and microglia with regard to genes related to phagocytosis and receptor-mediated endocytosis.

We conclude that a clear task division in the zebrafish brain between BLECs and microglia is accomplished and that BLECs and microglia work side by side to maintain the removal of extracellular components from the brain. Interestingly, within the mammalian liver, there is a similar division of labor between liver sinusoidal endothelial cells and the liver resident macrophages (Kupffer cells), which together form the reticuloendothelial system within this organ, clearing the blood plasma from endogenous and exogenous waste (Kmiec, 2001; Smedsrod et al., 1990). We suggest that BLECs and microglia possibly function in a similar way in the brain and collaborate with each other, since both cell types have high affinities for a wide range of substrates.

In conclusion, our results provided here add further insight into how endothelial cells contribute to the maintenance of brain homeostasis. Next to microglia, BLECs play a critical role in the cleaning system of the brain parenchyma and are the first described SEC population within the brain meningeal layer in vertebrates that cooperates with microglia to remove particle waste from the brain. Whether a functionally related cell type exists in mammalian systems, or whether this cell-type is zebrafish specific needs to be addressed in future studies.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

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