Stem Cell Reports
Report
ISSCR
OPEN ACCESS

PAX6-positive microglia evolve locally in hiPSC-derived ocular organoids

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https://doi.org/10.1016/j.stemcr.2021.12.009

SUMMARY

Microglia are the resident immune cells of the central nervous system (CNS). They govern the immunogenicity of the retina, which is considered to be part of the CNS; however, it is not known how microglia develop in the eye. Here, we studied human-induced pluripotent stem cells (hiPSCs) that had been expanded into a self-formed ectodermal autonomous multi-zone (SEAM) of cells that partially mimics human eye development. Our results indicated that microglia-like cells, which have characteristics of yolk-sac-like linage cells, naturally develop in 2D eye-like SEAM organoids, which lack any vascular components. These cells are unique in that they are paired box protein 6 (PAX6)-positive, yet they possess some characteristics of mesoderm. Collectively, the data support the notion of the existence of an isolated, locally developing immune system in the eye, which is independent of the body's vasculature and general immune system.

INTRODUCTION

The central nervous system (CNS) is inaccessible to immune cells in general circulation owing to the presence of the blood-brain barrier. Instead, microglial cells in the brain and spinal cord provide the CNS with its immune protection, acting against inflammation, degeneration, tumor growth, injury, trauma, and ischemic brain injury following cerebral infarction (Li and Barres, 2018; Tremblay et al., 2011). Microglia deliver immune protection for the posterior eye, including the neural retina, and have an involvement with some sight-threatening conditions, such as age-related macular degeneration, uveitis, glaucoma, and retinal degeneration (Rashid et al., 2019). Despite the key role of microglia in facilitating our immune defenses, their developmental origin has attracted controversy, and the question remains unresolved as to whether they emerge from the ectoderm or the mesoderm (Alekseeva et al., 2019; Ginhoux and Prinz, 2015). It is also uncertain whether or not microglia can form in isolation from the vasculature in tissues such as the eye.

Experiments on mouse embryos have indicated that adult microglia derive from primitive macrophage precursors that originate in the yolk sac before embryonic day 8, which thereafter proliferate (Ginhoux et al., 2010). A recent discovery by Muffat and associates has shown that microglia-like cells can be generated from human-induced pluripotent stem cells (hiPSCs) via myeloid differentiation (Muffat et al., 2016). Here, we show that PAX6-positive microglia-like cells, which we hereafter refer to as PAX6-positive microglia (PPM) cells, develop in hiPSC-derived eye-like self-formed ectodermal autonomous multi-zone (SEAM) organoids (Hayashi et al., 2016). This points to the existence of a local immune system in the eye that is isolated from the general immune system. Our novel findings did not investigate the development of microglia precisely since the SEAM is a 2D model and not a 3D model. However, given that the SEAM lacks vascular components, our data suggest that human microglia may develop in the absence of a blood supply.

RESULTS

Microglia-like cells evolve in hiPSC-derived SEAMs

Typical four-zoned eye-like SEAM organoids (Figure 1A) were generated from 201B7 hiPSCs and subjected to immunostaining for markers of immune cells. This revealed that retinal and retinal pigment epithelium cells in SEAM 2, along with ocular surface cells in SEAM 3, were positive for *CD11b*, transmembrane protein 119 (TMEM119), and CX3C chemokine receptor 1 (CX3CR1) (Figures 1B–1E; Videos S1a and S1b). Previous reports have disclosed that CNS macrophages and microglia express CD11b and CX3CR1 and that microglia are positive for TMEM119 (Bennett et al., 2016; Ford et al., 1995; Martin et al., 2017). This supports the contention

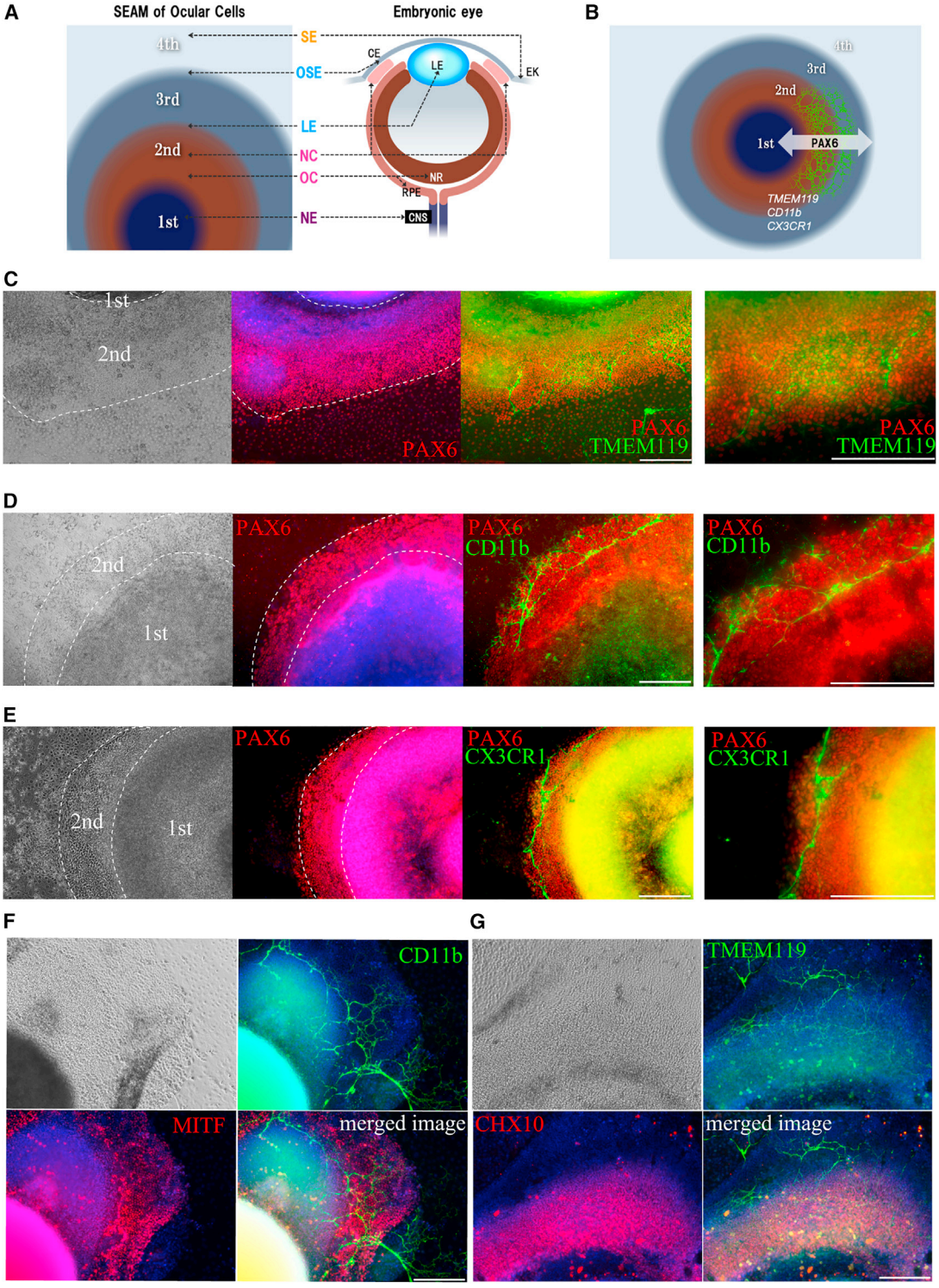

Figure 1. Spontaneously generated PPM cells in a 2D SEAM of human eye development

(A) A self-formed ectodermal autonomous multi-zone (SEAM) derived from hiPSCs contains cells of ectodermal lineage that mimic anterior and posterior eye development *in vivo*. CNS, central nervous system; NE, neuroectoderm; OC, optic cup; NR, neuroretina; NC, neural crest; LE; lens; OSE, ocular surface ectoderm; SE, surface ectoderm; CE, corneal epithelium; EK, epidermal keratinocyte.

(B) Schematic showing the expression pattern of microglia-related genes in the SEAM.

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that microglia-like cells evolve in hiPSC-derived eye-like SEAMs. Perivascular macrophages and the microglia marker CD68 were not detected in the SEAM (Figure S1A) (Fiala et al., 2002) nor were the Muller and astrocyte markers glial fibrillary acidic protein (GFAP), RLBP1, and CHX10 (Figures S1B–S1D) at 4 weeks (Suga et al., 2014). CD11b- and TMEM119-positive cells were not detected at day 8 of SEAM development (Figures S1E and S1F) but became evident from day 10 onwards (Figures S1G and S1H), forming a circumferential network at the border of SEAMs 2 and 3. By day 14, circumferential and radial patternings of cells that stained positive for these microglial markers were apparent (Figures S1I and S1J) and persisted when the cultivation media was switched to one that supported SEAM differentiation into retina-like tissue (Figures 1F, 1G, and S1K). Similar results were found when another hiPSC line (D2) was studied and when human embryonic stem cells (KhES-1) were investigated (Figures 2A–2D). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) of the SEAM at days 14, 21, and 28 revealed the expression of a number of immune cells markers (Figures 2E and S2A). Together, the immunolabelling and qRT-PCR findings indicated that microglial-like cells emerge in SEAMs. qRT-PCR of the SEAM at day 7 of cultivation, used to probe early development, confirmed the expression of *PAX6*, an early developmental marker of the neuroectoderm and ocular cells (Figure 3A) (Chow et al., 1999; Zhang et al., 2010). Endoderm markers *GATA4* and *SOX17*, mesoderm markers *NKX2-5*, *SNAL1*, and *TBX3*, meso-endoderm marker *T*, and *PU.1* (an essential factor required for the development of the yolk sac and myeloid system [Kierdorf et al., 2013]) were not detected (Figure 3A). It was also found that almost all cells were stained PAX6 or p63, a surface ectodermal marker (Yoh and Prywes, 2015), at the 4-week culture point just before cell sorting (Figures S2B–S2F).

Gene expression, cytokine stimulation, and phagocytosis

To further interrogate PPM cells, they were isolated from 28-day-expanded SEAMs using fluorescence-activated cell sorting (FACS) according to the expression of a combination of CD11b and CD45. CD11b-positive cells include microglia and CNS macrophages, with CD45 labeling used to distinguished PPM cells from macrophages based on the high levels of CD45 expression in macrophages compared with microglia (Ford et al., 1995). This analysis revealed that 0.33% ± 0.12% of cells (n = 8) were CD11b-positive PPM cells (Figure 3B). After PPM cells were cultured for just 24 h for cell recovery, to probe cell function, these were stimulated alongside immortalized human microglia cells (abm, Richmond, QC, Canada) with lipopolysaccharide (LPS), interferon gamma (IFN-γ), and transforming growth factors β1 and β2 (TGF-β1 and -β2) (Figure S3A). RNA sequencing revealed that the expression levels of several genes associated with primary microglia were higher in PPM cells than in immortalized human microglial cells (Figure 3C) (Ayata et al., 2018; Galatro et al., 2017; Gautier et al., 2012; Gosselin et al., 2017; Hickman et al., 2013). *PAX6* expression levels, along with those of microglial cell immune genes, were elevated in PPM cells (Figures 3D and 3E). The expression levels of microglia-associated genes in PPM cells also fluctuated with the stimulation conditions, which has potential functional relevance. Cytokine levels in the culture medium after the stimulation of PPM cells revealed the expression of interleukin (IL)-6 and IL-8, but not IL-1β, IL-4, IL-10, or tumor necrosis factor (TNF)-α (Figures 3F and S3B). IL-8 expression was significantly higher in the immortalized human microglia group than in the PPM group ($p < 0.0001$), whereas the opposite was true for IL-6, with higher expression levels in PPM cells ($p < 0.0001$). IL-6 levels were significantly higher in LPS- and IFN-γ-stimulated conditions for both PPM and immortalized microglial groups when compared with non-stimulated and LPS-only stimulated conditions ($p = 0.003$, $p = 0.02$). The amount of IL-8 secreted from PPM cells was less than that from immortalized human microglia cells. Expression levels of vascular epithelial growth factor A (*VEGFA*), which increases when microglia are stimulated (Ding et al., 2018), did not change following cytokine stimulation in immortalized human microglial cells but did increase when the PPM cells were stimulated. *VEGFA* expression in PPM cells was significantly higher after TGF-β1 stimulation compared with no cytokine stimulation ($p = 0.04$). *TGF-β2* expression, moreover, was significantly higher in PPM cells than in immortalized human microglia cells ($p < 0.0001$) (Figure 3G). TGF-β2 secretion of the TGF-β2 stimulated PPM cells was significantly higher than that found for combined LPS- and IFN-γ stimulation ($p = 0.0062$). A phagocytosis test of PPM cells revealed that the PPM cells did not phagocytose the microspheres (Figure S3C).

(C–E) Immunostaining of PAX6 (red) in zones 1–3 of a 4-week, cultivated 201B7 hiPSC-derived SEAM along with staining of nuclei (blue) and TMEM119 (C, green), CD11b (D, green), and CX3CR1 (E, green).

(F and G) Immunostaining after 7 weeks of differentiation for retinal culture: (F) CD11b (green) and MITF retinal pigment epithelium cells (red) and (G) TMEM119 (green) and CHX10 neuro-retinal cells (red). Images are representative of three independent experiments. Scale bar, 200 μm.

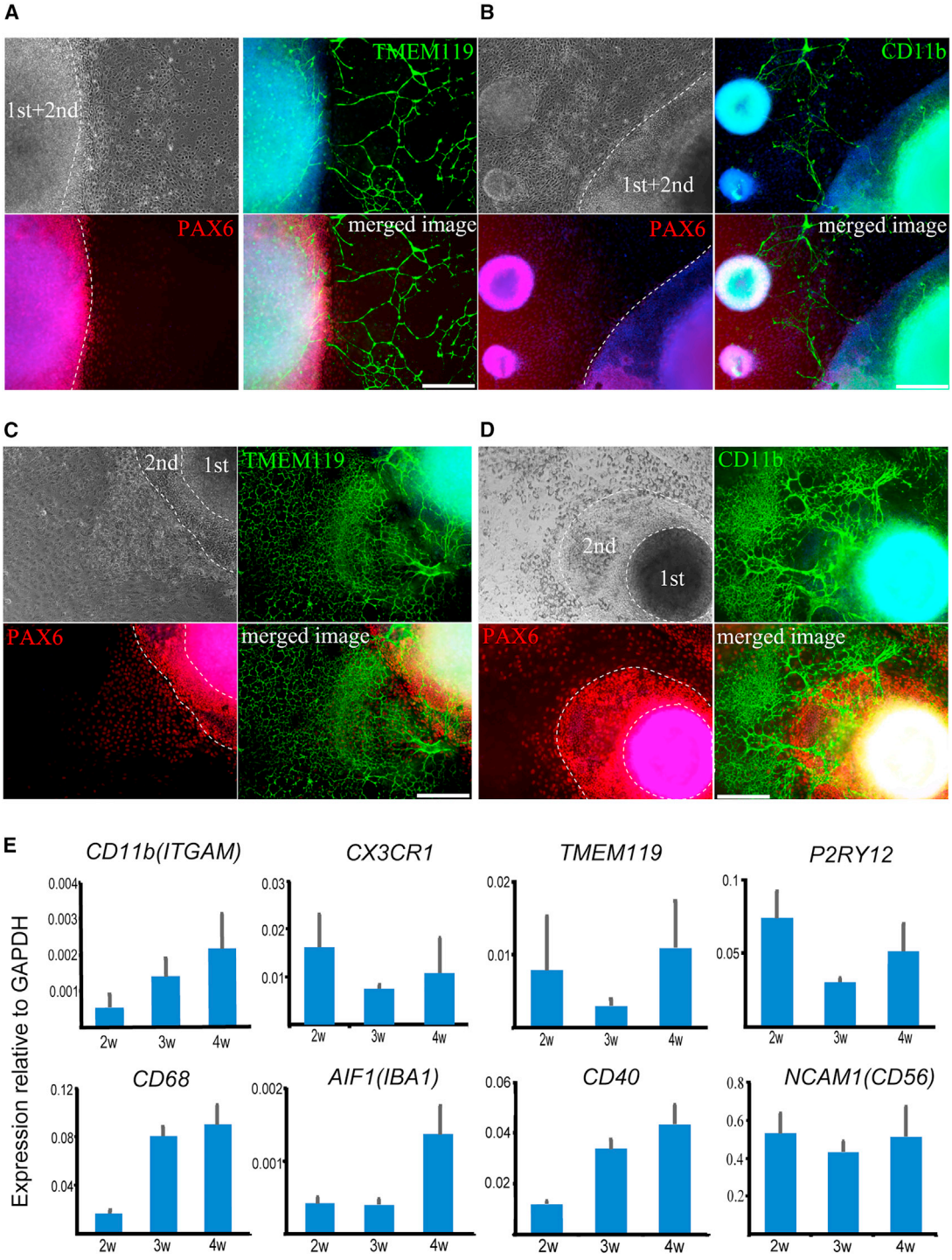

Figure 2. Immunostaining of differently derived SEAMs and expression of immune cell markers

(A and B) hES KhES-1-derived SEAMs after 4 weeks of culture immunostained with (A) TMEM119 (green) and PAX6 (red) and (B) CD11b (green) and PAX6 (red).

(C and D) 1383D2 hiPSC-derived SEAMs after 4 weeks of culture immunostained with (C) TMEM119 (green) and PAX6 (red) and (D) CD11b (green) and PAX6 (red). Images are representative of multiple independent experiments. Nuclei are in blue. Scale bar, 200 μm.

(E) Gene expression analysis of immune-related markers in SEAMs after 4 weeks of culture (six independent experiments). Error bars show standard deviation (SD).

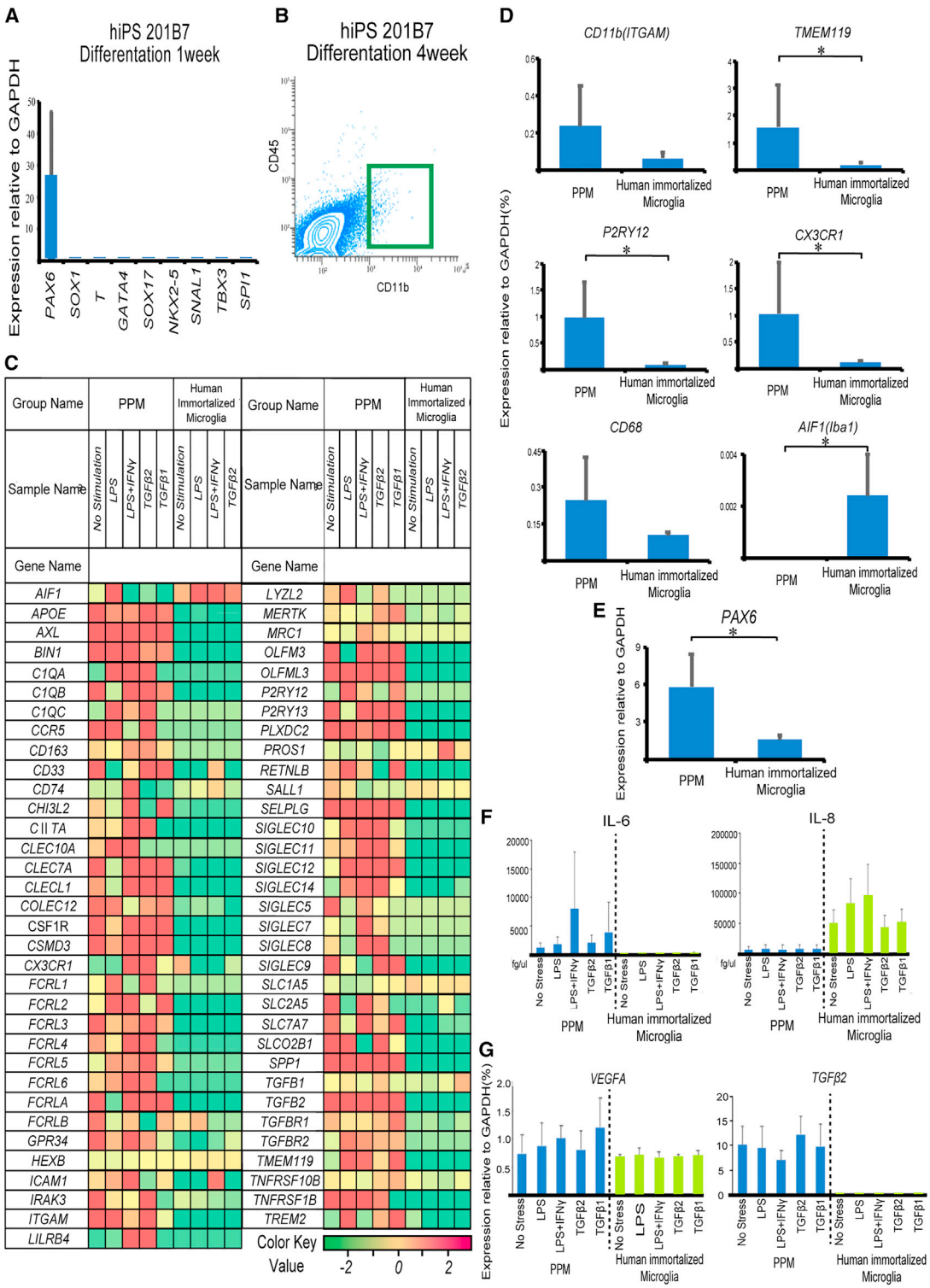

Figure 3. Developmental and immune cell markers in a SEAM, and isolation and analysis of PPM cells

(A) Gene expression analysis of development-related markers in a SEAM after 1 week of culture (six independent experiments).

(B) Flow cytometric analysis of CD11b and CD45 in differentiated hiPSCs after 27–28 days of culture, with PPM cells indicated (box). Data are representative of seven independent cell-sorting experiments.

(C) RNA-seq data of PPM cells and human immortalized microglia. Both cell types were stimulated for 24 h.

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Single-cell RNA sequencing

Despite the fact that there was some heterogeneity in the data, which was likely caused by cellular immaturity or limitations of sorting, single-cell RNA sequencing showed that, overall, PPM cells are more similar to yolk-sac-derived, myeloid-based progenitors (YSMP) cells than other human embryonic hematopoietic cells, such as microglia and macrophages (Figures 4A, 4B, S4A, and S4B; Table S1) (Bian et al., 2020). Zhilei Bian et al. reported human tissue-resident macrophage, microglia, YSMPs, and other immune cells from a human embryo at Carnegie stages 11–23. We compared our single-cell RNA sequencing data of PPM cells with the human embryo immune cell data they reported, as detailed in the Supplemental experimental procedures. They defined a yolk-sac-derived progenitor population, which appeared in the yolk sac at CS11, showed much weaker transcriptomic erythroid features than mouse erythromyeloid progenitors, and had a myeloid-biased nature, as YSMP. In particular, PPM cells expressed the PAX6 and YSMP markers (Figures 4C–4E). The PPM cells were divided into several clusters, which was thought to be due to immaturity of the cells. Cumulatively, the data point to a unique type of immune cell, referred to here as a PPM cell, that evolves in the eye (and in the retina especially) locally and in isolation from the general immune system.

DISCUSSION

In this study, we provided the possibility that ocular microglia-like cells emerge naturally and locally in the eye and are PAX6-positive. The origin of ocular microglia, the predominant innate immune cell system in the sensory retina as part of the CNS, has been the subject of considerable debate for decades. The conventional theory holds that microglia develop in the yolk sac and travel to the brain (possibly including the retina, a part of the CNS) through the bloodstream during early development before the closure of the blood-brain barrier (Alliot et al., 1999). Arguing against this notion, however, is the fact that microglia are present in the avascular retina of birds (Navascues et al., 1994) and that they appear in human and mouse retinas prior to vascularization (Diaz-Araya et al., 1995; Santos et al., 2008). Here, we used a previously described technology (Hayashi et al., 2016), in which hiPSCs form 2D SEAM eye-like organoids, and show that SEAMs include PPM cells, which emerge spontaneously under simple and natural differentiation conditions in the absence of any factors that mimic blood circulation. Thus, PPM cells that develop in hiPSC-derived SEAM organoids do so in the absence of a vasculature.

Stimulation of PPM cells by LPS did not elicit any cytokine response, and in this regard, they are unlike normal microglia (Lund et al., 2006). LPS stimulation increases IL-6 expression in microglia generated from both primary mouse microglia and from hiPSCs via the mesoderm, leading us to conclude that PPM cells are distinct from bone-marrow-derived or mesoderm-derived microglia. The present investigation also reveals that PPM cells and immortalized human microglia cells differ in their responses to inflammation stimulation. The eye is said to have partial immune privilege, and the introduction of LPS into the vitreous cavity does not induce ocular inflammation (Stein-Streilein, 2013). This is consistent with the finding that PPM cells, as the sole immune gatekeepers of the eye's posterior segment, do not respond to LPS stimuli.

Direct regulation of autoreactive regulatory T cells is the major mechanism by which TGF-β maintains tolerance (Gorelik and Flavell, 2000). TGF-β2 is the chief immune-modulating factor in the aqueous humor of the eye (a fluid similar to plasma) and acts to generate tolerogenic antigen-presenting cells. Bone marrow cells, on the other hand, can be activated to produce TGF-β1 (Cousins et al., 1991; Stein-Streilein, 2013). TGF-β2 plays a pivotal role in the immune system in maintaining tolerance, primarily by regulating lymphocyte proliferation, differentiation, and survival (Stein-Streilein, 2013). *TGFB2* expression in PPM cells is elevated after TGF-β2 exposure, whereas IL-6, IL-8, and *VEGFA* levels remain unchanged. Exposure to TGF-β1, which is constantly maintained at low levels in the eye, stimulated inflammation and upregulated the expression level of *VEGFA* in PPM cells. Similarly, exposure to IFN-γ (which is present at low levels in the eye) resulted in inflammatory stimulation, with a high expression of IL-6 and a low expression of TGF-β2 and IL-8. This suggests that IFN-γ-stimulated microglia may induce $Foxp3^{+}$ $CD8^{+}$ regulatory T cells in ocular tissue (Nakagawa et al., 2010). Based on these results, we conclude that PPM cells may perform similar functions as M2-type-activated microglia, and that this may impact upon immune tolerance and suppression in ocular tissue. Similar tendencies were observed in immortalized human microglia, although with lower expression levels of *TGF-β2* than

(D and E) Gene expression analysis of (D) microglia-related markers and (E) *PAX6* in PPM cells and human immortalized microglia cells (data from four independent experiments). *$p < 0.05$ by the Mann-Whitney rank-sum test.
(F) IL-6 and IL-8 levels after stimulation of PPM cells in 10–16 independent experiments.
(G) Gene expression analysis of *VEGFA* and *TGFB2* after stimulating PPM cells (data from seven to ten independent experiments). Error bars show SD.

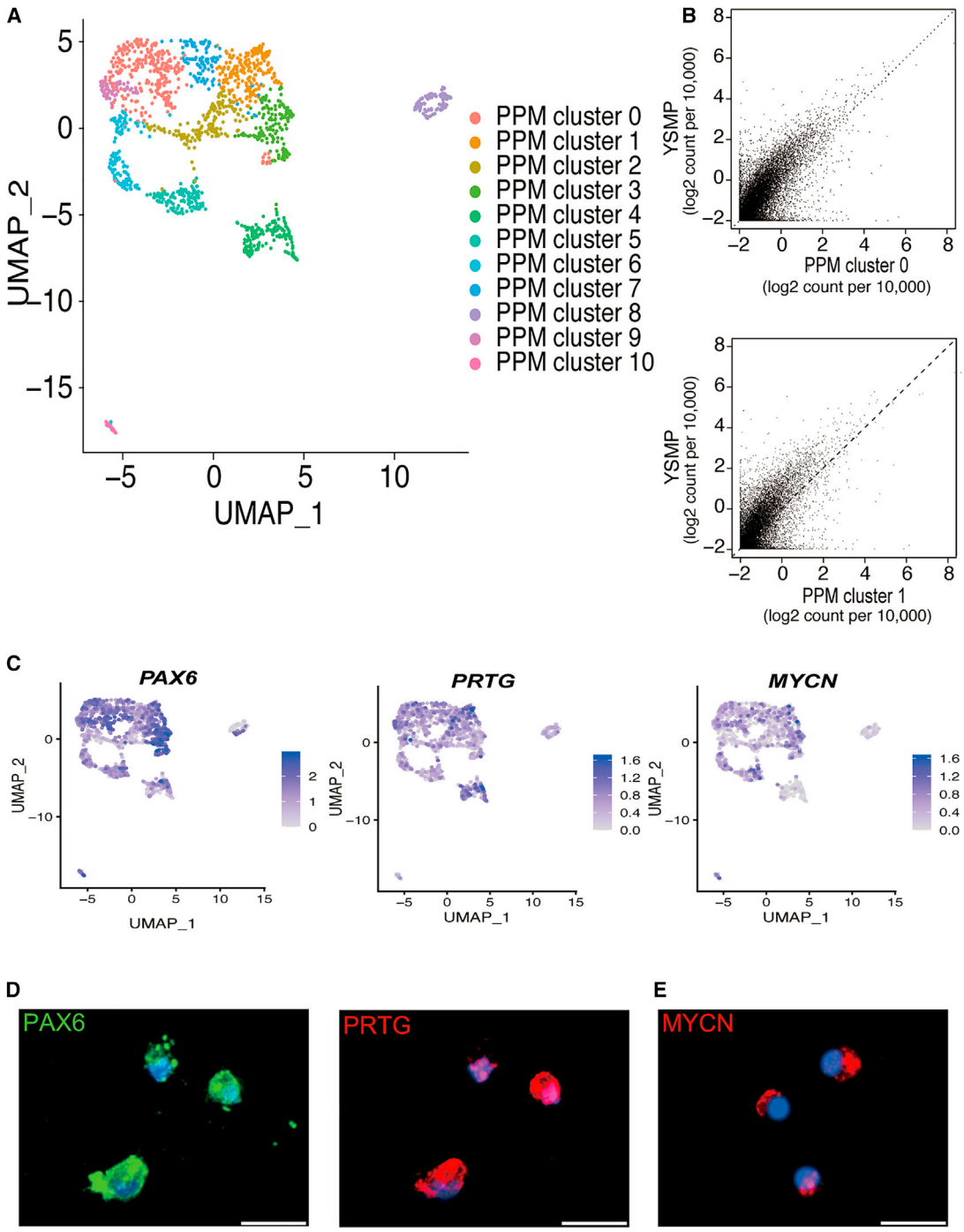

Figure 4. Single-cell RNA-seq analysis of CD11b + cells in a SEAM

(A) Uniform manifold approximation and projection (UMAP) plot visualizing 1,498 CD11b + cells and colored based on 11 cluster types.

(B) Scatterplot showing the gene expression of PPM cluster_0 and 1 identified in CD11b + scRNA-seq and of a YSMP cluster in scRNA-seq of embryonic macrophages.

(C) Feature plots of *PAX6*, *PTRG*, and *MYCN* expression. The expression levels of each cell type are colored in the UMAP plot.

(D and E) Immunostaining of (D) PAX6 (green) and PRTG (red) and (E) MYCN (red). CD11b + cells were sorted by FACS and cytospin after 4 weeks of culture. Images represent three independent experiments. Nuclei are in blue. Scale bar, 25 μm.

are found in PPM cells. PPM cells are limited in phagocytosis tests and some functions, which could be attributed to immaturity or other causes. Further attempts at maturation and examination of their functions may contribute to a better understanding of the immune system of the eye. Additionally, this model is a 2D model, and a 3D model of the entire eye (including cornea, conjunctiva, neural retina, retinal pigment epithelium [RPE], lens, etc.) is yet to be created; however, a 3D model of the neural retina (Eiraku et al., 2011; Zhong et al., 2014) has been created. The development of a 3D model of human ocular development will enable us to study the immune system more thoroughly.

A gene expression analysis revealed that PPM cells have unique properties. For example, they express microglia-specific genes, such as *CD11b*, *TMEM119*, and *CX3CR1*. Single-cell gene expression investigations further point to similarities to YSMP cells, with the microglial population positive for PAX6 (this was supported by immunostaining of isolated microglial cells with cytospin). *PAX6* is considered to be an ocular master control gene (Chow et al., 1999; Halder et al., 1995) and is necessary for eye development in flies to humans (Hill et al., 1991; Nakayama et al., 2015). It is required for the differentiation of most retinal lineages, and when PAX6 is inactivated in retinal progenitor cells, they do not form retinal cells (Marquardt et al., 2001). PAX6 is also highly expressed in the anterior segment structures of the eye that are derived from surface ectoderm, such as the lens vesicle and corneal and conjunctival epithelia (Shaham et al., 2012; Terzic and Saraga-Babic, 1999). *PAX6*, moreover, is necessary and sufficient for the differentiation of human embryonic stem cells (ESCs) to neuroectoderm, and while it is dispensable for mouse ESC development, it is a generic neuroectodermal specification factor in primates (Zhang et al., 2010). Thus, PPM cells that emerge in hiPSC-derived SEAMs likely have a neuroectodermal origin but also possess some features of mesodermal cells. This suggests that some retinal microglia are neuroectodermally derived cells, which retain some properties of the mesoderm. This notion is complementary to, rather than contrary to, previous reports, which show that some retinal microglia are likely to be neuroectodermally derived cells with mesodermal properties (Ginhoux et al., 2010) and that microglia that repopulate the adult mouse brain after experimental depletion do so via the proliferation of cells that are positive for an ectodermal neural stem cell marker (Elmore et al., 2014). The findings significantly enhance our understanding of the origin of microglia that regulate the immune system of the CNS, especially the retina, and raise the possibility of locally developing immune systems involving cells such as resident macrophages in the brain or other organs.

EXPERIMENTAL PROCEDURES

Detailed methods are provided in the Supplemental experimental procedures.

Flow cytometry and cell sorting

SEAMs cultured in DM for 4 weeks were dissociated using Accumax (Life Technologies) for 10 min at 37°C and resuspended in an ice-cold cell-staining buffer (420201, BioLegend, San Diego, CA, USA), after which the harvested cells were filtered using a cell strainer. They were stained with a CD11b-fluorescein isothiocyanate (FITC) antibody (101206, BioLegend); FITC rat IgG2b (400605, BioLegend); phycoerythrin (PE)/cyanine7 anti-human CD45 (304016, BioLegend); PE/Cy7 mouse IgG1 (400126, BioLegend); an APC anti-human CD45 antibody (304,037, BioLegend); and APC mouse IgG1 (400120, BioLegend) for 30 min on ice (Ford et al., 1995; Sedgwick et al., 1991). After washing three times with PBS, the stained cells were sorted using a FACSAria II instrument (BD Biosciences), and the data were analyzed using BD FACSDiva software (BD Biosciences). CD11b-positive cells, which did not express high levels of CD45, were harvested. After sorting, some of the cells were used for cytospin, while the remaining cells (now with the identity of PPM cells) were seeded on poly-D-lysine-coated (1.3 g/cm^2) and LN511E8-coated (0.5 μg/cm^2) dishes at the density of 1.6–2.4 × 10^5 cells/cm^2 and cultured in microglial culture medium (MCM; DMEM/F12 [1:1] supplemented with 10% FBS [Japan Bio Serum, Hiroshima, Japan], 1% GultaMAX [Thermo Fisher Scientific], and 1% penicillin-streptomycin solution). The sorted PPM cells were adjusted to a density of 2 × 10^5 cells/mL, after which 200 μL of the cell suspension was centrifuged at 1,000 RPM for 5 min in a Cytospin 4 Cytocentrifuge (Thermo Fisher Scientific).

Cell stimulation

To examine the effect of inflammatory agents, PPM cells and immortalized microglia cells were cultured for 24 h and further stimulated for 24 h with LPS (1 μg/mL) (Sigma-Aldrich), LPS (1 μg/mL) and IFN-γ (200 ng/mL) (Wako), TGF-β2 (5 ng/mL) (R&D Systems, Minneapolis, MN, USA), or TGF-β1 (5 ng/mL) (Wako) added to the culture media. The medium was then collected in protein low-adhesion tubes, and the cells were stored in QIAzol reagent (Qiagen, Valencia, CA, USA).

Resource availability

Details are provided in the Supplemental resource availability.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2021.12.009.

AUTHOR CONTRIBUTIONS

N.S. and K.M. designed the study; N.S. designed and performed the experiments and analyzed the data; T.K. performed hESC differentiation and analyzed the data; T.T., S.S., A.O., and Y.M. assisted in analyzing single-cell RNA sequencing (RNA-seq) data; N.S., K.M., A.J.Q., and K.N. analyzed the data and wrote the manuscript;

R.H., M.T., and K.N. provided conceptual advice regarding the study design.

CONFLICT OF INTERESTS

The authors declare no competing interests.

ACKNOWLEDGMENTS

We thank Y. Ishikawa, Y. Kobayashi, S. Shibata, S. Inoue, Y. Yasukawa, and M. Morita of Osaka University for technical assistance. We acknowledge the NGS core facility of the Genome Information Research Center at the Research Institute for Microbial Diseases of Osaka University for assistance in RNA sequencing and data analysis. This research was supported by AMED under grant number 20gm1210004, by JSPS KAKENHI under grant number JP17K11475, and by the Integrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives, Osaka University.

Received: September 15, 2021
Revised: December 13, 2021
Accepted: December 14, 2021
Published: January 13, 2022

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