Adult Human Peripheral Blood Mononuclear Cells Are Capable of Producing Neurocyte or Photoreceptor-Like Cells That Survive in Mouse Eyes After Preinduction With Neonatal Retina

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Key Words. Peripheral blood mononuclear cell • Adult stem cell • Pluripotency • Subretinal transplantation • Retinal degeneration

ABSTRACT

Adult human peripheral blood mononuclear cells (hPBMCs) exhibit pluripotency in vitro and so may be a valuable cell source for regenerative therapies. The efficacy of such therapies depends on the survival, differentiation, migration, and integration capacity of hPBMCs in specific tissues. In this study, we examined these capacities of transplanted hPBMCs in mouse retina as well functional improvement after transplant. We isolated hPBMCs and preinduced them for 4 days in media preconditioned with postnatal day 1 rat retina explants. Preinduction increased the proportions of hPBMCs expressing neural stem cell, neural progenitor cell, or photoreceptor markers as revealed by immunofluorescent staining, flow cytometry, and quantitative real-time polymerase chain reaction. Preinduced hPBMCs were transplanted into the subretinal space of retinal degenerative slow (RDS) and retinal degeneration 1 (RD1) mice. At 1, 3, and 6 months after transplantation, treated eyes of RDS mice were collected and cell phenotype was studied by immunofluorescent staining, flow cytometry, and quantitative real-time polymerase chain reaction. Preinduced hPBMCs survived in the subretinal space; migrated away from the injection site and into multiple retinal layers; and expressed neural stem cell, neuronal, and photoreceptor markers. Finally, we assessed RD1 retinal function after subretinal transplantation and found significant improvement at 3 months after transplantation. The ease of harvesting, viability in vivo, capacity to express neuronal and photoreceptor proteins, and capacity for functional enhancement suggest that hPBMCs are potential candidates for cell replacement therapy to treat retinal degenerative diseases.

SIGNIFICANCE

This study provides support for the use of peripheral blood mononuclear cells (PBMCs) as a potential source of pluripotent stem cells for treating retinal degeneration. First, this study demonstrated that PBMCs can differentiate into retinal neuron-like cells in vitro and in vivo. Second, some transplanted cells expressed markers for neural progenitors, mature neurons, or photoreceptors at 1, 3, and 6 months after subretinal injection. Finally, this study showed that PBMC transplantation can improve the function of a degenerated retina.

INTRODUCTION

Neurodegenerative diseases are largely untreatable conditions affecting millions of people worldwide. Protection or replacement of host neurons by cell-base therapy may be a novel way to treat these disorders [1–3]. Recently, it was reported that peripheral blood stem cells (PBSCs) can differentiate into lineages other than typical blood cell lines under appropriate culture conditions, including cardiomyogenic, endothelial, neuroectodermal, and even keratinocyte-like cell lineages [4–6]. Peripheral blood stem cells are part of the mononuclear fraction (peripheral blood mononuclear cells, or PBMCs). These PBMCs are easier and safer to collect than other types of stem cells; furthermore, isolation of PBMCs involves few ethical concerns compared with embryonic stem cells. Peripheral blood can also be obtained regardless of patient age and autografted without rejection, suggesting that they may be an ideal source of pluripotent stem cells for cell-based regenerative therapy. It appears that PBMCs are composed of several distinct subpopulations; however, it is possible that this apparent heterogeneity results from examination of the same...
cell population at different stages of differentiation [7]. In some studies, cell stage was related to cell size (with stem cells usually smaller) [8].

The retina is part of the central nervous system (CNS) and contains a multitude of distinct cell types with well-defined functions and highly segregated anatomic localization [9, 10]. Although retina offers numerous advantages for transplantation of stem cells compared with other regions of the CNS, such as ease of access and an immune privileged microenvironment [11], numerous challenges remain to stem cell therapy in the adult retina, such as limited survival, physical barriers to migration, and possible lack of appropriate trophic factors. Alternatively, the retinal microenvironment may be favorable for the differentiation and integration of transplanted cells [12, 13] if they are first preinduced toward correct lineages pathways. Moreover, a pathological host retina may be more amenable to these processes due to compensatory mechanisms activated by damage or cell loss (e.g., trophic factor upregulation) [14, 15].

Preinduction by brief exposure to an embryonic or neonatal growth environment may improve survival and tissue-specific differentiation in vivo. To examine the viability and function of PBMCs after preinduction, we injected preinduced PBMCs into the subretinal space of retinal degenerative slow (RDS) and retinal degeneration 1 (RD1) mice. The RDS mouse, a well-established rodent model of retinitis pigmentosa, harbors a dominant spontaneous mutation in peripherin 2 (PRPH2), resulting in slow degeneration of rods and cones by 12 months of age [16]. The RD1 mouse, with a mutation in rod photoreceptor-specific cyclic guanosine monophosphate phosphodiesterase 6, is a more acute model of retinitis pigmentosa. The rods of RD1 mice start to degenerate at approximately postnatal day 10 and are almost completely absent by postnatal day 12 [17].

In the current study, we addressed several critical issues related to the feasibility of PBMC transplant therapy for retinal diseases, namely survival, migration, and functional reconstruction in vivo. First, we demonstrate that PBMCs can differentiate into retinal neuron-like cells in vitro and in vivo. Second, we report that some transplanted cells express markers for neural progenitors, mature neurons, or photoreceptors at 1, 3, and 6 months after subretinal injection. Finally, we show that PBMC transplantation can improve the function of degenerated retina. This study provides support for the use of PBMCs as a potential source of seed cells for treating retinal degeneration.

**Materials and Methods**

**Experimental Animals**

This study was approved by the Medical Ethics Committee of Zhongshan Ophthalmic Center, Sun Yet-sen University, Guangzhou, China (protocol no. [2008] 15). All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Ethics Committee (Animal Welfare Assurance no. 2011-015). Thirty 12-month-old RDS mice were randomly divided into three groups (n = 10), each subjected to subretinal injection with a different batch of preinduced human peripheral blood mononuclear cells (hPBMCs). Ten 12-month-old RDS mice were used as the control group and injected subretinally with serum-free Dulbecco’s modified Eagle’s medium (DMEM). Forty 12-month-old RD1 mice were used for electroretinography (ERG) and polymerase chain reaction (PCR) detection. These mice were randomly divided into four groups (n = 10), three of which received subretinal transplantation of preinduced hPBMCs and the other no treatment (blank control group). In addition, six C57 mice were used as positive controls for ERG studies.

**Isolation and Preinduction of hPBMCs**

Adult peripheral blood was obtained from three healthy individuals (ages 24, 30, and 50 years) who provided written informed consent. Peripheral blood was centrifuged through a Ficoll-Hyphaque (Haoyang Biotech, Tianjin, China, http://www.tbdsscience.com) density gradient at 2,000 rpm (approximately 246g) for 20 minutes and PBMCs collected from the interface between the plasma layer and the Ficoll-Hyphaque layer. Isolated hPBMCs were seeded at 4–5 × 10^6 cells per well in the bottom wells of six-well transwell plates (catalog no. 3450; Corning Inc., Corning, NY, http://www.corning.com) below inserts preseeded 24 hours earlier with retinal tissue from 1-day-old Sprague-Dawley rats. The retinal tissue was cultured in a neural stem cell medium composed of DMEM/F12 (90 ml/100 ml; Gibco, Grand Island, NY, http://www.invitrogen.com), human basic fibroblast growth factor (hFGF; 10^3 ng/100 ml; PeproTech, Rocky Hill, NJ, http://www.peprotech.com), human stem cell factor (hSCF; 200 ng/100 ml; PeproTech), human epidermal growth factor (hEGF; 10^3 ng/100 ml; PeproTech), B27 stem cell culture supplement (50×; 2 ml/100 ml; Gibco), L-glutamine (LG; 3%, 1 ml/100 ml; Whiga Technology, Guangzhou, China, http://whiga.biomart.cn), fetal bovine serum (FBS; 7 ml/100 ml; SuluiQing Biotech, Huzhou, China, http://www.hsjq.com.cn), and gentamicin (150 μg/100 ml; Tianxin Pharmaceuticals, Guangzhou, China, http://www.tianxin.com.cn) to produce a conditioned medium for hPBMC differentiation. The hPBMCs and retinal tissue were cocultured at 37°C under a 5% CO_2 atmosphere for 4 days.

**Immunofluorescent Staining and Flow Cytometry**

Samples from the same batch of freshly isolated hPBMCs and preinduced PBMCs were characterized by immunostaining with human-specific antibodies against markers for various lineages: neural stem cell (using a human nestin, 1:200; Millipore, Temecula, CA, http://www.emdmillipore.com), neural progenitor cell (vimentin, 1:300; Abcam, Cambridge, MA, http://www.abcam.com; bIII-tubulin, 1:100; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), mature neuron (microtubule-associated protein 2 [MAP2], 1:250; Abcam; synapsin, 1:250; Abcam), Müller glia (glial fibrillary acidic protein [GFAP], 1:250; Abcam), photoreceptor (rhodopsin, 1:250; Abcam), and multiple blood cell lineages (hemopoietic stem cell marker CD34, 1:250; Abcam; natural killer [NK] cell marker CD11b, 1:250; Epitomics, Burlingame, CA, http://www.epitomics.com; monocyte marker CD14, 1:100; NOVOS, Ankara, Turkey, http://www.novos.com.tr; leukocyte marker CD45, 1:250; Invitrogen, Carlsbad, CA, http://www.invitrogen.com; T-cell marker CD3, 1:250; Invitrogen; NK cell marker CD16, 1:250; Invitrogen; B-cell marker CD19, 1:250; Invitrogen).
Staining was examined qualitatively by microscopy and quantitatively by flow cytometry. For microscopic examination, cell samples were collected, washed, and resuspended in phosphate buffered saline (PBS, $10^6$ cells per milliliter), transferred to cupsules containing cytospin slides (Shandon Cytospin 4, Thermofisher Scientific, Waltham, MA, http://www.thermofisher.com), and centrifuged at 300 rpm (approximately 10g) for 2 minutes. Cells on the cytospin slides were fixed in 4% paraformaldehyde at room temperature (RT) for 5 minutes, permeabilized by Triton X-100 (1:1,000, Whiga Technology) at RT for 2 minutes (except for staining of cell-surface CD molecules), and incubated with primary antibodies at the appropriate dilutions overnight at 4°C. Antibodies against cell surface markers (CD45, CD3, CD16, or CD19) were fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated (Invitrogen). Immunolabeling of the other markers was revealed by incubation with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:1,000; Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com) at 37°C for 1 hour. Immunolabeled cells were then counterstained with Hoechst 33342 (1:1,000; Sigma-Aldrich), covered with antifade mounting medium (Applygen Technologies Inc., Beijing, China, http://www.applygen.com), and examined by laser scanning confocal microscopy (Carl Zeiss, Jena, Germany, http://www.zeiss.com). Negative control slides prepared in parallel were treated with human serum in place of primary antibodies. In other samples, the fractions expressing the various markers were measured by flow cytometry. For cell surface markers, cells were harvested, washed in PBS, and then incubated for 30 minutes with FITC-conjugated primary antibodies against CD3, CD16, CD19, or CD45 in the dark, or with unlabeled primary antibodies against CD34, CD11b, or CD14 at room temperature. The latter groups were incubated with Reagent A (catalog no. GAS003, Invitrogen) for 15 minutes at RT and then with a FITC- or PE-conjugated secondary antibody for 30 minutes in the dark at RT (CD34 and CD11b were FITC-labeled, CD14 was PE labeled). For intracellular antigens (nestin, MAP2, vimentin, βIII-tubulin, synapsin, GFAP, rhodopsin), cells were first incubated with Reagent A (catalog no. GAS003, Invitrogen) for 15 minutes at RT, then Reagent B was added together with the recommended antibody titer for 30 minutes at RT. These labeled cells were washed in PBS, and incubated in an FITC- or PE-conjugated secondary antibody for 30 minutes at RT. All processess followed the supplier’s protocols. The fractions of labeled cells were measured using a FACSARia flow cytometer (Becton Dickinson, Franklin Lakes, NJ, http://www.bd.com).

Quantitative Real-Time Polymerase Chain Reaction
Freshly harvested and preinduced hPBMCs were washed twice with PBS and approximately $10^7$ cells from each group collected, resuspended in 1 ml TRIZol (Invitrogen), and sent to the Shanghai KangChen Bio-tech Institute for quantitative real-time polymerase chain reaction (qRT-PCR). The experimental protocols were developed by KangChen Bio-tech Institute. Briefly, total RNA was extracted and tested for quality control. RNA concentration and purity were evaluated by measuring the optical density using a Nanodrop ND-1000 spectrophotometer (Merinton, Ann Arbor, MI, http://www.merinton.com). Denaturing agarose gel electrophoresis was used to test RNA integrity and gDNA contamination. Then, approximately 1 μg RNA from each sample was reverse transcribed into cDNA, which subsequently served as the template for qRT-PCR. The primers (supplemental online Table 1) for qRT-PCR were designed using Primer 5.0 software. Reaction mixtures containing 2 μl cDNA were prepared in 2× PCR master mix (Arraystar, Rockville, MD, http://www.arraystar.com) and subjected to qRT-PCR analysis using the Viia 7 Real-time PCR System (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). The thermocycle reaction was 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. Threshold cycle (Ct) values were obtained for each gene and relative expression analyzed using the standard curve method. Values were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The average relative expression ± SD is reported.

Chloromethyl-Benzamidodialkylcarbocyanine Labeling of Cells
In experiments examining transplanted cell survival, differentiation, and integration at 1 or 3 months after injection, all cells were prelabeled with the fluorescent marker chloromethyl-benzamidodialkylcarbocyanine (CM-Dil). In some studies [18, 19], however, CM-Dil remained for only 2–3 months after transplantation, so cells transplanted for 6 months were not Dil-labeled before transplantation but rather labeled using an anti-human mitochondrial marker by immunofluorescent staining. After preinduction for 4 days with postnatal day 1 (P1) rat retinal tissue, hPBMCs in the bottom wells were collected, washed with PBS, resuspended in serum-free DMEM at $10^6$ cells per milliliter, and incubated with CM-Dil (5 μl per 1 ml of DMEM; catalog no. V22888; Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) at 37°C for 5 minutes and at 4°C for an additional 15 minutes. Cells were gently resuspended every 5 minutes during incubation. Incubation at 4°C allows the dye to label the plasma membrane but reduces the rate of endocytosis, thus reducing dye localization in cytoplasmic vesicles. The CM-Dil-labeled cells were washed with PBS, resuspended in serum-free DMEM at $5 \times 10^5$ cells per milliliter, and injected into the ocular cavity as described.

Subretinal Transplantation
Mice were anesthetized by intraperitoneal injection of 4.3% chloral hydrate (430 mg/kg body weight; from the pharmacy of Zhongshan Ophthalmalic Center [ZOC], Guangzhou, China, http://www.gzsoc.com) and were prepared for transplantation by local anesthesia using dicaine hydrochloride eye drop (from the pharmacy of ZOC), pupil dilation using compound tropicamide eye drop (from the pharmacy of ZOC), and insertion of a preset conjunctival suture (catalog no. 8065698001; Alcon, Hünenberg, Switzerland, http://www.alcon.com) at 2 o'clock on the right eye ball (treated eye). The bulbar conjunctiva was then bluntly separated from the sclera. A volume of anterior chamber fluid was removed to reduce intraocular pressure and the preinduced cell suspension injected through a 33-gauge microinjector needle (Hamilton Company, Reno, NV, http://www.hamiltoncompany.com) angled toward the optic nerve. A 15-degree angle was maintained as the sclera was perforated 1.5 mm posterior to the superotemporal limbus, and 5–6 $\times 10^5$ cells in 2 μl of serum-free culture medium was injected into the subretinal space. After injection, the preset conjunctival suture was knotted. The right eyes of the control group were injected with an equal volume of serum-free DMEM. Successful subretinal transplantation was confirmed by local retinal detachment at the injection site. Tobramycin and dexamethasone eye drops (from the pharmacy of ZOC) were
tightly administered three times per day in the week after injection. The retinas reattached approximately 3 days after the operation. No cataract, vitreous hemorrhage, or endophthalmitis was observed after subretinal injection. At 1, 3, and 6 months after transplantation, migration and differentiation of exogenous cells and retinal function were examined as described next.

**Immunofluorescent Staining of Frozen Sections**

Cell-specific marker expression by injected cells was examined in air-dried frozen sections (5 μm thick) of whole eyeball harvested 1, 3, or 6 months after transplantation. All sections were fixed in 4% paraformaldehyde (Whiga Technology) at RT for 5 minutes, washed with PBS, permeated by 0.1% Triton-X (Whiga Technology), blocked with goat serum (Boster Biological Technology, Wuhan, China, http://www.boster.com.cn), then incubated overnight at 4°C with human-specific nestin, MAP2, vimentin, βIII-tubulin, or rhodopsin primary antibodies. Sections harvested 6 months after transplant were also incubated with an Alexa Fluor 488-conjugated primary antibody against human mitochondrial protein (1:50; Millipore). Immunolabeling of sections at 1 and 3 months after transplant was visualized with a goat antirabbit FITC-conjugated secondary antibody (1:1,000; 37°C for 1 hour), whereas sections at 6 months after transplant were incubated with a donkey antirabbit Alexa Fluor 594-conjugated secondary antibody (1:500; 37°C for 1 hour; Invitrogen). Cell nuclei were counterstained with Hoechst 33342 (1:1,000; Sigma-Aldrich) for 5 minutes. Labeled sections were coverslipped with antifade mounting medium (Applygen Technologies Inc.) and viewed under a laser scanning confocal microscope (Carl Zeiss).

**Granzfeld ERG**

To assess retinal function, ERG responses of RD1 mice were recorded 1, 3, and 5 months after subretinal transplantation (1 month, n = 30; 3 months, n = 19; 5 months, n = 9). Mice were dark-adapted for at least 12 hours before ERG. In dim red light, mice were anesthetized with 4.3% chloralhydrate (from the pharmacy of ZOC), the pupils dilated with tropicamide (from the pharmacy of ZOC), and Goniovisc (HUB Pharmaceuticals, Rancho Cucamonga, CA, http://www.bio-glo.com) applied regularly to the corneas to prevent dehydration and allow for optimal electrical conductivity. Each mouse was positioned on a heating pad with a ground electrode inserted in the tail and a reference electrode subcutaneously in the cheek. The positions of the cornea and corneal electrode were adjusted for the best light stimulation and electrode contact. Each eye was stimulated with green light and ultraviolet (UV) light at the same time. ERG wavelets were recorded 20 times using a Phoenix Granzfeld ERG (Phoenix Research Laboratories Inc., Pleasanton, CA, http://www.phoenixreslabs.com), and the average amplitudes of a- and b-waves were analyzed.

**PCR of Treated Retinas After Transplantation**

To test for the presence of living hPBMC-derived cells in the subretinal space, we measured expression of the human mitochondrial cytochrome b gene. After ERG measurements, a subgroup of treated mice was sacrificed by cervical dislocation (1 month, n = 10; 3 months, n = 10; 5 months, n = 9), and the treated eyes were harvested. The retinas were gently dissected from the treated eyeballs and washed with PBS. The tissues were lysed in TRIzol and the RNA was extracted. Total RNA was reverse transcribed into cDNA, which was used as the template for PCR. The human-specific primer set (forward: GCTTGCAACTATAGCAGC; reverse: GGACTGTCTACTGAGTAGCC) was designed using Primer 5.0 software. Reactions containing 1 μl of cDNA were prepared in 2× SYBR Premix Ex Taq II (Tiangen Biotech, Beijing, China, http://www.tiangen.com) and subjected to PCR analysis using ABipcr (Applied Biosystems). The reactions were performed at 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The PCR product was analyzed by 2% agarose gel electrophoresis.

**Statistical Analysis**

The flow cytometry, qRT-PCR, and b-wave amplitude results at 3 and 5 months (treated eyes vs. contralateral eyes) after transplantation were compared by two-tailed t test for paired samples. The b-wave amplitude results of C57 and the RD1 mice (3 and 5 months after transplantation) were compared using a two-tailed test for independent samples. Statistical analysis was performed using SPSS 13.0 software (IBM Corporation, Armonk, NY, http://www.ibm.com). Data are expressed as mean ± SD. Statistical significance was set at p < .05.

**RESULTS**

**Changes in hPBMC Morphology During Preinduction**

Freshly isolated hPBMCs were spheroid in suspension (Figs. 1A, 1B). To induce differentiation toward retinal lineages, hPBMCs
were cultured for 4 days in the bottom wells of transwell plates below inserts preseeded 24 hours earlier with retinal tissue from 1-day-old Sprague-Dawley rats. On the second or third day in coculture, floating cell masses were observed but few cells exhibited shape changes indicative of differentiation (Fig. 1C). After 4 days of incubation, several morphologically distinct cell subtypes were observed: spheroid suspended mononuclear cells, cells with one or two short protrusions (Figs. 1D, 1E). Total cell numbers decreased by approximately one third after 4 days in coculture with rat retinal tissue.

Protein and RNA Expression Patterns of Preinduced hPBMCs

The expression levels of neural stem cell, photoreceptor, mature neuronal, and glial markers were examined in freshly isolated and preinduced hPBMCs by immunostaining (Fig. 2), flow cytometry (Table 1; supplemental online Figs. 1, 3), and qRT-PCR (Table 2; supplemental online Fig. 4).

Immunostaining (Fig. 2) revealed that very few cells in the freshly isolated population expressed βIII-tubulin, MAP2, rhodopsin, synapsin, GFAP, nestin, CD34, or CD44. A larger fraction expressed CD14, CD11b, CD3, CD16, or CD19, whereas most expressed the neural progenitor marker vimentin and almost all expressed the leukocyte marker CD45. After preinduction, greater proportions expressed neural stem cell markers, neuronal markers, or a photoreceptor marker. These changes were quantified by flow cytometry (Table 1), which revealed significantly greater proportions of cells expressing the neural stem/precursor cell markers nestin, vimentin, or βIII-tubulin (p < .05). In addition, the fraction expressing the photoreceptor marker rhodopsin and those expressing one of several blood cell markers (CD14, CD44, or CD45) increased after preinduction (p < .05). In contrast, the cell fractions expressing one of the immunocyte markers CD11b, CD16, or CD19 were smaller after preinduction (p < .05).

The results of qRT-PCR (Table 2) revealed that RNA expression levels of MAP2, rhodopsin, and CD19 increased (p < .05) after preinduction, whereas expression of vimentin decreased (p < .05).

Phenotypic Expression of the Transplanted Cells

Immunofluorescent staining of frozen sections (Figs. 3A–E; supplemental online Fig. 5) from RDS mouse eyeballs obtained 1 month after injection with preinduced hPBMCs revealed that some transplanted cells (labeled with CM-Dil) expressed nestin, vimentin, βIII-tubulin, MAP2, or rhodopsin. Most of these cells were clustered in the inner nuclear layer (INL). In sections prepared 3 months after hPBMC injection (Figs. 3F–J; supplemental online Fig. 6), most transplanted cells (labeled with CM-Dil) expressed nestin, vimentin, βIII-tubulin, MAP2, or rhodopsin. Cells were distributed in the INL and the inner plexiform layer (IPL), some dispersed as single cells and others still aggregated with other transplanted cells. At 6 months after hPBMC injection (Figs. 3K–O; supplemental online Fig. 7), many transplanted cells (expressing a human mitochondrial protein) still expressed nestin, vimentin, βIII-tubulin, MAP2, or rhodopsin. Most were dispersed as single cells and had spread into the INL and the ganglion cells layer (GCL). However, a subgroup hPBMC-derived (human mitochondrial marker [+] cells were found that did not express neural markers.

Figure 2. Immunofluorescent staining of freshly isolated (A) and preinduced (B) peripheral blood mononuclear cells. The freshly isolated population rarely expressed βIII-tubulin, MAP2, rhodopsin, synapsin, GFAP, nestin, CD34, or CD44. A larger fraction expressed CD14, CD11b, CD3, CD16, or CD19, whereas most expressed the neural progenitor marker vimentin and almost all expressed the leukocyte marker CD45. After 4 days of preinduction, many more cells expressed a neural stem cell (nestin, βIII-tubulin, vimentin), neuronal (MAP2, synapsin), or photoreceptor marker (rhodopsin). Scale bars = 20 μm. Abbreviations: GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2.
Table 1. Changes in cell-specific protein marker expression after preinduction as measured by flow cytometry

| Antigen | Before induction (% of total, mean ± SD) | After induction (% of total, mean ± SD) | t     | p value |
|---------|-----------------------------------------|----------------------------------------|-------|---------|
| Nestin  | 0.60 ± 0.79                             | 2.93 ± 1.06                            | −8.03 | .01*    |
| βIII-tubulin | 6.16 ± 8.20                             | 8.70 ± 8.88                            | −5.27 | .03*    |
| Vimentin | 45.90 ± 6.16                             | 80.06 ± 14.01                          | −5.74 | .03*    |
| Rhodopsin| 1.43 ± 1.81                              | 23.10 ± 2.31                           | −37.28| .00*    |
| MAP2    | 2.47 ± 4.19                              | 2.83 ± 4.22                            | −4.16 | .05     |
| GFAP    | 0.03 ± 0.06                              | 2.73 ± 1.80                            | −2.67 | .12     |
| Synapsin| 2.00 ± 3.29                              | 4.37 ± 5.06                            | −2.305| .15     |
| CD34    | 3.40 ± 2.55                              | 3.97 ± 1.24                            | −0.55 | .63     |
| CD14    | 3.56 ± 1.17                              | 10.41 ± 0.67                           | −7.42 | .02*    |
| CD44    | 0.57 ± 0.98                              | 3.53 ± 0.55                            | −9.33 | .01*    |
| CD45    | 67.70 ± 13.72                            | 78.03 ± 13.43                          | −16.89| .00*    |
| CD11b   | 4.60 ± 3.01                              | 3.13 ± 2.51                            | 5.16  | .03*    |
| CD3     | 32.33 ± 13.59                            | 26.53 ± 27.02                          | 0.75  | .53     |
| CD16    | 15.42 ± 5.56                             | 4.3 ± 2.11                             | 4.41  | .02*    |
| CD19    | 16.37 ± 3.36                             | 1.07 ± 0.06                            | 7.887 | .01*    |

*p < .05 by paired sample t tests.
Abbreviations: GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2.

Table 2. Changes in RNA expression after preinduction as measured by quantitative real-time polymerase chain reaction

| Genes     | Before preinduction (mean ± SD) | After preinduction (mean ± SD) | After/before | p value |
|-----------|---------------------------------|--------------------------------|--------------|---------|
| Nestin    | $6.00 \times 10^{-3} \pm 1.57 \times 10^{-3}$ | $1.55 \times 10^{-3} \pm 1.21 \times 10^{-4}$ | 25.86 | .165   |
| βIII-Tubulin | $2.29 \times 10^{-4} \pm 1.23 \times 10^{-4}$ | $7.03 \times 10^{-3} \pm 5.90 \times 10^{-4}$ | 3.08  | .293   |
| Vimentin  | $1.54 \pm 3.44 \times 10^{-1}$ | $3.91 \times 10^{-3} \pm 1.75 \times 10^{-2}$ | 0.25  | .027*  |
| Rhodopsin | $4.62 \times 10^{-3} \pm 1.45 \times 10^{-3}$ | $1.95 \times 10^{-3} \pm 5.51 \times 10^{-3}$ | 4.21  | .049*  |
| MAP2      | $1.90 \times 10^{-1} \pm 1.23 \times 10^{-1}$ | $6.20 \pm 1.51$ | 32.59 | .023*  |
| GFAP      | $7.33 \times 10^{-3} \pm 1.20 \times 10^{-3}$ | $2.41 \times 10^{-3} \pm 1.24 \times 10^{-3}$ | 32.86 | .083   |
| Synapsin  | $1.34 \times 10^{-3} \pm 9.06 \times 10^{-4}$ | $5.49 \times 10^{-3} \pm 1.47 \times 10^{-3}$ | 4.11  | .081   |
| CD34      | $4.27 \times 10^{-1} \pm 8.02 \times 10^{-2}$ | $3.94 \times 10^{-1} \pm 8.99 \times 10^{-2}$ | 0.92  | .772   |
| CD14      | $1.79 \times 10^{-3} \pm 7.42 \times 10^{-4}$ | $7.12 \times 10^{-3} \pm 5.64 \times 10^{-3}$ | 3.99  | .204   |
| CD44      | $6.26 \times 10^{-2} \pm 1.63 \times 10^{-2}$ | $2.61 \times 10^{-1} \pm 1.07 \times 10^{-1}$ | 4.16  | .109   |
| CD45      | $2.92 \times 10^{-1} \pm 1.41 \times 10^{-1}$ | $3.81 \times 10^{-1} \pm 2.22 \times 10^{-1}$ | 1.30  | .445   |
| CD11b     | $3.96 \times 10^{-1} \pm 9.97 \times 10^{-3}$ | $3.94 \times 10^{-1} \pm 1.65 \times 10^{-1}$ | 9.96  | .064   |
| CD3       | $9.73 \times 10^{-3} \pm 1.54 \times 10^{-3}$ | $9.59 \times 10^{-3} \pm 3.27 \times 10^{-3}$ | 0.99  | .954   |
| CD16      | $6.36 \times 10^{-1} \pm 9.22 \times 10^{-2}$ | $3.62 \times 10^{-1} \pm 4.77 \times 10^{-2}$ | 0.57  | .077   |
| CD19      | $2.68 \times 10^{-2} \pm 1.27 \times 10^{-2}$ | $1.36 \times 10^{-1} \pm 3.02 \times 10^{-2}$ | 5.07  | .038*  |

*p < .05 by paired sample t tests.
Abbreviations: GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2.

PCR of Treated Retinas

The human mitochondrial cytochrome b gene was still expressed in mouse retina 1, 3, and 5 months after transplantation as shown by electrophoretograms of PCR products (Fig. 4).

Granzfeld ERG

To test for functional recovery, RD1 mice were dark-adapted overnight, and then presented with green light and UV flashes simultaneously during ERG measurements. The blank control group (RD1 mice without treatment, n = 10; Figs. 5A, 5B) showed flat ERG responses at all time points (1 month, 3 months, and 5 months). Similarly, neither hPBMC-injected nor untreated contralateral eyes exhibited measureable ERG light responses 1 month after subretinal transplantation (n = 30 mice; Figs. 5C, 5D; supplemental online Fig. 8A, 8B). At 3 months after transplantation, all treated mice examined had clear responses in both eyes (n = 19 mice; Figs. 5E, 5F; supplemental online Fig. 8C, 8D). The b-wave amplitude did not differ significantly between treated and untreated contralateral eyes (23.44 ± 15.58 μV vs. 17.33 ± 12.49 μV; p > .05). At 5 months after transplantation, however, none of the untreated contralateral eyes (zero of nine mice) exhibited a light response, whereas four of nine treated eyes did (Figs. 5G, 5H; supplemental online Fig. 8E, 8F; b-wave amplitude...
of 24.93 ± 4.12 µV), which was not significantly different from the mean response in the treated eyes at 3 months after transplantation ($p > .05$). Thus, transplantation at least partially rescued retinal function at 3 and 5 months, although function as measured by b-wave amplitude was still below that of healthy C57 mice (Figs. 5I, 5J; supplemental online Table 2; supplemental online Fig. 2).

**DISCUSSION**

The possibility of using cell-based therapies to treat retinal degenerative diseases has prompted numerous studies to identify the ideal donor cell source [1–3, 20–22]. Pluripotent stem cells have several advantages over other cell types: (a) pluripotency may allow for the generation of multiple cell types to repair or replace damaged tissues; (b) self-renewal could provide a long-term therapeutic effect; and (c) many types, such as bone marrow-derived stem cells, can secrete a variety of trophic factors that delay the progression of degeneration or promote regeneration [23–28].

The clinical application of stem cell therapies requires a source that is readily available and safe and can be developed commercially for large-scale production. Human PBMCs exhibit high phenotypic plasticity [4], can be easily isolated from autologous blood at any donor age, and are less likely to contain malignant cells compared with bone marrow-derived stem cells [29, 30], and there are no serious ethical issues associated with harvesting (unlike fetal stem cell harvesting). Stem cells can also be guided along specified lineages in different microenvironments [13, 31–33]. Here, we demonstrate that preinduced PBMCs are able to survive in the adult mouse retina and migrate into multiple cell layers. Finally, we show that preinduced PBMC transplantation can improve the function of degenerated retina. Thus, preinduced PBMCs fulfill many of the requirements of an ideal cell source for transplantation.

In this study, explant cultures of P1 rat retina (consisting mainly of undifferentiated early retinal precursor cells) provided an “early retinal” environment for the initial lineage specification of hPBMCs [13]. The various extracellular signals controlling retinal progenitor cell fate are actively expressed in the P1 retina, thus providing the growth factors and cytokines for hPBMC differentiation into neuronal precursors, retinal neurons,
or photoreceptors. In addition, differentiation into photoreceptors in the retina may have been promoted by compensatory mechanisms activated by the loss of endogenous photoreceptors due to the RDS and RD1 mutations.

Flow cytometry was performed to assess the change in phenotype frequency distribution and qRT-PCR to reveal alterations in the expression levels of specific mRNAs after preinduction. After induction, there were significant increases in protein and RNA expression of rhodopsin, a marker of photoreceptors, indicating that this medium efficiently induced hPBMC differentiation into photoreceptor-like cells, possibly aided by B27 supplement [34]. Nestin, βIII-tubulin, and MAP2 expression levels also increased significantly, demonstrating that hPBMCs could differentiate toward the neuronal lineage. Nestin is a neural stem cell marker, βIII-tubulin a neural progenitor marker, and MAP2 a marker of mature neurons; these proteins are expressed in succession during differentiation [35, 36]. According to the sequence of RNA and protein expression changes, we speculate that after 4 days of preinduction, hPBMCs had differentiated fully into neural progenitors (expressing nestin and βIII-tubulin) and were in the process of transforming into mature neurons that express MAP2. Transplantation of cells in this transitional state on day 4 [37] may have enhanced in vivo differentiation, maturation, and integration [38].

The proportions of CD14-, CD44- and CD45-positive cells were also higher after preinduction compared with freshly isolated hPBMCs. Usually, CD14 is used as a marker of monocytes and CD45 as a marker of leukocytes, but recent studies revealed a population of stem cell-like hPBMCs expressing CD14 and CD45 that could differentiate along multiple limb-bud mesodermal lineages [39–41]. Thus, although this medium promoted differentiation, it did not deplete the source population of multipotent CD14- and CD45-positive cells. Indeed, increased expression suggests that these stem-like cells continued to proliferate during preinduction. In contrast, the fractions expressing the monocyte marker CD11b or the NK marker CD16 were smaller after preinduction, suggesting that the neural stem cell medium was not suitable for survival of mature immunocytes and that they were eliminated by apoptosis during preinduction.

There were some discrepancies between flow cytometry and qRT-PCR results. Vimentin expression increased after preinduction as measured by flow cytometry but decreased as measured by qRT-PCR. We suggest that mesenchymal cells also expressed vimentin and that the gene was downregulated before the preexisting proteins degraded. Conversely, expression of CD19 increased as measured by qRT-PCR but decreased as measured by flow cytometry. CD19 is a B-cell marker, and B cells can respond to xenogenic proteins. Some of the extracellular factors released by rat retinal tissue in coculture could have been recognized by B cells, resulting in proliferation, maturation, and eventual apoptosis as the environment was unsuitable for terminal immunocyte survival.

The electrophoretogram of PCR production revealed that hPBMCs were still alive in the mouse retina 1, 3, and 5 months after transplantation. Cells double-stained by CM-Dil (or antihuman mitochondrial protein) and antibodies against rhodopsin, nestin, βIII-tubulin, vimentin, or MAP2 were observed in the retina of RDS mice at 1, 3, and 6 months after transplantation. Moreover, the distribution of transplanted cells became wider (from subretinal space to GCL) and more dispersed, with few cell clusters and greater numbers of individual isolated cells. These observations indicate that preinduced hPBMCs can survive in the pathological retina for at least 6 months and differentiate into neural cells and photoreceptors. They also migrated from the injection site to the INL, IPL, and GCL. However, the lack of an outer nuclear layer indicates that preinduced hPBMCs could not completely reconstruct the damaged retina of the host at 6 months.

Grahnfeld ERG results showed partial functional recovery at 3 and 5 months after transplantation. Considering the immunofluorescence staining, retinal PCR, and ERG results, we speculate that hPBMCs were alive and differentiated 1 month after transplant, but most were still aggregated at the injection site and...
had not yet integrated in the host retina. By 3 months after transplantation, most hPBMCs had differentiated, dispersed, and migrated into the INL and IPL. The positive light response in some transplanted retinas at 3 and 5 months indicates that a fraction of hPBMCs had integrated into the host retina and formed functional connections with host cells. Surprisingly, the mean b-wave amplitude of treated eyes at 3 months did not differ from that of untreated contralateral eyes, suggesting that hPBMCs may migrate to the contralateral eye through the optic nerve [42]. This possibility remains to be examined in our model. At 6 months after transplantation, hPBMCs were still alive and scattered throughout the host retina. Moreover, four transplanted eyes showed light-evoked b-waves on the ERG at 5 months postinjection, whereas the four contralateral eyes did not. Thus, injection of preinduced hPBMC appeared to partially rescue retinal function, at least in some mice. However, five of nine mice exhibited no light response in either eye, indicating that the surviving cells were insufficient in number to maintain or improve retinal function, or had not functionally integrated into the host retinal circuitry. Moreover, even in light-responsive treated eyes, b-wave amplitude was not as large as in adult C57 mouse eyes. Overall, the results of Granzfeld ERG suggest that preinduced hPBMCs could act as seed cells to promote functional recovery in some cases.

Recruitment of retinal function after transplantation depends on the survival, differentiation, migration, and integration of the transplanted cells (PBMCs) and possibly also on release of trophic factors. Further studies are required to assess whether the effects reported here are due to functional integration or a trophic response. Although only modest recovery was achieved, the transplantation method could be further improved to increase the probabilities of survival, appropriate differentiation, and host integration. For instance, greater recovery or preservation of function may require repeated cell injections or purification of a more dynamic cell population before transplantation.

CONCLUSION

Our findings indicate that PBMCs preinduced with retinal tissue from 1-day-old Sprague-Dawley rats can differentiate into early-stage retinal precursor cells, primarily rhodopsin-positive photoreceptor-like cells, and survive in the subretinal space of degenerated mouse retina for at least 6 months. Furthermore, the transplanted cells exhibited the protein expression phenotypes of retinal neurons and photoreceptors, dispersed from the injection site, and migrated into several host retinal layers. Our study also demonstrates that retinal function can be restored 5 months after preinduced hPBMC transplantation.

Future studies are needed to (a) analyze the signaling mechanisms for PBMC differentiation in vitro, (b) identify all subpopulations derived from transplanted PBMCs, (c) determine how long transplanted cells can survive and maintain differentiation capacity after transplantation into the subretinal space, (d) assess the mechanisms responsible for functional integration, and (e) optimize the transplantation method.

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AUTHOR CONTRIBUTIONS

B.X., Y.Z., and Y.P.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; J.H.: collection and/or assembly of data; W.L., W.W., and M. Zhang: administrative support, provision of study material or patients; K.L.: administrative support; H.Z. and M. Zhao: provision of study material or patients; X.L.: conception and design; B.H.: conception and design; final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.
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