

Competency of iPSC-derived retinas in MHC-mismatched transplantation in non-human primates

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SUMMARY

Transplantation of embryonic/induced pluripotent stem cell-derived retina (ESC/iPSC-retina) restores host retinal ganglion cell light responses in end-stage retinal degeneration models with host-graft synapse formation. We studied the immunological features of iPSC-retina transplantation using major histocompatibility complex (MHC)-homozygote monkey iPSC-retinas in monkeys with laser-induced retinal degeneration in MHC-matched and -mismatched transplantation. MHC-mismatched transplantation without immune suppression showed no evident clinical signs of rejection and histologically showed graft maturation without lymphocytic infiltration, although immunological tests using peripheral blood monocytes suggested subclinical rejection in three of four MHC-mismatched monkeys. Although extensive photoreceptor rosette formation was observed on histology, evaluation of functional integration using mouse models such as mouse ESC-retina (C57BL/6) transplanted into rd1(C3H/HeJ, MHC-mismatched model) elicited light responses in the host retinal ganglion cells after transplantation but with less responsiveness than that in rd1-2J mice (C57BL/6, MHC-matched model). These results suggest the reasonable use of ESC/iPSC-retina in MHC-mismatched transplantation, albeit with caution.

INTRODUCTION

Stem-cell-based therapy is a promising approach for restoring and reconstructing visual function in patients with retinal degeneration. The development of innovative protocols for retinal organoid induction from mouse embryonic stem cells (ESCs) (Eiraku et al., 2011), followed by those using human ESCs and induced pluripotent stem cells (iPSCs) (Kuwahara et al., 2015; Meyer et al., 2011; Nakano et al., 2012; Tu et al., 2019; Zhong et al., 2014), has accelerated research on the clinical application of stem-cell-based therapies, with further improvement in differentiation protocols (Kuwahara et al., 2019; Reichman et al., 2017).

In the past decade, transplantation of photoreceptor precursors in retinal degeneration models with a residual outer nuclear layer (ONL) revealed that donor cells can restore the function of host retinas through both direct integration and/or supplying the missing proteins in a phenomenon called “material transfer” (Barber et al., 2013; Gasparini et al., 2022; Gonzalez-Cordero et al., 2013; MacLaren et al., 2006; Ortin-Martinez et al., 2017; Pearson et al., 2012, 2016; Santos-Ferreira et al., 2016; Singh et al., 2013, 2016; Waldron et al., 2018; Warre-Cornish et al., 2014). We and other groups have reported the efficacy of transplantation

of retinal sheets or purified photoreceptor precursors from stem-cell-derived retinas for end-stage retinal degeneration with no remaining ONL (Assawachananont et al., 2014; Barnea-Cramer et al., 2016; Iraha et al., 2018; Mandai et al., 2017; Ribeiro et al., 2021; Shirai et al., 2016; Singh et al., 2013; Tu et al., 2019). In particular, we 3-dimensionally identified *de novo* host-graft synapses using a reporter mouse line expressing GFP in rod bipolar cells, reporter grafts expressing the synaptic marker CtBP2:tdTomato in rod photoreceptors, and immunostaining of postsynaptic marker Cacna1s. We also showed that host retinal ganglion cells (RGCs) at the grafted area respond to light using multi-electrode arrays (MEAs) and that transplanted animals better respond to light in behavioral tests (Mandai et al., 2017; Matsuyama et al., 2021). The grafted photoreceptor cells mostly form rosettes but are consistently photoresponsive, even without the addition of 9-*cis* retinal supplementation (Yamasaki et al., 2022). Based on this proof of concept, a clinical trial using human iPSC-retinas for transplantation in patients with retinitis pigmentosa was recently conducted in Japan (jRCTa050200027).

The management of immunological rejection is another critical issue in cell-based therapies. The major histocompatibility complex (MHC) or the human leukocyte

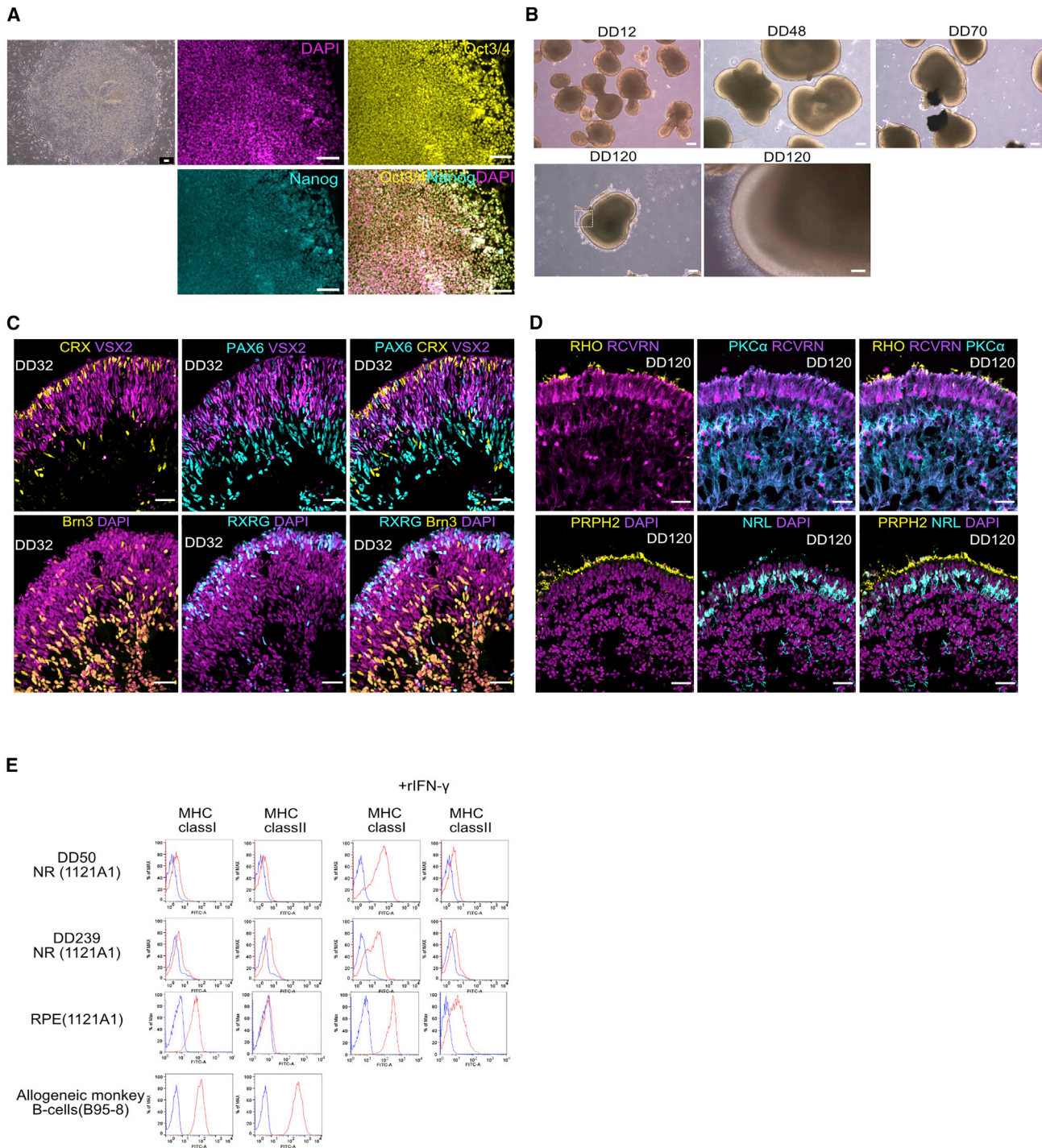


Figure 1. Characterization of monkey 1121A1 MHC homozygous HT1 iPSC retinas

(A) Bright-field image and immunostaining images of the pluripotent stem cell markers in 1121A1-HT1 mkiPSCs.

(B) Bright-field images of DD12–DD120 mkiPSC-retinas during differentiation. Magnified view of a DD120 mkiPSC-retina (white dotted box) presenting fluffy inner segment-like structures.

(C) Immunostaining of DD32 mkiPSC-retinas. (Top panels) PAX6-positive cells are located basal to VSX2-positive retinal progenitor cells. CRX-positive photoreceptor precursor cells are mostly present on the apical side. (Bottom panels) RXRG-positive cone photoreceptor precursor cells and Brn3-positive retinal ganglion cells are observed on the apical and basal sides, respectively.

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antigen (HLA) is a key genetic element in the immune response after transplantation (Petersdorf, 2013). The immunological tolerability of HLA-matched allogeneic transplantation of iPSC/ESC-derived tissues reduces immune cell infiltration and increases graft survival (Morizane et al., 2017; Shiba et al., 2016; Sugita et al., 2016). A bank project to prepare stocks of donor-derived iPSC lines homologous to HLA loci that are highly frequent among the Japanese population now increases the chances of HLA matching for transplantation (Umekage et al., 2019). However, it is expensive to prepare sufficient cell lines.

The eyes are unique immune-privileged sites (Streilein, 2003) that suppress immune responses and actively induce tolerance to foreign antigens (Keino et al., 2018); additionally, they have low immunogenetic properties (Hori et al., 2003). Recently, Yamasaki et al. reported the immunological properties of human ESC/iPSC-retinas, showing low immunogenicity and active features of immunosuppression, partly by the secretion of transforming growth factor β (TGF- β) (Yamasaki et al., 2021). In fact, the long-term survival of transplanted human fetal retinas/retinal cells without systemic immunosuppression has also been reported in patients with retinitis pigmentosa (Das et al., 1999; Radtke et al., 2002). To further assess the immune tolerability of allogeneic iPSC-retina transplantation with or without MHC matching, we optimized a differentiation protocol for the MHC homologous monkey iPSC-line (mkiPSC) and compared the immune responses of the host and the graft development between MHC-matched and -mismatched transplantations using the monkey model of laser-induced photoreceptor degeneration (Shirai et al., 2016). We also studied whether functional integration was achieved with or without MHC matching using mouse ESC (mESC) retinas of C57BL/6 for transplantation into the late-stage retinal degeneration mouse models with C57BL/6J or C3H/HeJ genetic backgrounds.

RESULTS

Monkey iPSC-derived retinal organoids show low immunogenicity and the potential to functionally integrate after transplantation in rats

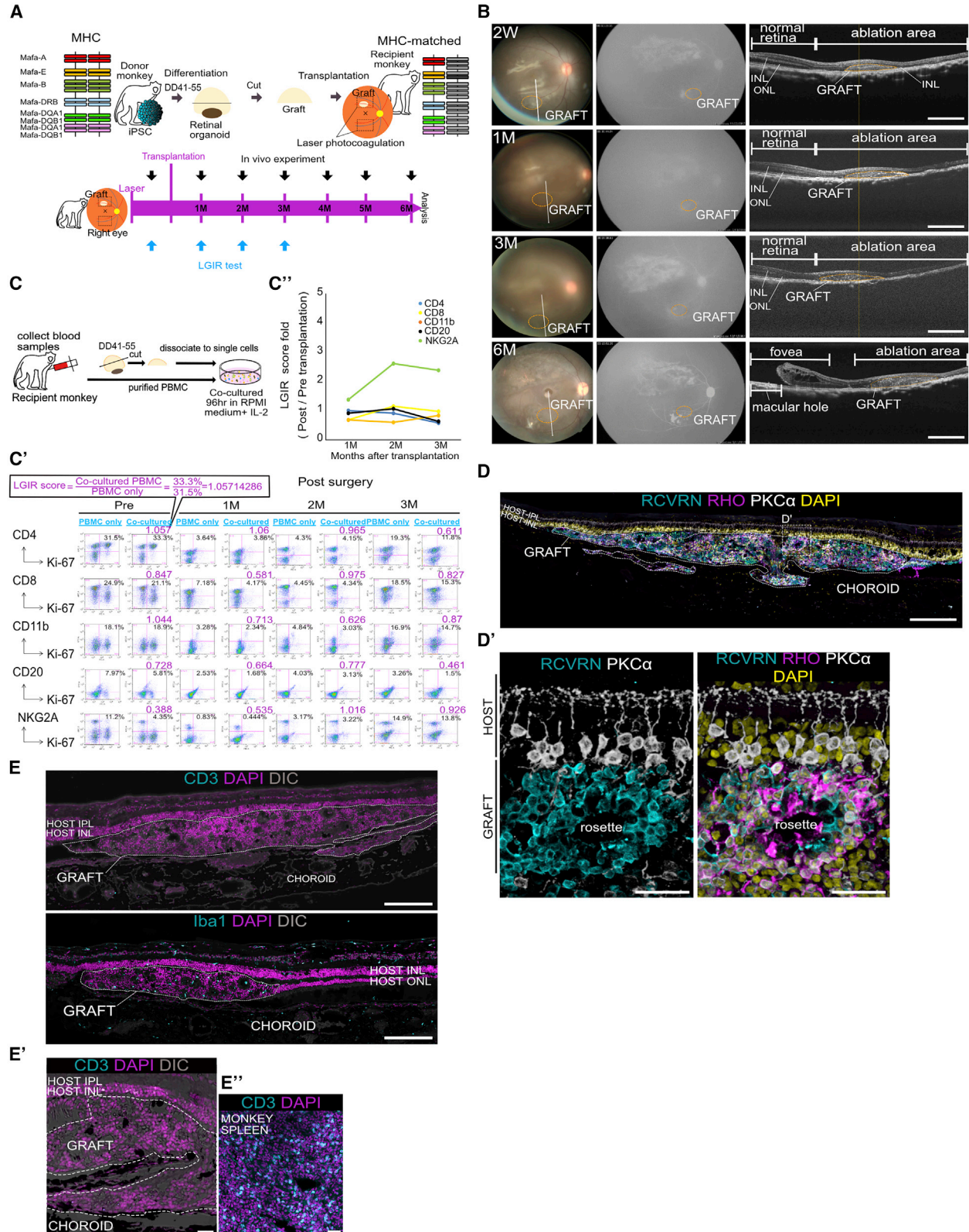
The 1121A1 MHC homozygote HT1 mkiPSC line was differentiated into retinas with a characteristic morphology

similar to previously reported hESC/iPSC-retinas, and fluffy cilia-like structures were observed on the apical surface at differentiation day 70 (DD70) and DD120 (Figures 1A and 1B). These organoids expressed the retinal progenitor markers PAX6 and VSX2 and photoreceptor precursor marker CRX on DD32. The cone marker RxRG and RGC marker Brn3 were observed in the apical and basal layers, respectively (Figures 1C and 1D). On DD120, PKC α -positive rod bipolar cells and Nrl-positive rod photoreceptor cells were present, and peripherin2 and rhodopsin were localized in the cilia (Figure 1D). In retinal mkiPSCs, on DD50 and DD239, MHC class I expression was lower than that in mkiPSC-retinal pigment epithelial cells (RPEs) or monkey B cells (positive controls) (Sugita et al., 2016), whereas treatment with interferon γ (IFN- γ) enhanced MHC class I expression in mkiPSC-retinal and RPE cells. MHC class II was expressed only in mkiPSC-RPE cells but not in mkiPSC-retinal cells (Figure 1E).

We then examined the competency of the mkiPSC-retina as a graft by transplanting them to 24-week-old SD-Foxn1 Tg (S334ter) 3LavRrrc nude rats, in which most of the rod photoreceptors were lost in the first several weeks of life (Seiler et al., 2014; Tu et al., 2019). We previously showed that ESC/iPSC-retinas transplanted at younger stages develop thick inner nuclear/plexiform layers (INLs/IPLs) to support the ONL, while more developed grafts tend to lose their integrity after transplantation; however, a thick INL may simultaneously hinder the host-graft contact (Assawachananont et al., 2014; Shirai et al., 2016). We therefore tested mkiPSC-retina transplantation at three developmental stages (DD30, DD41, and DD55). The grafts consistently developed rhodopsin-positive photoreceptors at DD126–DD137. As the DD30 grafts developed thick IPLs/INLs, we decided to use DD41–DD55 grafts for following transplantation studies (Figure S1A). We then tested the functional potency of mkiPSC-retinas by isolating dark-adapted grafted retinas from 59- to 60-week-old rats (DD291–DD299) and recorded light responses from the host RGCs using MEA recording systems ($n = 4$). In contrast to the rarely detected light response in RGCs of degenerated SD-Foxn1 Tg (S334ter) 3LavRrrc rats aged ≥ 60 weeks (Tu et al., 2019; Yamasaki et al., 2022), two of four transplanted retinas showed evident RGC responses to light over the grafted area (Figure S1B). These

(D) Immunostaining of DD120 mkiPSC-retinas. A RCVRN-positive photoreceptor layer formed on the apical side, which was positive for Nrl. RHO and PRPH2 were localized in the inner segment-like structures on the apical surface. The rod bipolar cell marker PKC α was expressed in the inner cells.

(E) Expression of MHC class I and II by mkiPSC-retinas of immature (DD50) and more mature (DD239) mkiPSC-retinas. MHC class I expression was low in the mkiPSC-retinas (NR), which increased with rIFN- γ treatment, and there was almost no expression of MHC class II. The MHC expression levels of mkiPSC-RPE and B cells (positive control) are presented for comparison. Blue line; isotype control; red line: HLA class I or class II. Scale bars: (A) 100 μ m; (B) 200 μ m; bottom right panel, 50 μ m; (C and D) 30 μ m. mkiPSC, monkey iPSC; mkiPSC-retina, monkey iPSC-derived retina; DD, differentiation day; NR, neural retina; MHC, major histocompatibility complex.



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indicated that mkiPSC-retinas are useful to study host immune responses to allogenic grafts.

Transplanted MHC-matched grafts survived with no clinical signs of rejection and with negative lymphocyte graft immune reaction tests

We first transplanted mkiPSC-retina into an MHC-matched (10154C) monkey eye after focal laser ONL ablation (Figure 2A). *In vivo* imaging showed stable graft survival with no clinical signs of rejection, such as fluorescein leakage on fluorescein angiography (FAG) or inflammatory responses on optical coherence tomography (OCT) (Figure 2B). Intra-graft speckles on OCT are likely to represent photoreceptor rosettes with inner/outer segment (IS/OS) components and microglia (MG) in later months. We then conducted a lymphocyte graft immune reaction (LGIR) test, which evaluates the proliferation of recipient peripheral blood mononuclear cells (PBMCs) against mkiPSC-retinas (Sugita et al., 2016) (Figures 2C and S2). Up to 3 months postoperatively, only natural killer cells (NKG2A) exceeded the 2-fold LGIR score, which was judged as negative for rejection (Figures 2C', 2C'', and S2C). Immunohistochemical staining was positive for mature retinal cell markers, including photoreceptor cells (recoverin and rhodopsin) and rod bipolar cells (PKC α) (Figure 2D). The photoreceptor cell layers developed mostly in a rosette form, showing enhanced rhodopsin expression in the IS/OS-like structures, while some cells expressed rhodopsin on cell membranes. Some photoreceptors in the rosettes were observed to contact the host bipolar cells (Figure 2D'). This was considered important, as we

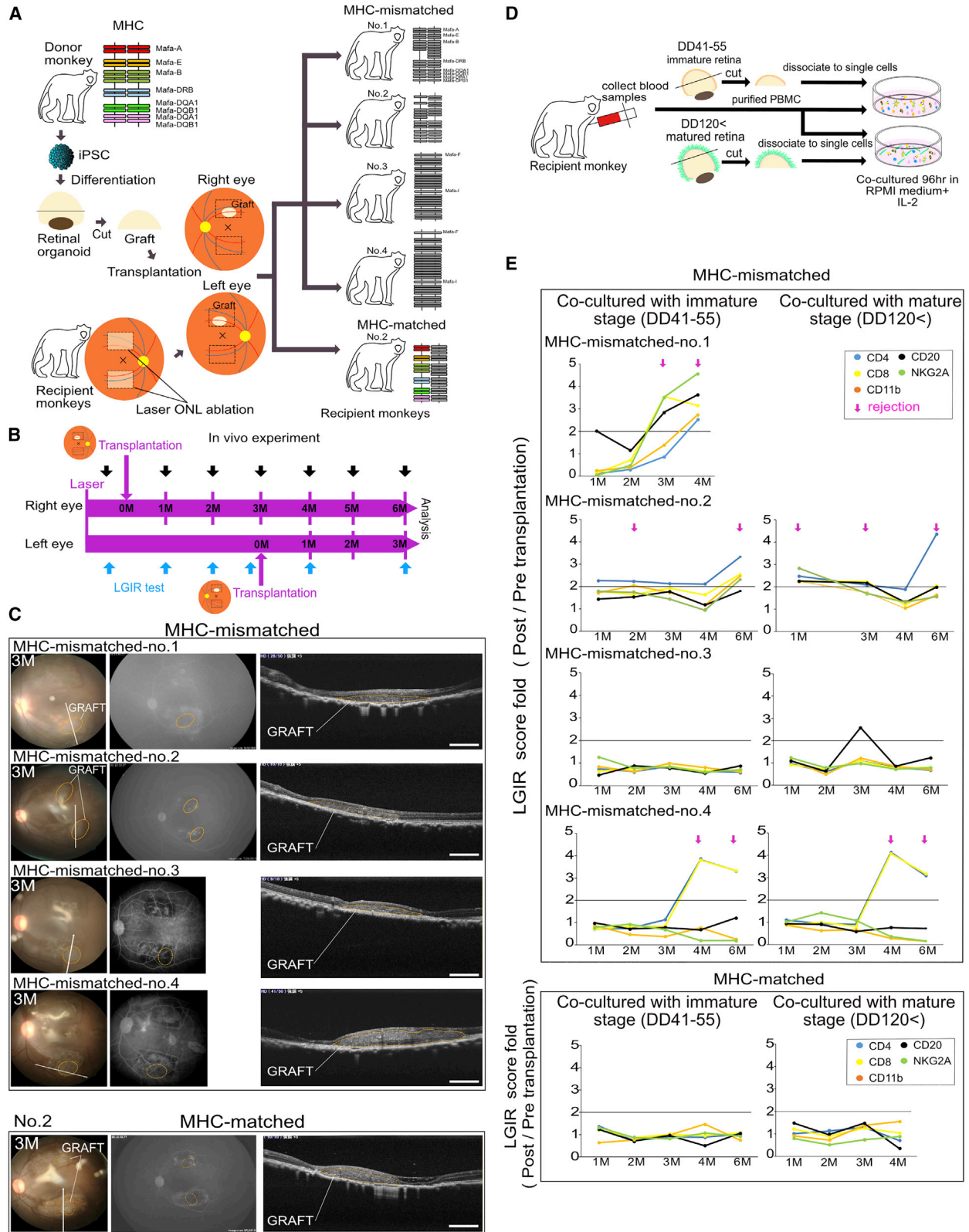
previously observed that contact between the host bipolar cells and graft photoreceptor cells, even in rosette forms, was correlated with host RGC light responses (Yamasaki et al., 2022). Infiltration of CD3-positive T cells in or around the graft were not observed, even in the displaced portion of the choroid. In contrast, ionized calcium-binding adapter molecule 1 (Iba1)-positive MG cells were distributed throughout the graft and inside the graft photoreceptor rosettes (Figures 2E–2E'').

LGIR test revealed subclinical rejection in MHC-mismatched transplantation

Next, we conducted transplantation in four MHC-mismatched monkeys and an additional MHC-matched monkey following the refined study design presented in Figures 3A and 3B. In clinical settings, some patients may wish to receive transplantation in both eyes or do so multiple times; however, a second transplantation may trigger rejection. To test this, the second eye was planned for mkiPSC-retina transplantation 3 months after the first transplantation. A maximum of two retinal grafts were transplanted when the situation allowed for optimization of the multiple sheet transplantation in preparation for clinical application. Table 1 presents the list of monkeys with the observed characteristic features. The haplotypes of the monkeys are summarized in the Table S1. All transplanted eyes (MHC matched or mismatched and without immune suppression) showed no evident clinical signs of rejection, even with repeated transplantation (Figure 3C, and other images in Figures S3 and S4). However, LGIR tests judged that three MHC-mismatched monkeys had subclinical rejection (mismatched

Figure 2. Graft maturation and immune responses in MHC-matched transplantation

(A) Summary of experimental outline for MHC-matched transplantation (top) and *in vivo* examinations and LGIR tests (bottom). (B) Time course of color fundus photographs and FAG and OCT images after transplantation. Orange circles in fundus images indicate the transplanted graft areas. FAG shows hyper-fluorescence due to RPE atrophy in the laser photocoagulated area. Each OCT image shows the sectional view of the line indicated by the white arrow in the color fundus image. The eye developed a macular hole following the vitrectomy surgery. Grafts survived up to 6 months without evident clinical signs of rejection. (C–C'') Schematic images of LGIR test (C). Flow cytometry analysis of recipient PBMCs in LGIR test. Fluorescence-activated cell sorting (FACS) plot results of the proliferative population (Ki-67-positive) in each immune cell type as labeled on the left with LGIR score shown above each FACS plot (C'). Ratio changes in LGIR scores of recipients PBMCs at 1, 2, and 3 month posttransplantation (C''). LGIR score increased by more than 2-fold only in NKG2A, and overall LGIR judgment was negative. (D and D') Immunostaining of RCVRN (photoreceptors), RHO (rod photoreceptors), and PKC α (rod bipolar cells) after transplantation. Low-magnification image of the transplantation site. Dashed white line indicates the area of the transplant, part of which strays into the choroid (D). Graft photoreceptor cells mostly form rosettes. Magnified view of the photoreceptor rosette adjacent to the host INL (white dotted box in D) (D'). Some photoreceptors express RHO at the outer segment-like structures inside the rosette, whereas others express rhodopsin on the cell membrane. Host PKC α + bipolar cells contact the graft photoreceptor cells. (E and E') Immunostaining of CD3 (T cells) and Iba1 (MG). Low-magnification image of the transplantation site (E). The graft is outlined with a dotted white line. There is no evident infiltration of lymphocytes within or around the grafts. MG are observed throughout the graft, mostly inside the rosette. Magnified view of the displaced graft in the choroid in the white dotted box in (E), with no infiltration of lymphocytes (E'). Positive immunostaining for CD3 in a monkey spleen (E''). Scale bars: (B) 1 mm; (D and E) 200 μ m; (D', E', and E'') 30 μ m. DD, differentiation day; LGIR, lymphocyte graft immune reaction; FAG, fluorescein angiography; OCT, optical coherence tomography; MG, microglia; INL, inner nuclear layer; PBMC, peripheral blood mononuclear cell.



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nos. 1 and 2 after the first transplantation and mismatched no.4 after the second transplantation; [Figures 3D and 3E](#)). As the grafts were transplanted at the retinal progenitor stage and further developed after transplantation, the LGIR test was conducted against the mkiPSC-retinas of both DD41–DD55 and DD120 <. The results were mostly consistent between the immature and developed retinas.

Transplanted grafts developed photoreceptors and expressed synapse markers irrespective of MHC matching

We then evaluated graft maturation using immunohistochemistry. A low-magnification image of the left eye (3 months after transplantation) of mismatched no.2 is representatively shown. Recoverin-positive photoreceptors were mostly deleted in the ONL-ablated area, and the graft was placed within the lesion ([Figure 4A](#)). Transplanted mkiPSC-retinas consistently developed recoverin/rhodopsin-positive photoreceptors in the left (3 months after transplantation) and right eyes (6 months after transplantation) of all MHC-matched and mismatched monkeys, except for the unexamined right eye of mismatched no.2, which developed retinal detachment after oil removal/cataract surgery at 5 months ([Table 1](#)). Well-organized photoreceptor rosettes expressed rhodopsin/cone opsins and peripherin II at the OS-like structures inside the rosette, whereas some disorganized rosettes expressed opsins on the cell membrane ([Figures 4B and 4C''](#)). The ratio of the well-organized rosettes varied from eye to eye, indicating that there may still be room for optimization in mkiPSC-retinal differentiation. Rarely, part of the mkiPSC-retinas developed a retina with a full IPL/INL/ONL, with the presence of photoreceptor synapses positive for CtBP2 and mGluR6 (mismatched no. 4; [Figures 4D and 4E'](#)). Pairs of pre- and postsynaptic markers CtBP2 and mGluR6 were also observed on the margin of the graft photoreceptor rosettes at the host-graft interface and inside the graft (MHC-matched no. 2; [Figures 4F–4F'''](#)).

Presence of activated MG was observed in MHC-matched and -mismatched transplantations

Next, we performed histological assessment of immune cell infiltration. Although subclinical immune rejection was indicated in three of four mismatched monkeys by LGIR, histologically, we observed no evident infiltration of CD3-positive T cells into the subretinal graft in these eyes. However, clusters of CD3-positive cells were observed in the displaced graft in the choroid, and the strayed graft was absent only in the MHC-mismatched monkey that was negative for LGIR ([Figures 5A and 5B'](#); [Table 1](#)).

We then took a closer look at the MG by staining sections with a marker for Iba1. MG were present in both the MHC-mismatched and -matched grafts. Interestingly, most MG in the subretinal graft had ramified shapes ([Figures 5C and 5D](#)), while those in the displaced grafts in the choroid presented ameboid shapes ([Figures 5A' and 5B'](#)). We then co-stained an activation marker, MHC class II, with Iba1 to determine whether there was site-specific MG activation in three regions: the transplant, untreated, and laser injury sites ([Figures 5C', 5C'', 5D', and 5D''](#)). MHC class II-positive MG were not observed in the untreated area, but some MG seemed activated in laser injury and transplant sites. We analyzed the degree of MG migration in the subretinal graft by quantifying the area of Iba1-positive regions within the grafts. There was no evident difference between the eyes due to the large variance ([Figures 5E and 5F](#)).

These results suggest that MG activation or accumulation was not evidently different between MHC-matched and -mismatched transplantation; however, activated MG after laser injury may trigger rejection in mismatched transplantation.

mESC-retina transplantation in an MHC-mismatched strain elicited host light responses to a lesser degree than matched transplantation

MHC class I molecules are expressed by neurons and contribute to synapse elimination or modification during

Figure 3. LGIR tests detected subclinical rejection in repeated MHC-mismatched transplantation

(A) Schematic overview of MHC homozygote mkiPSC-retina transplantation in 4 MHC-mismatched monkeys and 1 matched monkey. (B) Experimental schedule of *in vivo* experiments and LGIR tests. The second transplantation (left eye) was planned 3 months after the first transplantation (right eye). (C) *In vivo* images of the color fundus photographs and FAG and OCT images of the eyes that received the second transplantation 3 months after transplantation (see [Figures S3 and S4](#) for the first eye images and for the different timings). Orange circles indicate graft areas. Each OCT image shows the sectional view of the line indicated by the white arrow on the color fundus image. (D) Schematic image of LGIR test for the repeat experiment. Recipient PBMCs are co-cultured with mkiPSC-retinas at two developmental stages (DD41–DD55 and DD120<). (E) Line plotting results for LGIR tests following transplantation. Vertical lines indicate post/pre-transplantation LGIR scores. Horizontal lines indicate months after transplantation. Magenta arrows show the time points when the post/pre-LGIR scores were above 2-fold in 2 or more cell types after transplantation (subclinical rejection). LGIR test, lymphocyte graft immune reaction test; DD, differentiation day; FAG, fluorescein angiography; OCT, optical coherence tomography. Scale bars: (C) 1 mm.

**Table 1. Summary of LGIR test and immunological features of transplanted eyes in histology**

MHC	Mismatched								Matched		
Monkey number	1		2		3		4		1	2	
Operate eye	R	L	R	L	R	L	R	L	R	R	L
Postsurgical events	no		retinal detachment after cataract/oil removal surgery	no	cataract	no	no	retinal damage	no	no	no
Immunohistology	cryosection		cryosection	paraffin	cryosection	paraffin	cryosection	paraffin	cryosection	cryosection	paraffin
LGIR test	positive		positive		negative		positive		negative	negative	
Iba1-positive cell infiltration in choroidal tissue	+	+	ND	+	+	+	+	+	+	+	+
Iba1-positive cell infiltration in graft	+	+	ND	+	+	+	+	+	+	+	+
Graft maturation	+	+	ND	+	+	+	+	+	+	+	+
Strayed graft cells in choroid	+	–	ND	+	–	–	+	+	+	+	–
T cell infiltration in choroidal graft tissue	+	–	ND	+	–	–	+	+	–	–	–
T cell infiltration in subretinal graft	+/–	–	ND	–	–	–	–	–	–	–	–

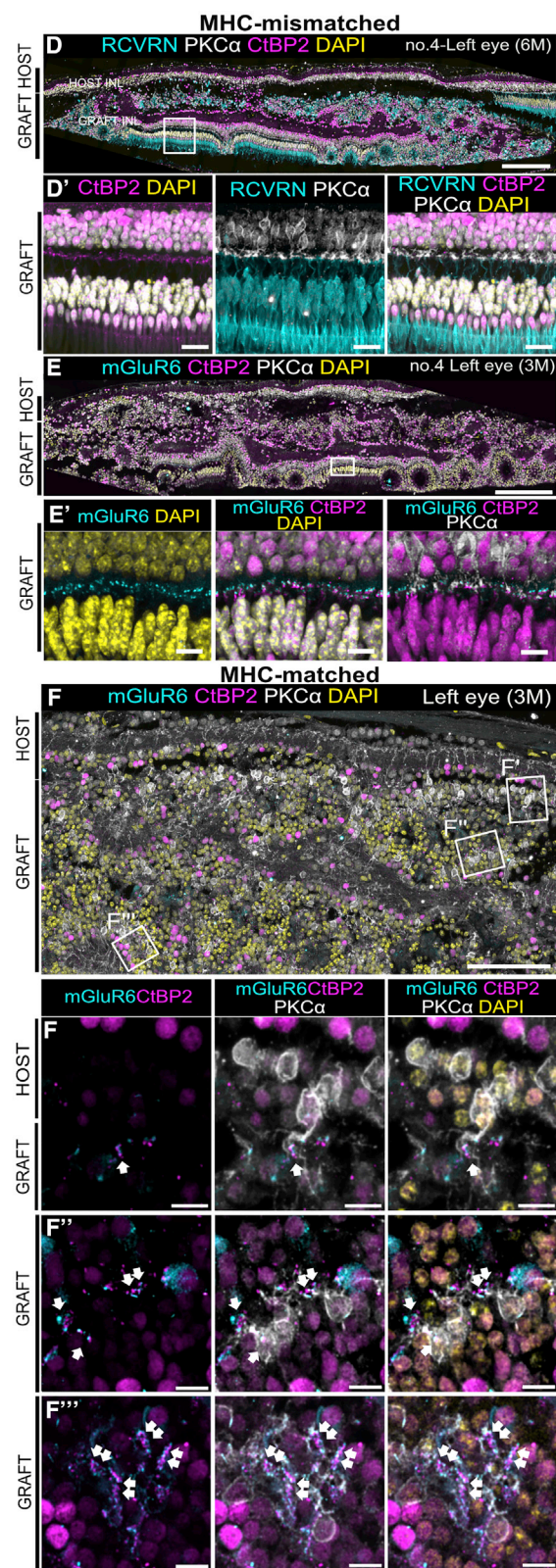
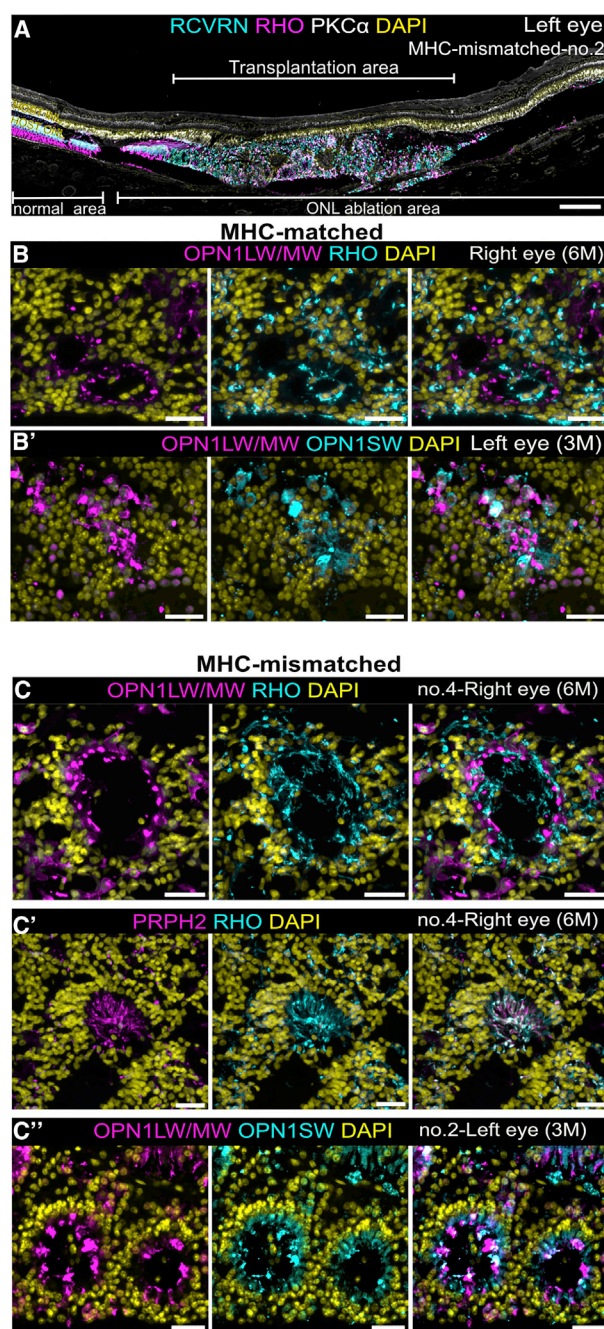
ND, not determined. Indicated immunohistology was not evaluated in these eyes (mismatched 2R had retinal detachment). Immunohistology features the state of 6 months after transplantation in the right eye and 3 months after transplantation in the left eye.

CNS development (Huh et al., 2000), and MG are known to play a role in synapse maintenance (Wang et al., 2016). Thus, although MHC matching may not affect graft development, it may still affect functional host-graft integration. To investigate this, we used the mESC-retina of the C57BL/6J line that expresses CtBP2:tdTomato under the Nrl promoter (Matsuyama et al., 2021) and transplanted them into 10- to 12-week-old rd1-2J (C57BL/6J) and C3H/HeJ mice with end-stage retinal degeneration (Figure 6A). The transplanted mice were administered indomethacin per our routine protocol; however, no immunosuppressive drugs were administered. The transplanted grafts developed recoverin- and rhodopsin-positive photoreceptor rosettes in both strains (Figure 6B). Host bipolar cells extended dendrites to graft rod photoreceptor cells expressing the reporter CtBP2:tdTomato in synaptic terminals (Figures 6B and 6B'), some of which were also stained for the postsynaptic marker mGluR6 (Figures 6C–6C'). CD3-positive lymphocyte infiltration was not evident at 14 or 28 days after transplantation in either strain, while Iba1-positive MG were present similarly around and inside photoreceptor rosettes in both strains (Figure 5S). Retinas were then isolated for MEA recordings 8–10 weeks after transplantation. Figure 6D shows an example of a peristimulus time histogram of the host RGC light responses

overlaid on the mapped graft location. The probabilities of host RGC responses to different light intensities from all retinas in each group are plotted in Figure 6E. We then modeled the response probability using a logistic regression as described previously (Matsuyama et al., 2021), considering the host (whether the transplanted animal was rd1-2j [matched] or C3H [mismatched]), stimulus strength (weak, medium, strong), L-AP4 treatment (before, L-AP4, after), sample bias, and spontaneous RGC firing (spiking rate before light stimulation). The transplanted retinas showed a higher probability of RGC responses to light than the untransplanted controls in both the rd1 and C3H samples, although the response probability was generally lower in C3H, i.e., mismatched, retinas (Figure S6).

DISCUSSION

Among cell-based therapies for degenerative diseases with a genetic background, allogeneic transplantation is the primary approach in which immunological control is an essential issue. We have previously shown that mESC/iPSC- and hESC-retinas can form synapses with host bipolar cells and significantly enhance light responses in host



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RGCs (Matsuyama et al., 2021; Yamasaki et al., 2022). In this study, we specifically investigated immunological responses in primates by optimizing a differentiation protocol for mkiPSC-retinas using homologous MHC cell lines to transplant them in MHC-matched or -mismatched monkeys. mkiPSC-retinas were functionally similar to hESC/iPSC-retinas in xeno-transplantation in rat retinal degeneration models, eliciting light responses in the host RGCs in roughly half of the transplanted rat retinas. The reason for this limited ratio is considered partly due to the presence of graft bipolar cells because genetic deletion of graft ON-bipolar cells in hESC-retinas further improved RGC responsiveness when transplanted in rats (Yamasaki et al., 2022).

Although the eye is an immune-privileged site, it is well estimated that in diseased eyes, the immunological environment is also altered when the blood-retinal barrier is impaired. Here, laser-induced ONL ablation disrupted the blood-retinal barrier (Berkowitz et al., 1991); however, transplants consistently developed rod- and cone-opsin-positive photoreceptor cells in both MHC-matched and -mismatched transplantations, even without immune suppression. However, using the LGIR test, three of four mismatched monkeys were judged to have subclinical rejection. LGIR has already been used in our clinical study of hiPSC-RPE transplantation for neovascular age-related macular degeneration (AMD), which sensitively detected immune rejection associated with a very early clinical manifestation (Sugita et al., 2020). Histologically, CD3 lymphocytes were rarely observed in the subretinal graft but were detected in or around the strayed grafts only in the choroid in mismatched monkey eyes. As we sometimes experienced technical difficulties inserting multiple grafts in these experiments, the strayed grafts could have been a surgical artifact, in addition to the effect of laser injury. Notably, strayed graft in choroid was absent only in the MHC-mismatched monkey that was negative for LGIR. This suggests that displaced allogenic grafts evoked immune responses outside the immune-privileged ocular site and that the LGIR test sensitively reflected these mild

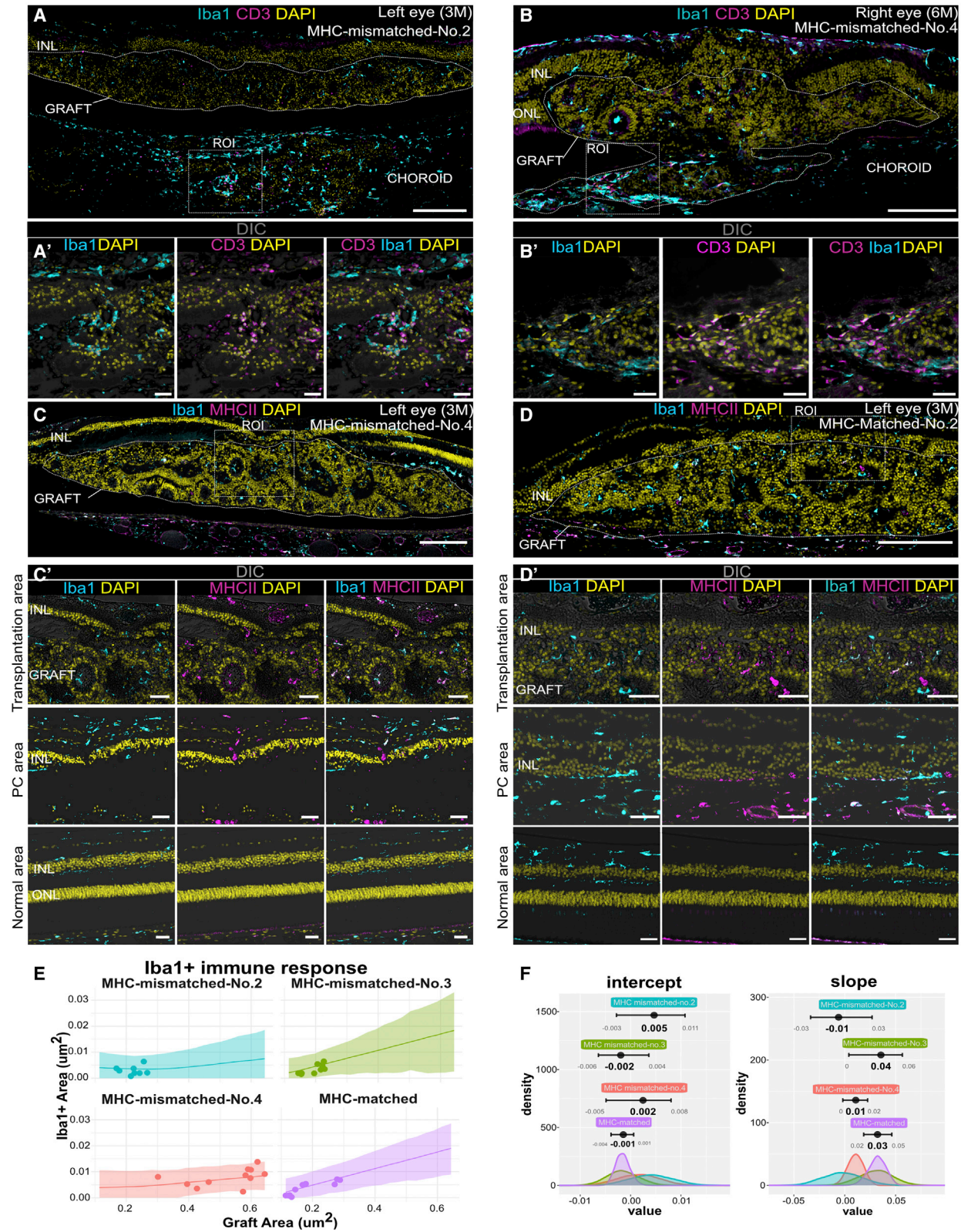
immune responses (Table 1). Inversely, disruption of the blood-retinal barrier by laser injury or mechanical surgical trauma could alter the immune-privileged environment and may increase the risk of subretinal graft rejection. From our limited results, we could not conclude that a second transplantation may boost the immune response. Altogether, despite the low immunogenicity and suppressive nature of hESC/iPSC-retinas, they may evoke an immune response, and LGIR may be a sensitive tool that detects such subclinical rejection. The use of immunosuppressants based on LGIR results may help prolong the survival of the graft. Additionally, LGIR tests using immature and developed retinas suggested that the risk of rejection did not seem to change according to the developmental stage of the graft after transplantation. It is also notable that in our LGIR tests, lymphocyte proliferation was initially suppressed shortly after transplantation in three of four mismatched monkeys. This may be consistent with our previous report that hESCs/iPSCs and monkey retinas actively suppress lymphocyte activation *in vitro* (Yamasaki et al., 2021).

Histologically, MG were consistently present in the grafts of both matched and mismatched transplantations. Retinal MG are important for immune modulation, such as antigen presentation, phagocytosis of dead cells, and pruning synapses in neural networks (Rathnasamy et al., 2019). MG in the graft may also eliminate damaged or non-integrating cells in the graft and contribute to host-graft or intra-graft synapse remodeling; however, they may also evoke host immune responses to donor-specific antigens. Here, MG were activated at the laser injury site in both MHC-mismatched and -matched eyes, implying that the blood-retinal barrier was disrupted by laser treatment. This may make MG more responsive to antigens in mismatched transplantations, and modulation of MG may reduce the risk of rejection.

A fully developed retina is rarely observed, typically appearing when the graft is young enough to develop a thick IPL/INL, such as in a DD30 graft that develops thick

Figure 4. Maturation of photoreceptors and photoreceptor synapses in the grafts for MHC-mismatched transplantation

(A) The low magnification image of the left eye of MHC-mismatched no. 2 representatively shows the ONL-ablated area and the graft. (B and C) Graft photoreceptor rosettes in MHC-matched (B and B') and mismatched (C–C'') eyes. OPN1SW/LW/MW and PRPH2 are localized at the outer segment-like structures inside the well-organized photoreceptor rosettes and are expressed on the cell membrane in disorganized rosettes. (D and D') Rarely, the graft develops an almost full retina with IPL and INL, including synapse formation within the graft, as suggested by the localization of the presynaptic marker CtBP2 at the contact site between graft bipolar dendrites and graft photoreceptor axons (mismatched no. 4). CtBP2 has a nuclear background staining. Magnified view of the box in (D) (D'). (E and E') The pre- and postsynaptic markers CtBP2 and mGluR6 were localized at the photoreceptor synapses in the same eye in (D). Magnified view of the box in (E) (E'). (F–F'') Pairs of mGluR6 and CtBP2 (arrows) on the margin of the graft photoreceptor rosettes, adjacent to the host INL (F') and within the graft (F''). Magnified views of boxes in (F) (F'–F''). Scale bars: (A, D, E, and F) 200 μ m; (B, B', and C–C'') 30 μ m; (D', E', and F'–F'') 20 μ m.



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IPLs/INLs and large photoreceptor rosettes. These thick INL/IPL structures seem to block host-graft synaptic contact; therefore, we used a later-stage graft that could still form a photoreceptor layer, albeit mostly in a rosette form (Assawachananont et al., 2014; Shirai et al., 2016). These rosettes are considered disadvantageous in terms of retinoid cycling as they do not contact RPEs. However, host RGCs are ready to respond to light in MEA experiments without 9-*cis* retinal supplementation (Yamasaki et al., 2022), which implies that visual pigments are restored in photoreceptor cells, even in rosettes, after dark adaptation. This also suggests that the photoreceptor cells in rosettes can function *in vivo*. Additionally, Müller cells, which aid in retinoid recycling in cone photoreceptors, and horizontal cells, which are part of the photoreceptor triad synapses, are present in ESC/iPSC retina grafts (Matsuyama et al., 2021; Yamasaki et al., 2022). This implies that ESC/iPSC-retina provides a supportive environment for graft photoreceptor function. Interestingly, in our previous study, the expression of glial fibrillary acidic protein (GFAP) and/or HLA class I was enhanced in the non-integrating, reversely oriented parts of the rosettes. In addition, long-surviving graft photoreceptors presented hemispherical structures with the OSs correctly oriented toward the RPE, implying that the reversed parts of photoreceptor rosettes are prone to degeneration over time (Tu et al., 2019; Yamasaki et al., 2022).

Whether host and graft cells can form *de novo* synapses is a major concern in retinal sheet transplantation. We have shown the presence of host-graft synapse formation in mice with reporter bipolar cells and in a graft with the CtBP2-tdTomato reporter at the photoreceptor synaptic terminal (Mandai et al., 2017; Matsuyama et al., 2021). In another study, we used hESC-retinas with genetically reduced ON-bipolar cells, in which ON-bipolar cells of mostly host origin contacted graft photoreceptors presenting various pre- and postsynaptic markers (Yamasaki et al., 2022). These results suggest the potency of mkESC/iPSC-

retinas to form host-graft synapses in monkey eyes, and our results showed that mkIPSC-retinas express photoreceptor synapse markers. However, it was difficult to clearly define the host-graft synapses in the present experimental setting.

Although MHC-mismatched transplantation seems acceptable, MHC molecules in the CNS are known to participate in synaptic pruning and the plasticity of the developing visual cortex in an activity-dependent manner (Zhang et al., 2013). Thus, we studied the functional aspects of MHC-matched and -mismatched mouse models. The overall mouse transplantation results were consistent with those of the monkey transplantations: there was no lymphocyte infiltration even with MHC-mismatched transplantation, and synaptic markers were present in the grafts of both MHC-matched and -mismatched transplantations, suggestive of host-graft synaptic contact. However, RGC light responsiveness was better in the C57BL/6 (matched) strain than in the C3H (mismatched) strain, possibly due to the MHC-matching process or differences in the pathological backgrounds of the host mice or in the host strain itself. If available, experiments using wild-type C3H grafts in rd1-2J and rd1(C3H) host mice would help clarify the effect of MHC matching on graft functional integration. We previously showed that C57BL/6J iPSC-retinas elicit host RGC light responses in immune-deficient NOG-rd1 mice, suggesting that host-graft functional integration is also achieved with MHC-mismatched transplantation, provided that immune rejection is controlled. Whether MHC matching affects the efficiency of functional integration requires further study.

In conclusion, we showed that in non-human primate (NHP) and mouse models, MHC-mismatched transplantation of ESC/iPSC-retinas could achieve graft maturation without evident clinical signs of rejection, even without systemic immune suppression, and that functional integration may be achieved. However, subclinical immune

Figure 5. Histological immune responses after transplantation

(A and B) Immunostaining of lymphocytes (CD3) and MG/macrophages (Iba1) at 3 months (MHC-mismatched no. 2, left eye) and 6 months (MHC-mismatched no. 4, right eye) after transplantation. Lymphocytes were barely observed in the subretinal graft, but lymphocytes and MG/macrophages were accumulated in the choroid near the graft (A, A', B, and B') or within the strayed graft in the choroid. Magnified view of the box in (A) and (B) (A' and B').

(C and D) Some MG co-localized with an activated marker MHC II in MHC-mismatched no. 4 and MHC-matched no. 2. Immunostaining of MG/MHC II in the graft, laser, and untreated areas, from top to bottom, with magnified view of graft site from the white box in (C) and (D) (C' and D'). MG in the normal area is not activated, while some MGs were positive for MHC II at grafted and laser sites.

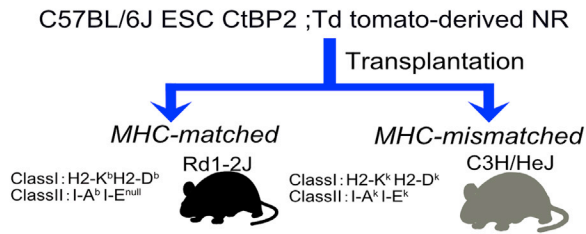
(E) Quantitative analysis of the glial area of Iba1-positive regions plotted against the areas of the grafts (dots). The model's (linear regression) posterior predictions are superimposed, with lines indicating the mean values and shaded areas indicating the 95% compatibility intervals.

(F) Posterior distributions for the intercept and slope of the linear regression, with bars on top indicating the mode and 95% compatibility intervals.

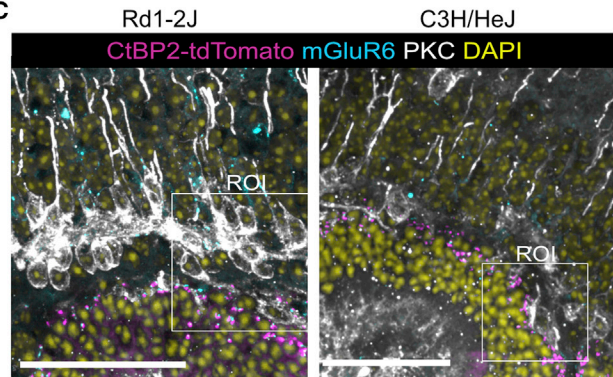
Scale bars: (A–D) 200 μ m; (A' and B') 30 μ m; (C' and D') 50 μ m.



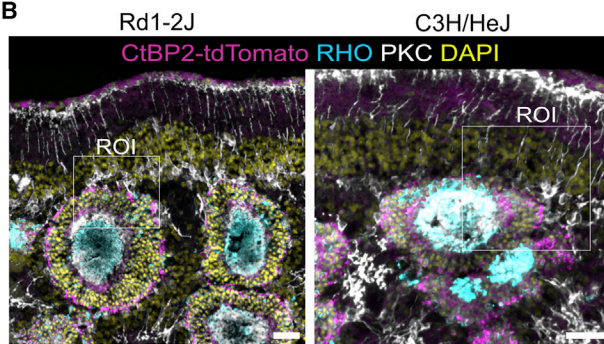
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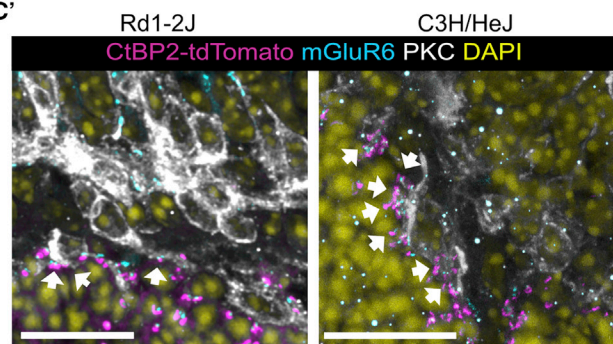
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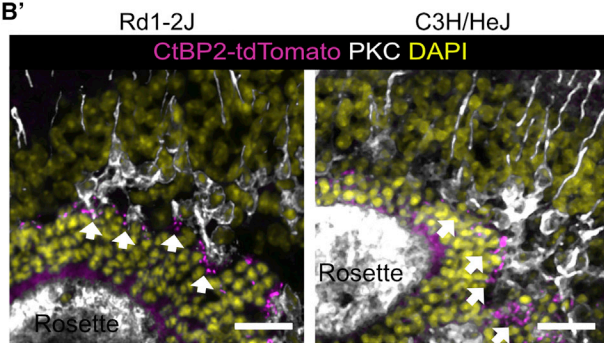
B



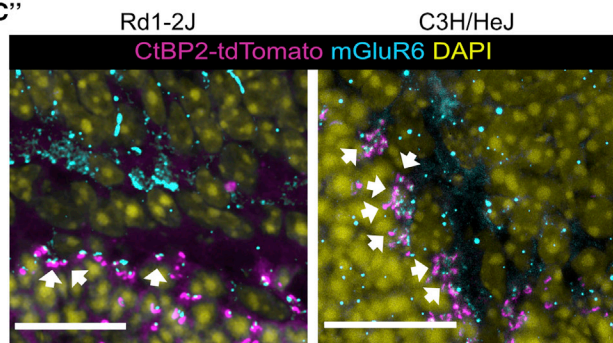
C'



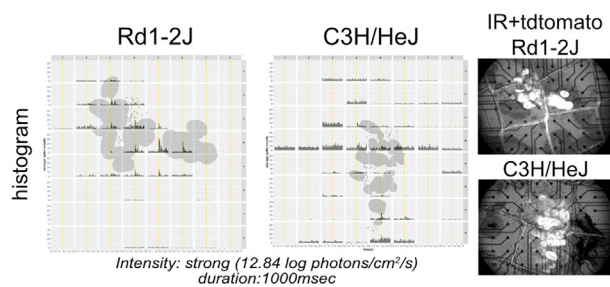
B'



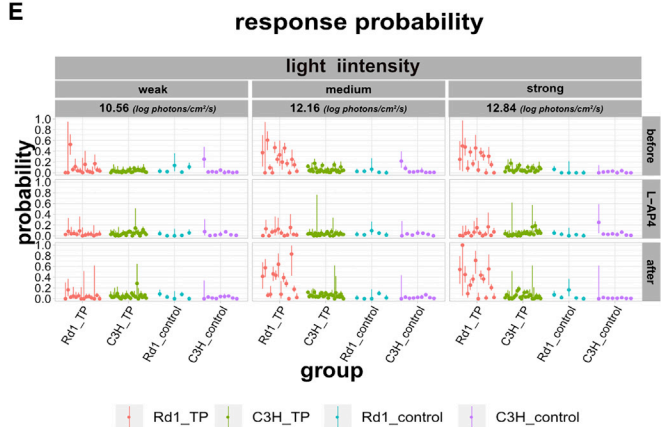
C''



D



E



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rejection has been implied by LGIR assays in MHC-mismatched transplantation in primates, which may affect long-term survival and functional integration. This suggests that with allogenic ESC/iPSC-retinal transplantation, even with stable graft survival, careful observation through LGIR monitoring is useful, and the effect of MHC matching on functional integration should be further investigated.

EXPERIMENTAL PROCEDURES

Animal experiments were approved by the RIKEN Biosystems Dynamics Research Ethics Committee (A2008-02-19). All animals were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for Animals in Ophthalmology and Vision Research.

Animals

Cynomolgus macaques (*Macaca fascicularis*) were obtained from Ina-Research or Eve Bioscience. SD-Foxn1 Tg (S334ter) 3LavRrrc nude rats were obtained from the Rat Resource and Research Center. C3H/HeJ mice were obtained from Japan SLC, and rd1-2J mice were obtained from Jackson Laboratory. Animals were exposed to a 12 h light/dark cycle, and mice and rats had free access to food and water.

MHC typing of monkeys

Genotyping of two MHC-controlled monkeys (10154C, 9471 B) and two other monkeys (DrpZ11-34C-F, DpC16-8B-J) was conducted by Ina-Research, and the other monkeys were genotyped by Tokai University based on a previous report (Shiina et al., 2015).

Retinal organoid induction from mkiPSCs

The 1121A1 mkiPSC (HT1 homo) line was established and provided by the Center for iPS Cell Research and Application (CiRA) (Okamoto and Takahashi, 2011; Sugita et al., 2016). For retinal differentiation, based on previous reports, we adapted the serum-free floating culture of embryoid body-like aggregates through quick reaggregation (SFEBq) using induction-reversal culturing (Kuwa-

hara et al., 2015; Nakano et al., 2012), with modifications. The detailed culture protocol is described in the [supplemental experimental procedures](#).

LGIR test for monkeys

The detailed procedure of the LGIR test has been described previously and in the [supplemental experimental procedures](#) (Sugita et al., 2016). The LGIR test was conducted at the time points described for each experiment. PBMCs were isolated from the blood and stored at -80°C in CELLBANKER1 plus (Nippon Zenyaku Kogyo) freezing medium until use. Two different developmental stages of iPSC-retinas were used as immunogens: cells at DD41–DD55 as the retinal progenitor stage for transplantation, and cells at >DD120 as the more developed stage. We evaluated the ratio of Ki67-positive lymphocytes to other immune cell markers such as CD4-positive helper T cells, CD8-positive cytotoxic T cells, CD20-positive B cells, CD11b-positive monocytes/macrophages, and NKG2A-positive NK cells using flow cytometry. We evaluated the proliferating populations of these five types of immune cells among PBMCs co-cultured with mkiPSC-retinas. Compared with 0 W, it was considered as “rejection” when there was a 2-fold or greater Ki-67-positive population in at least two of the above cell types.

MEA recordings

As described previously and in the [supplemental experimental procedures](#), MEA recordings were conducted with isolated retinas 8–10 weeks after transplantation using the USB-MEA60-Up-System (Multi-Channel Systems, Reutlingen, Germany) (Iraha et al., 2018; Tu and Matsuyama, 2020; Tu et al., 2019).

The procedures for the focal photoreceptor ablation in the monkey eyes, transplantation of mkiPSC-retinas in the monkey eyes, *in vivo* imaging after transplantation, quantification of Iba1-positive cells, statistical analysis, retinal organoid induction from mESCs, transplantation in rd1 mice, immunostaining, and analysis of MEA data are described in the [supplemental experimental procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.stemcr.2022.09.014>.

Figure 6. C57BL/6J mouse ESC-derived retinas functionally integrate in the host rd1 C57BL/6J and C3H/HeJ mice

- (A) Schematic images of experimental design for transplantation of mouse ESC-derived retinal organoids in rd1-2J (C57BL/6), i.e., matched or mismatched, mice.
- (B and B') Immunohistology of graft cells 4 weeks after transplantation. ESC-retinas have matured in both the rd1-2J and C3H/HeJ strains, and the dendrite tips of the host PKC α -positive bipolar cells contact the CtBP2:tdTomato reporter expressed at the synaptic terminal of the graft photoreceptors. Magnified views of the white box in (B) (B').
- (C–C'') Pairs of graft CtBP2:tdTomato reporter and mGluR6 immunostaining at the host rod bipolar dendrites. Magnification of white box in (C) (C'').
- (D) Representative peristimulus histograms at each channel, with overlaid graft mapping of the electrodes in the retinas of both host strains, of before, during, and after washing of pharmacological ON-blocker treatment.
- (E) The probability that the RGCs would respond to light was plotted for each retina with three different light stimuli before, during, and after washing with L-AP4 treatment. This was performed in each group of two strains with and without transplantation (rd1-transplantation: n = 15; rd1-control: n = 5; C3H/HeJ-transplantation: n = 29; C3H/HeJ-control: n = 9).
- Scale bars: (B and C) 50 μm ; (B', C', and C'') 20 μm .



AUTHOR CONTRIBUTIONS

M.M., S.S., and M.T. designed and supervised this study. S.Y. and H.U. differentiated the mkiPSC-retinas. S.S. and H.U. conducted the LGIR tests. Y.K., H.U., T.W., and M.M. conducted the transplantation experiments. H.Y.T., H.U., and T.M. conducted the MEA experiments and analyses. H.U. and T.M. conducted the immunohistochemistry procedures and associated analyses. H.U., M.M., H.Y.T., T.M., and S.S. wrote the manuscript.

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CONFLICTS OF INTERESTS

S.Y. is an employee at Sumitomo Pharma Co., Ltd. M.T. is a founder and the president of Vision Care, Inc., and a board member of Sysmex.

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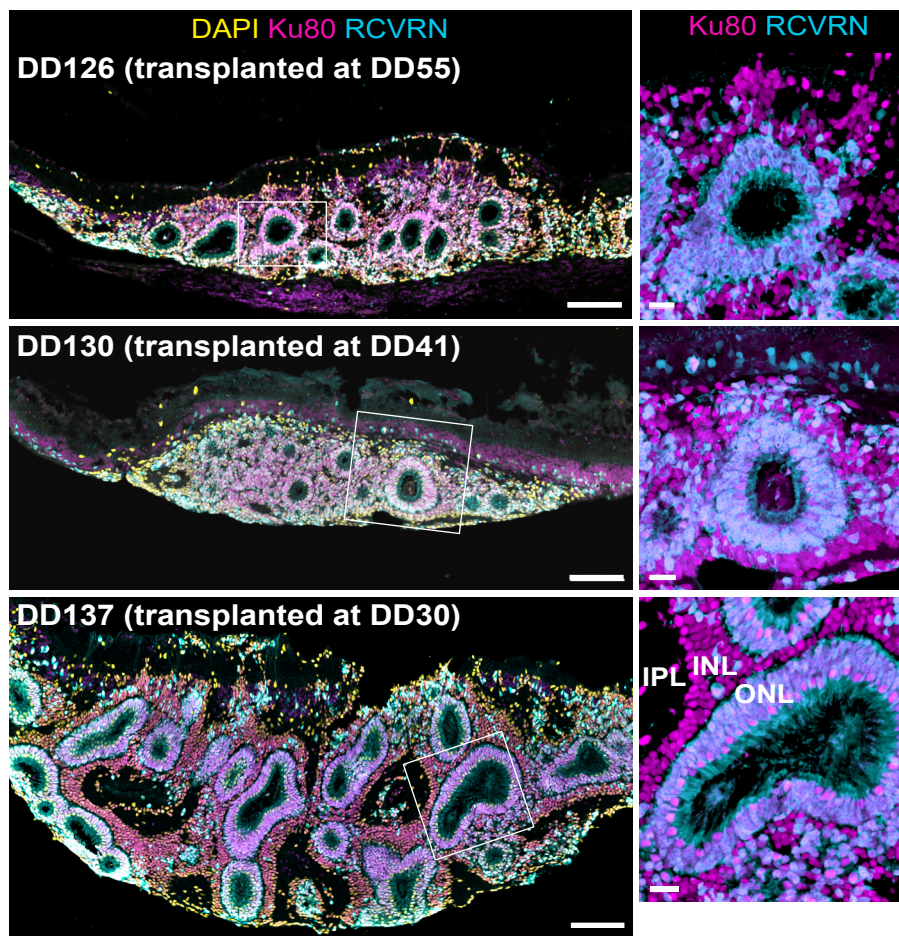
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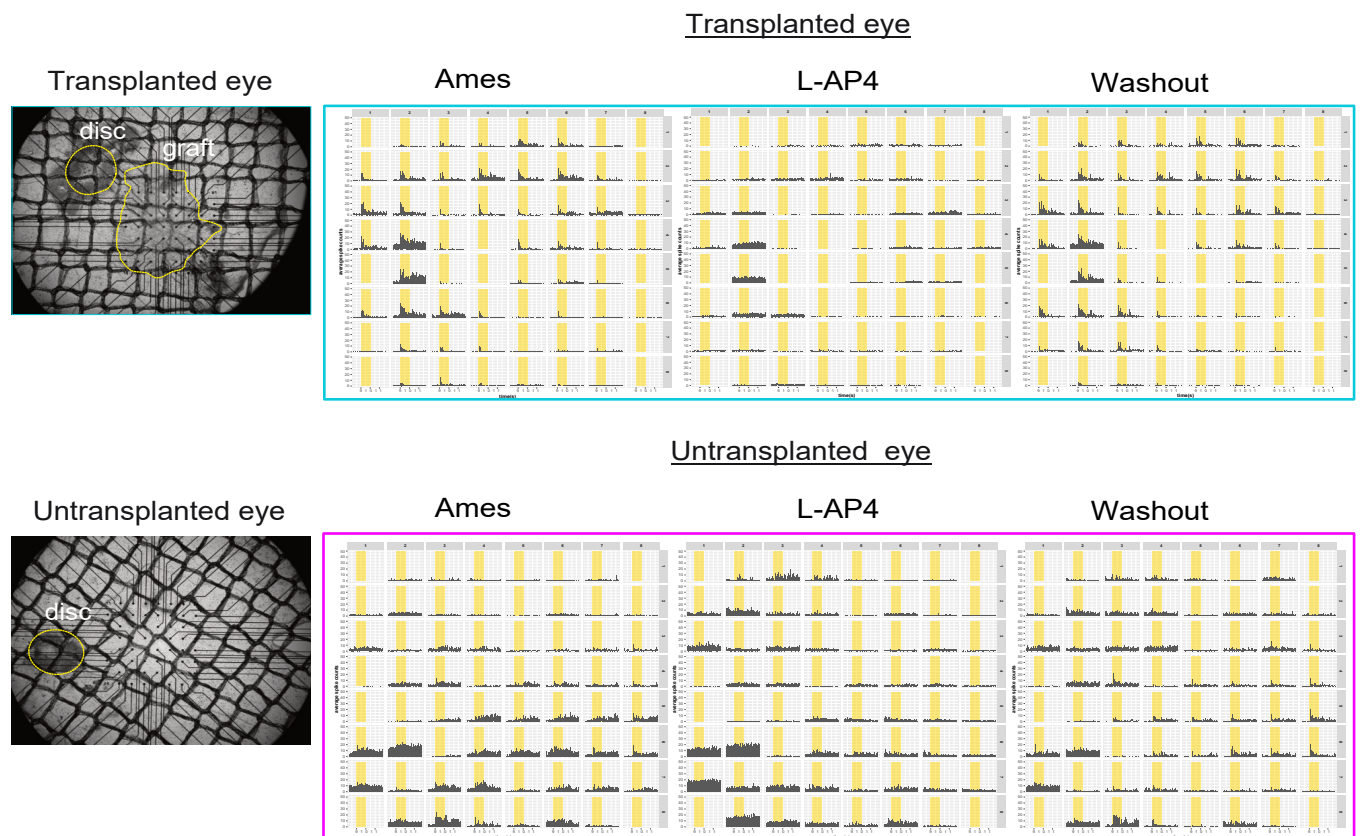
Competency of iPSC-derived retinas in MHC-mismatched transplantation in non-human primates

Hirofumi Uyama, Hung-Ya Tu, Sunao Sugita, Suguru Yamasaki, Yasuo Kurimoto, Take Matsuyama, Takashi Shiina, Takehito Watanabe, Masayo Takahashi, and Michiko Mandai

A



B



intensity: 12.84 log photons/cm²/s
duration: 1000 msec

Figure S1. Maturation and functional potency of 1121A1 monkey Major Histocompatibility Complex (MHC) homozygote HT1 iPSC-retinas after transplantation in rats with retinal degeneration, related to Figure1.

- A. The maturation of mkiPSC-retinas after transplantation in SD-Foxn1 Tg (S334ter) 3LavRrrc nude rats. Subretinal transplantation of mkiPSC-retinas at DD30, 41, and DD55 into the eyes of nude rats with retinal degeneration consistently resulted in recoverin-positive photoreceptor rosettes. The magnified image of the box in the left image is shown on the right. Transplantation of the DD30 graft results in a thick IPL/INL structure with large photoreceptor rosettes.
- B. The mkiPSC-retina elicited light responses after transplantation, as recorded by MEA. The isolated retina with transplants was mounted on MEA electrodes, and the other eye was used as a negative control. The spikes were recorded with a high-pass filter at 1 kHz. Peristimulus histograms are plotted as a sum of averaged responses of all detected RGCs with three repeated stimulations of 1000-msec 12.84 log photons/cm²/s after spike sorting at each channel under three conditions: before, during, and after ON-blocker L-AP4 treatment.

Scale bars: A; 100μm, magnified images; 20μm,

Abbreviations: DD, differentiation day; MEA, multiple electrode arrays; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell

A

mkiPSC-retina (Donor)

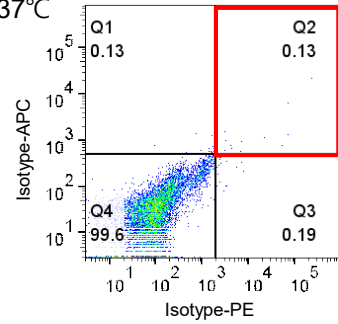
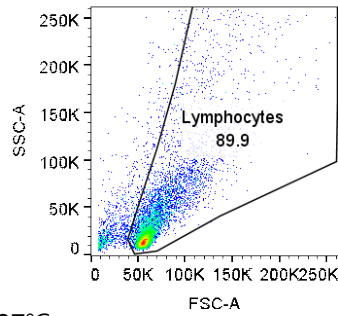
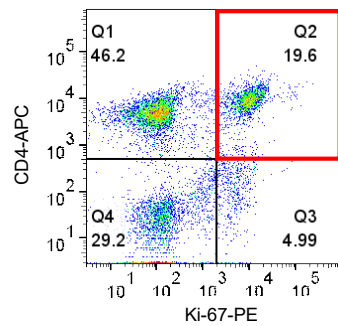
Cut

Neuron Dissociation
solution for 10 min at 37°C

NR single cells

Co-cultures

Recipient PBMC

BSet of isotype
control gating (<1%)
using control absDouble positive
cells (%)**C****LGIR score=**

$$\frac{\text{Co-culture PBMC}}{\text{PBMC only}} \text{ Double positive cells (\%)} = \text{LGIR score}$$

**LGIR score fold after transplantation
(months) =**

$$\frac{\text{Post}}{\text{Pre}} \text{ LGIR score}$$

Definition of LGIR test positive =At least 2 of 5 types of immune cells
with LGIR score fold higher than 2

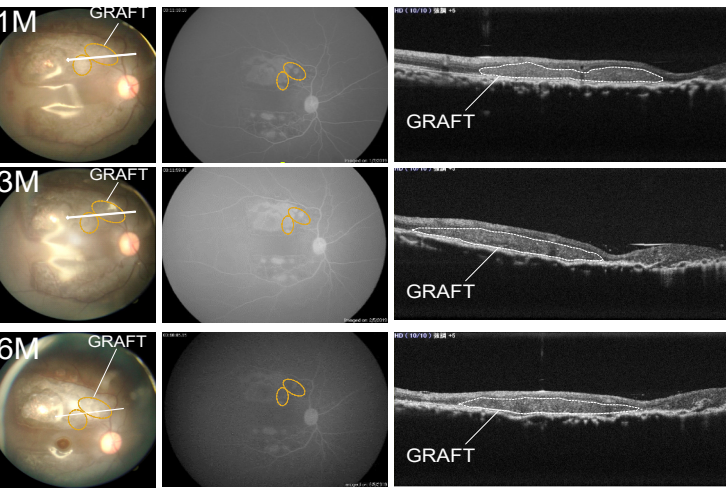
Figure S2. Overview of lymphocyte graft immune reaction (LGIR) test in this study related to Figures 2 and 3.

- A. Overview of LGIR test protocols. The neural retina was excised from the mkiPSC-retina and dissociated into single cells using neuron dissociation solution (Wako) for 10 min at 37°C, followed by co-culture with recipient PBMCs for 96 h.
- B. Detection of proliferative peripheral blood mononuclear cells (PBMCs) using flow cytometry. Gate was determined using isotype controls (<1%), and double positive populations of phycoerythrin (PE)-labeled Ki67 and Allophycocyanin (APC)-labeled lymphocyte markers were counted (%).
- C. Calculation of LGIR fold score after transplantation. The LGIR score was calculated as the percentage (%) of double-positive cells in the co-culture and PBMC only. LGIR test positivity was defined as at least 2 of 5 immune cell types with post/pre transplantation LGIR score greater than 2-fold.

Abbreviations: mkiPSC-retina, monkey iPSC-derived retina; LGIR, lymphocyte graft immune reaction; PBMC, peripheral blood mononuclear cell

MHC-matched-no.2

Right eye



Left eye

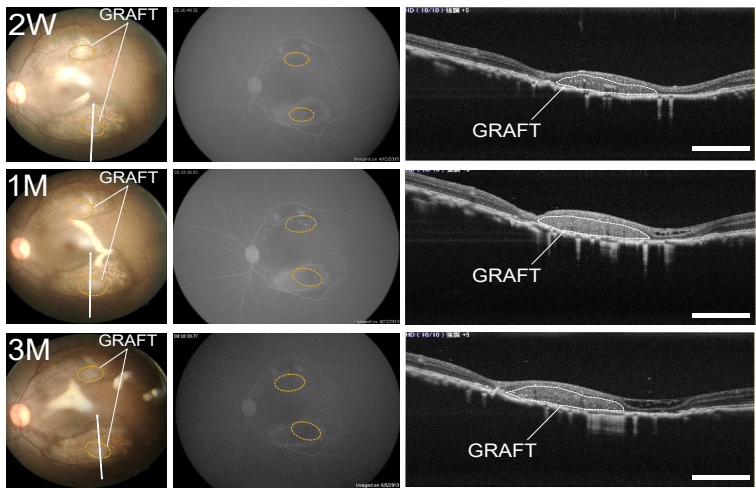


Figure S3. Time courses of in vivo imaging after monkey iPSC-retina transplantation of the MHC matched monkey related to Figure 3.

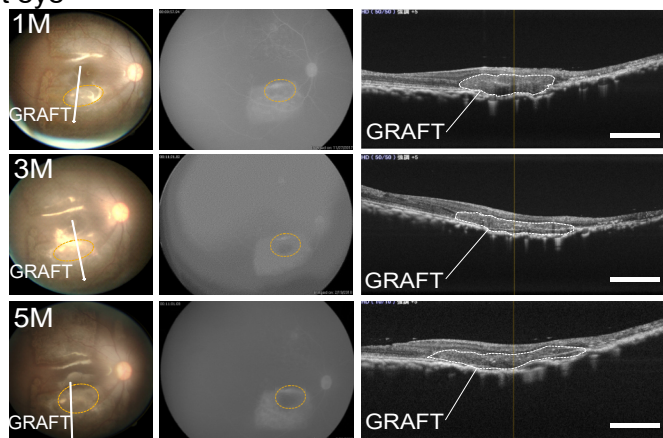
Time course of in vivo monitoring of transplanted grafts in MHC matched monkey no. 2. From left to right, OCT, fundus color photography, and FAG images are shown. The right and left eyes were presented for up to 6 and 3 months, respectively, after transplantation. Orange circles indicate the transplant area, and each OCT image shows the sectional view of the line indicated by the white arrow on the color fundus image. The transplanted grafts survived without signs of rejection until the end of observation.

Scale bars: 1mm

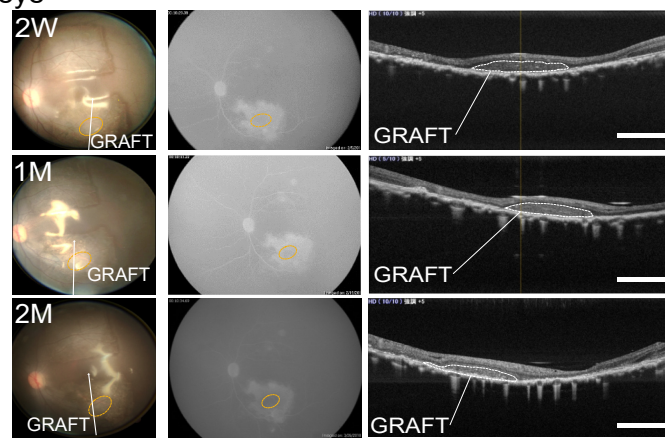
Abbreviations: mkiPSC-retina, monkey iPSC-derived retina; MHC, major histocompatibility complex; FAG, fluorescein angiography; OCT, optical coherence tomography

MHC-mismatched-no.1

Right eye

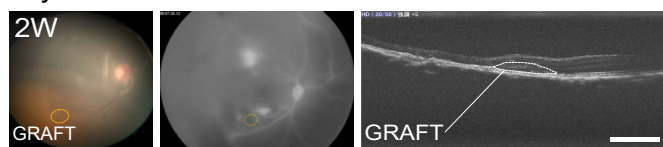


Left eye

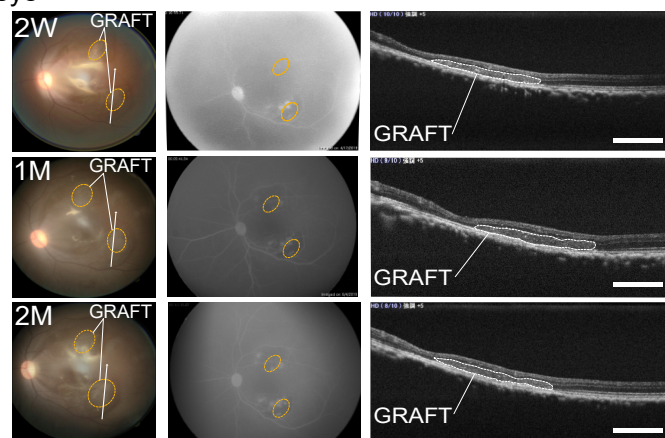


MHC-mismatched-no.2

Right eye

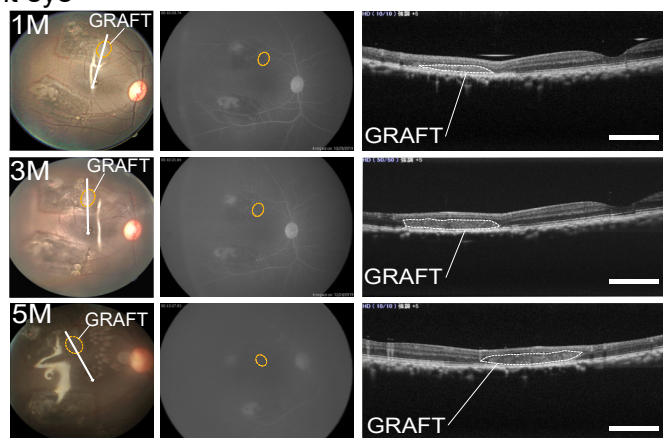


Left eye

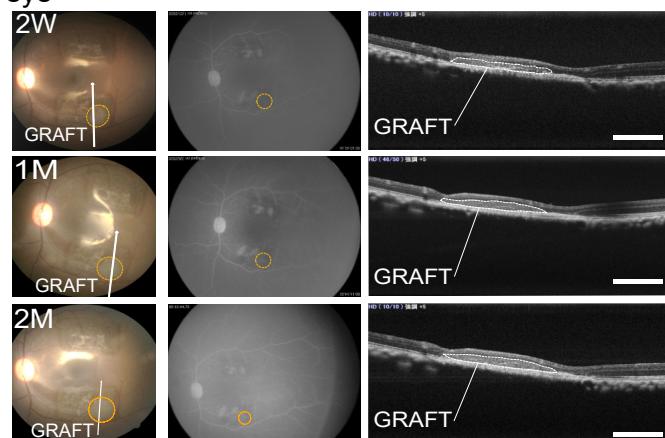


MHC-mismatched-no.3

Right eye

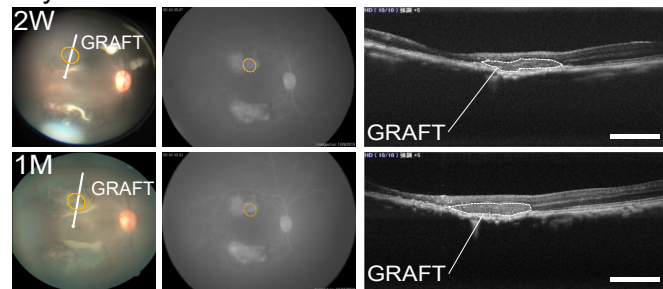


Left eye



MHC-mismatched-no.4

Right eye



Left eye



Figure S4. Time courses of in vivo imaging after mkiPSC-retina transplantation of MHC-mismatched monkeys related to Figure 3.

Time course of in vivo monitoring of transplanted grafts in MHC-mismatched-no.1-4. From left to right, OCT, fundus color photography, and FAG images are shown. The right and left eyes were presented for up to 5 and 2 months, respectively, after transplantation. Orange circles indicate the transplant area, and each OCT image shows the sectional view of the line indicated by the white arrow on the color fundus image. Transplanted grafts survived until the end of the observation period without signs of rejection. The right eye of MHC-mismatched-no.2 was imaged for up to 2 weeks, and the right eye of MHC-mismatched-no.4 was imaged for up to 1 month after transplantation due to a cataract.

Scale bars: 1mm

Abbreviations: mkiPSC-retina, monkey iPSC-derived retina; MHC, major histocompatibility complex; FAG, fluorescein angiography; OCT, optical coherence tomography

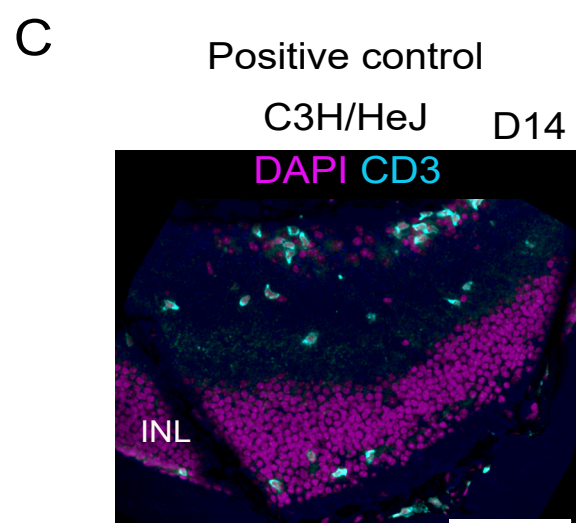
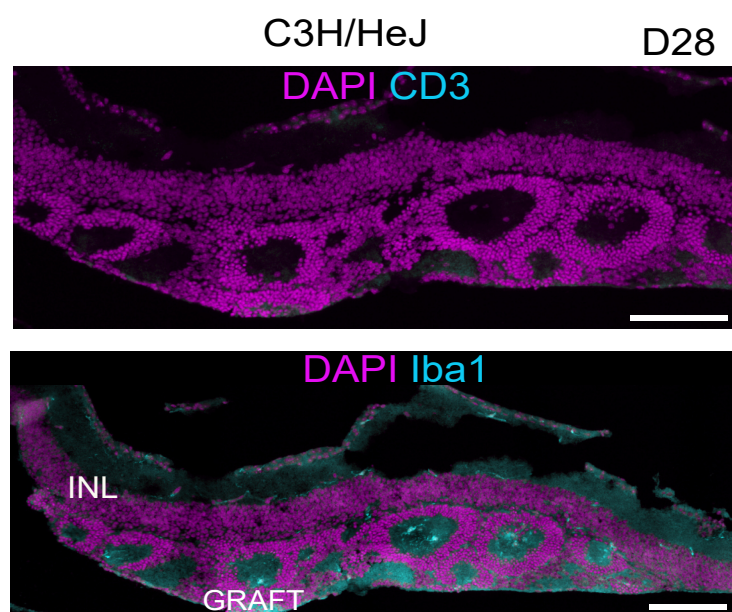
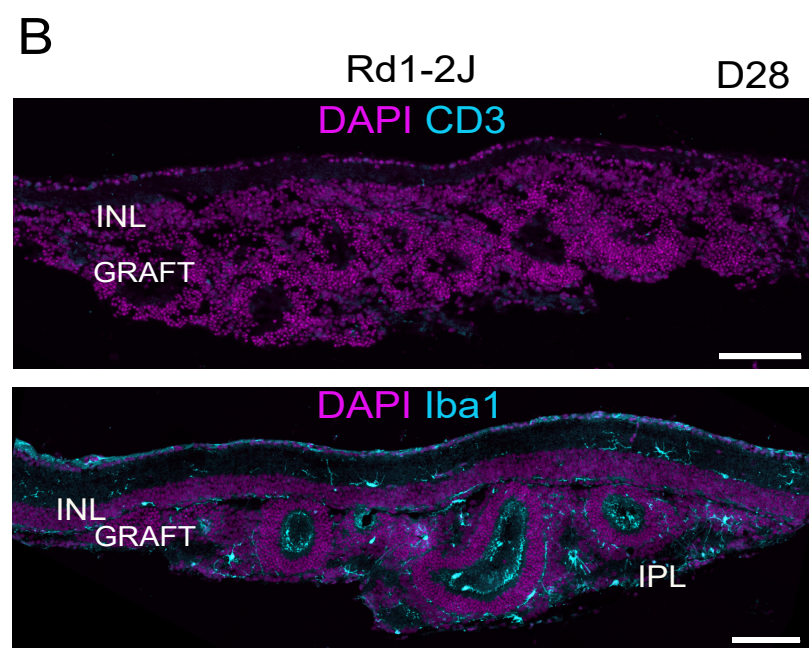
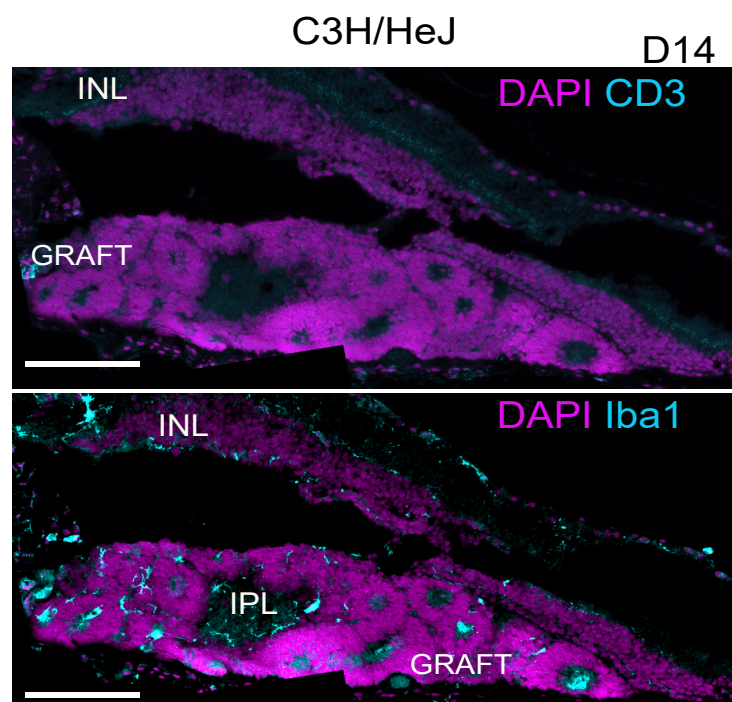
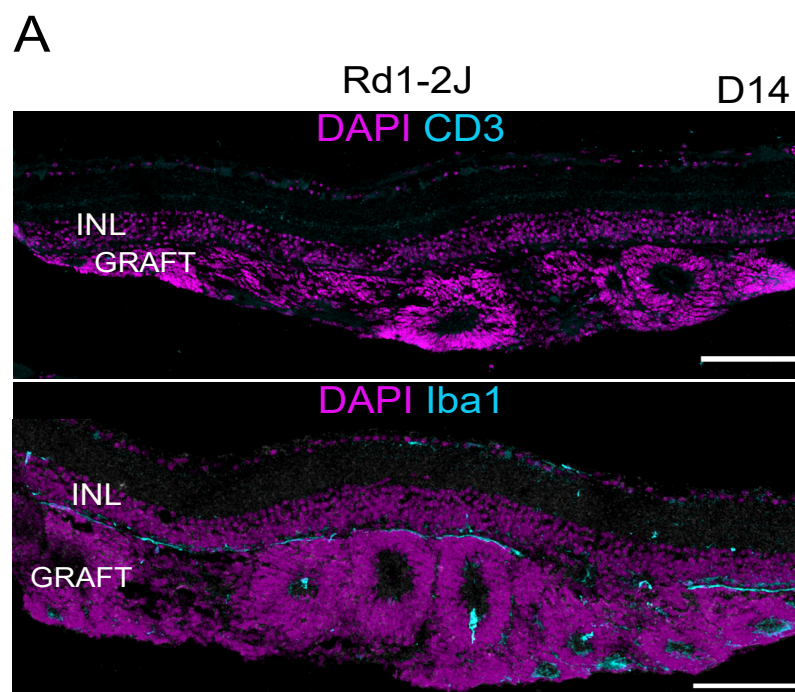


Figure S5. Immunostaining results of lymphocytes and microglia 14 and 28 days after transplantation in Rd1-2J and C3H/HeJ mice related to Figure 6.

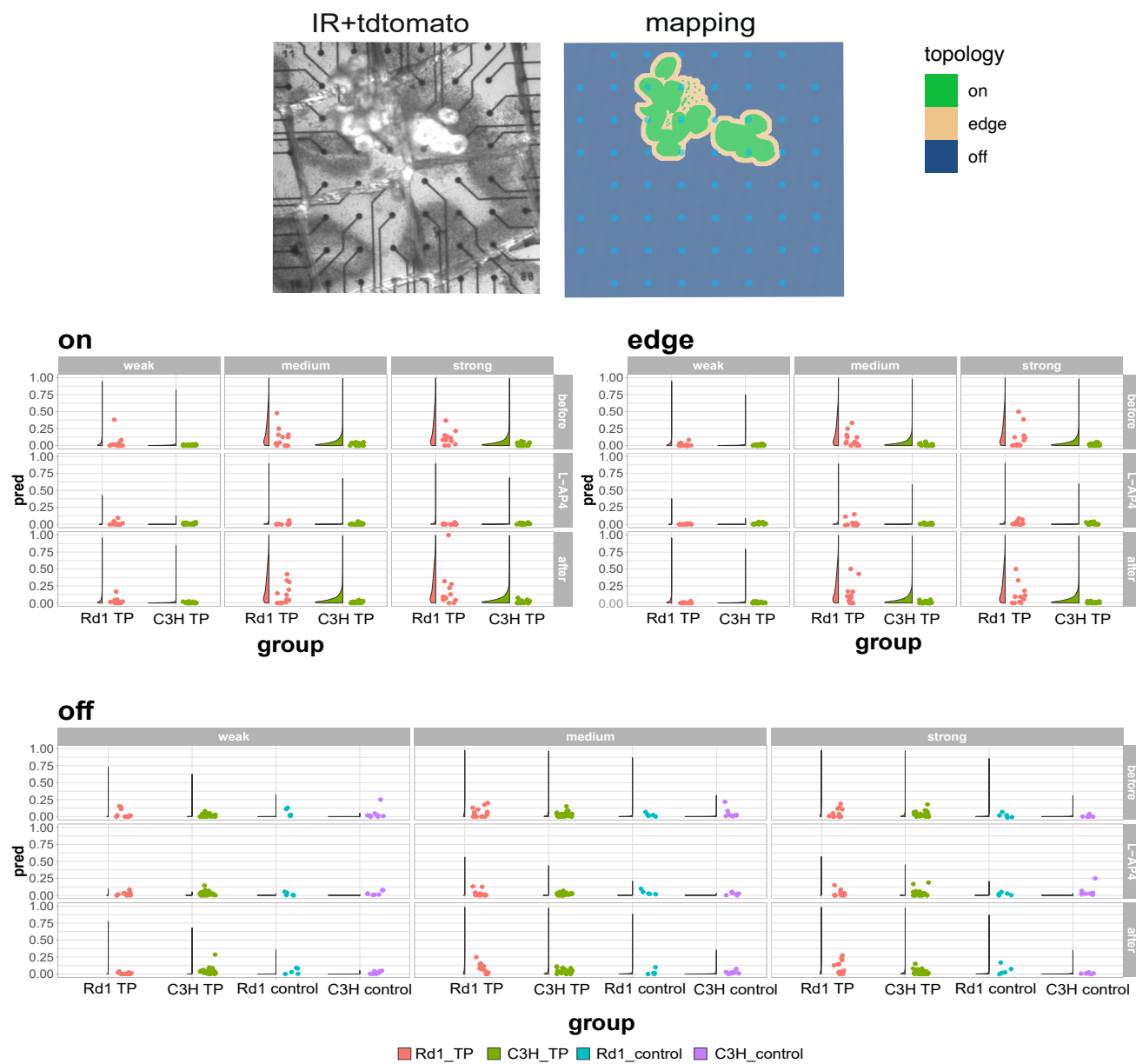
- A. Immunostaining of T-lymphocytes (CD3) and microglia (Iba1) 14 days after transplantation. CD3 infiltration was scarcely observed for either strain. Iba1 positive microglia were observed in the host and graft IPL, host-graft interface, and between and inside the graft photoreceptor rosettes.
- B. Immunostaining of T-lymphocytes (CD3) and microglia (Iba1) 28 days after transplantation. No infiltration of CD3-positive lymphocytes was observed in either of the strains. Microglia were observed in the host and graft IPL, host-graft interface, and between and inside the graft photoreceptor rosettes.
- C. Control staining of CD3 and Iba1 in eyes subretinally injected with Epstein-Barr virus-transformed B-cells (B95-8 B cells: JCRB Cell Bank, Lot No. 072795). Transplanted cells are mostly lost and T-cell infiltration was observed in the host retinal layer.

Scale bars: A-C: 100 μ m.

Abbreviations: IPL, inner plexiform layer; INL, inner nuclear layer

Rd1-2J

A



B

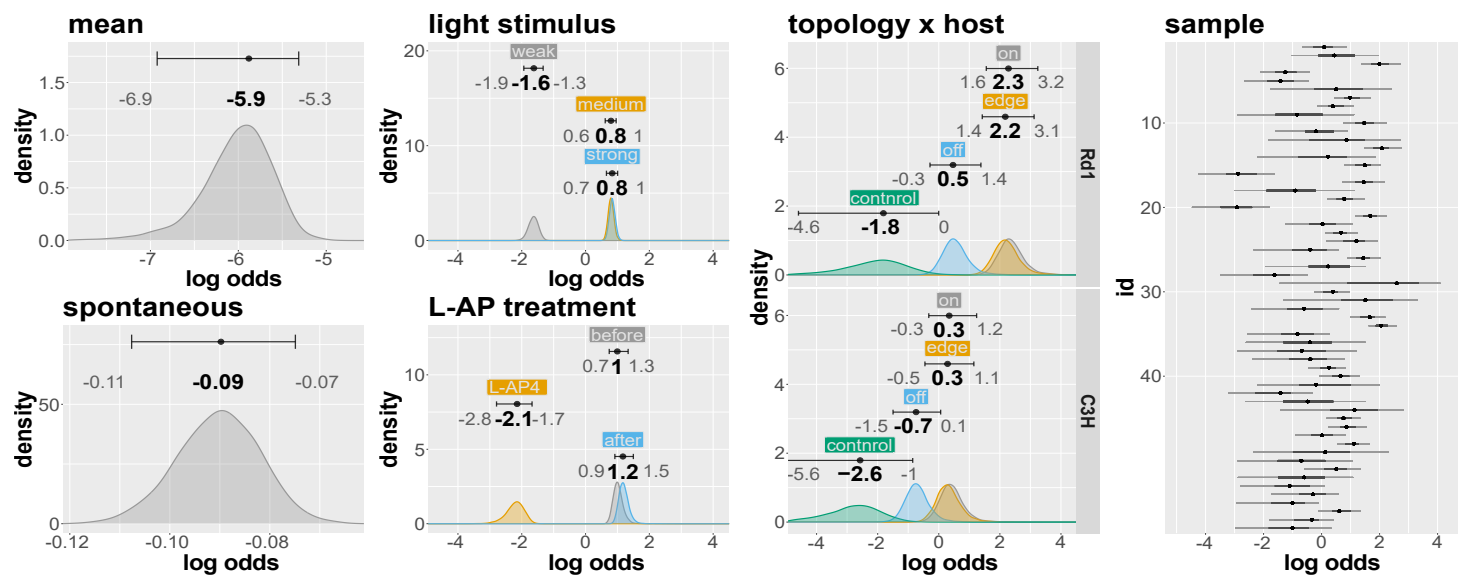


Figure S6. RGC responsiveness was modeled using hierarchical (multilevel) Bayesian inference in MHC-matched and mismatched transplantation in mouse rd1 models related to Figure 6.

- A. Summary of per-sample response probabilities broken down by areas with different graft coverages. We mapped the location of grafts in relation to the MEA probe by referring to Ctbp2::tdTomato on the IR-fluorescence images. The lower panel shows a summary of the response probabilities. (*on*, *edge*, *off*, and *control*, note that *off* and *control* are shown in the same panel for convenience). Dots (right half) represent the observed RGC response probability, and violin plots (left half) show posterior predictions from a logistic regression model. Our model predictions (violin plots) closely matched the observed data (dots), and the estimated fraction of outliers was relatively small (0.05 [95% interval: 0.05 to 0.06]; see *guess* parameter in methods for details), indicating that the data were largely explained by the proposed model.
- B. RGC responsiveness modeled using hierarchical (multilevel) Bayesian inference in MHC-matched and -mismatched transplantation in mouse rd1 models. Posterior distribution of logistic regression model for RGC responsiveness shown with mode and 95% compatibility intervals. We accounted for sample-to-sample variance, strength of stimulus light, L-AP4 treatment (before, L-AP4, after), and the interactions between graft coverage and host genotype, that is, combinations of the host (Rd1 or C3H) and graft coverage (*on*, *edge*, *off*, and *control*). The effect of these predictors is estimated as offsets from the overall “mean” by placing a sum-to-zero constraint for each predictor. In addition to these categorical variables, we used offsets from the mean spontaneous firing rate as covariates. Note that the horizontal values represent log odds, with higher values indicating a higher RGC response probability. The data in this figure were collected from 58 retinas (5 Rd1 control, 15 Rd1 transplanted, 9 C3H control, and 29 C3H transplanted retinas). The transplanted retinas showed an increased probability of RGC response to light compared to the untransplanted controls for both Rd1 and C3H samples (topology $\times$ host, control < off < edge, on, 95% interval was >0 for *off* – *control*, *edge* – *control*, and *on* – *control* for both Rd1 and C3H), although the response probability was lower for the C3H retinas in general (Rd1>C3H in *on*, *edge*, *off*, and *control*). The improvement was more robust in areas covered by or near the graft (95% interval >0 for *on* – *off* and *edge* – *off* for both Rd1 and C3H). Light responses were increased under *medium* (12.16 log photons/cm²/s) and *strong* light stimulation relative to *weak* stimulation (light stimulus) and largely abolished by ON-blocker L-AP4 treatment (L-AP4 treatment), indicating that these RGC

responses originate from photoreceptor cells. Finally, the spontaneous RGC firing was inversely correlated with light responsiveness (response probability).

Abbreviations: MHC, major histocompatibility; MEA, Multiple Electrode Array; RGC, Retinal ganglion cell

	MHC mismatched no.1		MHC mismatched no.2		MHC mismatched no.3	MHC mismatched no.4
	DrpZ11-34C-F		DpC16-8B-J		396693866	3094460102
Sex	Male		Male		Male	Male
Age	7Y		6Y5M		3Y2M	4Y1M
Mafa-F					F*02:05	F*02:07
Mafa-A	A1*098:02 A2:24:06	A1*094:01 A2:05:04	A1*008:02	A1*071:02 A8*01:01	A1*022:06/10 A4*14:03/04/13 A1*026:01	A1*089:06 A2*05:51 A1*102:03
Mafa-E	E-like4 E-like7	E-like4 E-like10	E-like9	E-like3 E-like10	E*02:03 E*02:01	E*02:03 E*02:05N E*02:06
Mafa-B	B*045:05 B*098:08 B*099:01 B*108:01	B*017:01 B*050:08 B*056:02:01 B*060:03:01 B*089:01:02 B*116:01 B*157:02	B*007:01:02 B*070:05 B*115:04:02 B*160:01	B*007*01:01 B*158:01 B*159:01	B*007:05/08/10 B*032:03 B*048:06 B*056:01 B*068:05/08/12 B*082:01/06	B*006:01 B*008:01 B*021:02 B*028:02 B*046:10/16 B*061:04 B*068:04/10 B*068:05/08/12 B*079:06 B*124:01/03
Mafa-I					I*01:31	I*01:02/40
Mafa-DRB	DRB1*W1:08 DRB*W3:01 DRB*W36:01	DRB1*03:18 DRB*W1:04 DRB*W2:02	DRB1*03:18 DRB*W1:04 DRB*W2:02	DRB1*03:18 DRB*W1:04 DRB*W2:02	DRB1*03:07 DRB*W001:02 DRB*W002:06 DRB*W049:01 DRB1*10:02	DRB1*03:34 DRB1*04:12 DRB*W007:01 DRB3*04:02
Mafa-DQA1	DQA1*26:03	DQA1*01:13	DQA1*24:10	DQA1*01:13	DQB1*16:01	DQB1*15:01
Mafa-DQB1	DQB1*18:07:02	DQB1*06:17:02	DQB1*18:26	DQB*06:17:02	DQB1*17:03	DQB1*06:27
Mafa-DPA1	DPA1*02:15:02	DQA1*07:04	DPA1*04:02	DQA1*07:04	DPB1*01:01/07/13	DPB1*01:04/08/10/15
Mafa-DPB1	DPB1*10:01	DPB1*21:01	DPB1*03:04	DPB*21:01	DPB1*18:02	DPB1*03:03/04/05

Table S1. HLA typing of monkeys in transplantation experiments, related to Figure 2 and 3

	MHC matched-no.1		MHC matched no.2	
	10154C		9471B	
Sex	Male		Male	
Age	8Y5M		6Y3M	
Mafa-F				
Mafa-A	A1*052:02 A4*01:04	A1*089*03 A2*05:50 A3*13:03:01	A1*052:02 A4*01:04	A1*089:03 A2*05:50 A3*13:03:01
Mafa-E	E-like5 E-like11	E-like3	E-like5 E-like11	E-like3 E-like10
Mafa-B	B*033:03 B*095*01 B*098:10	B*033:02 B*095:01 B*098:10	B*033:02 B*095:01 B*098:10	B*007:01:02 B*070:05 B*115:04:02 B*160:01
Mafa-I				
Mafa-DRB	DRB1*03:21 DR1*10:07	DRB*W6:04 DRB*W33:03	DRB1*03:21 DRB1*10:07	DRB1*03:18 DRB*W1:04 DRB*W2:02
Mafa-DQA1 Mafa-DQB1	DQA1*01:07 DQB1*06:08	DQA1*01:19 DQB1*06:01:02	DQA1*01:07 DQB1*06:08	DQA1*01:13 DQB1*06:17:02
Mafa-DPA1 Mafa-DPB1	DPA1*02:05 DPB1*15:04	DPA1*04:02 DPB1*03:04	DPA1*02:05 DPB1*15:04	DQA1*07:04 DPB1*21:01

Table S1. HLA typing of monkeys in transplantation experiments, related to Figure 2 and 3

No	Antibody	Species	Dilution	Catalog#	Source
1	MHC class II (HLA-DP, DQ, DR)	Mouse	1:100	M0775	Dako
2	CD3	Rabbit	1:100	ab5690	Abcam
3	CD4	Mouse	1:100	ab846	Abcam
4	Iba1	Rabbit	1:1000	019-19741	Wako
5	Iba1	Mouse	1:1000	MABN92	Millipore
6	Oct3/4	Rabbit	1:300	sc-98081	Santa Cruz
7	Nanog	Mouse	1:200	MABD24	Millipore
8	Crx	Rabbit	1:500	M231	Takara
9	RetP1	Mouse	1:1000	O4886	Sigma
10	OPSN1 SW	Goat	1:500	sc-14363	Santa Cruz
11	OPN1 MW/MW2/LW	Rabbit	1:500	AB5405	Millipore
12	ARR3	Goat	1:500	NBP1-37003	NOVUS
13	Recoverin	Rabbit	1:1000	AB5585	Millipore
14	PKCα	Goat	1:1000	AF5340	R&D systems
15	Brn3	Goat	1:500	sc-6026	Santa Cruz
16	Peripherin2	Rabbit	1:1000	18109-1-AP	Protein tech
17	CtBP2	Mouse	1:500	612044	BD
18	mGluR6	Sheep	1:2000	doi: 10.7554/eLife.5 9422	Dr. Jeannie Chen (University of Southern California)
19	mGluR6	Rabbit	1:500	NLS4655	NOVUS
20	RXRG	Mouse	1:200	Sc365252	Santa Cruz
21	Ku80	Goat	1:500	A5619	R&D
22	VSX2	Sheep	1:500	1180P	Exalpha

Table S2. Antibody information

No	Antibody	Species	Dilution	Catalog#	Source
23	Anti-human Ki-67, PE	Mouse	5µl/test	350504	BioLegend
24	Anti-human CD20, APC	Mouse	5µl/test	130-097-619	Miltenyi Biotec
25	Anti-human CD4, APC	Mouse	5µl/test	317416	BioLegend
26	Anti-human CD8a, APC	Mouse	5µl/test	17-0088	eBioscience
27	Anti-human NKG2A, APC	Recombina nt	5µl/test	130-098-809	Miltenyi Biotec
28	Anti-human CD11b, APC	Rat	5µl/test	13-091-241	Miltenyi Biotec
29	Anti-human HLA-class I, FITC	Mouse	10µl/test	F5662	Sigma-Aldrich
30	Anti-human HLA-class II, FITC	Mouse	10µl/test	MCA2497F	AbD Serotec

Table S2. Antibody information

Supplemental Experimental Procedures

Maintenance and retinal organoid induction from mkiPSCs

Mitomycin C-treated mouse embryonic fibroblast (MEF) cells were used as feeder cells, and mkiPSCs were maintained in a mixed medium of primate ES medium (ReproCELL) and mTeSR1 (STEMCELL Technologies) at a 2:1 ratio, supplemented with 1×10^3 U/ml mouse Lif protein (Merck Millipore) and 10 ng/ml bFGF (Wako). For retinal differentiation, we adapted a serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq) with induction-reversal culture, based on previous reports (1, 2). Briefly, the colonies were dissociated into single cells using TrypLE Select (Gibco). Subsequently, 1.2×10^6 cells/well were seeded in a V-shaped bottom 96-well Sumilon PrimeSurface plate (Sumitomo Bakelite) with a growth factor-free chemically defined medium (gfCDM)(3) supplemented with 30 nM sonic hedgehog smoothed agonist SAG (Enzo) and a 10^{-6} M-rock inhibitor, Y-27632 (Wako). On day 3, 1.5-nM recombinant human BMP4 (R&D Systems) was added, and the half-volume medium was changed every 2 days until day 12. The aggregates were transferred to a 10-cm low adhesive treatment Petri dish (Sumitomo Bakelite) in DMEM/F12 (Gibco) supplemented with the 5^{-6} M-FGFR inhibitor SU5402 (Wako) and 3^{-6} M-GSK-3 inhibitor CHIR99021 (Stemgent) until day 15. Thereafter, the medium was replaced with a retinal maintenance medium (Nukaya et al. WO19/017492, 054514, 054515) and changed every two or three days. For transplantation, the DD41-55 iPSC-retina with good morphology was selected, and a sheet with an approximate dimension of 0.5-mm x 1 mm was excised at the part containing the characteristic neural epithelium for transplantation and the LGIR test.

Focal photoreceptor ablation in monkey eyes

The monkeys were anesthetized for each of the experimental procedures (laser photocoagulation, graft transplanting surgery, and in vivo imaging experiments) via the intramuscular injection of ketamine hydrochloride (10 mg/kg) and xylazine (2 mg/kg), followed by an additional injection of ketamine hydrochloride (5 mg/kg) and xylazine (1 mg/kg) approximately every 30 min.

Focal photoreceptor degeneration was induced via laser photocoagulation based on our previous study (4). We used a 577 nm OPSL PASCAL laser system (Topcon Medical Laser Systems). Laser coagulation was performed in both the superior and inferior areas of the arcade retina in a rectangular shape. A QuadrAspheric (Volk, laser magnification x1.97) was used for the contact lens. The laser

power conditions were as follows: spot size, 100 μm ; laser power, 100 μW and 120 μW ; laser duration, 15 ms; and 3×3 or 4×4 square-shaped scanning patterns. The spot interval was set to 0 μm .

Flow cytometry analysis for MHC expression

Monkey iPSC-RPE in our previous reports (5) and Epstein-Barr virus-transformed B cells (B95-8 B cells: JCRB Cell Bank, Lot No. 072795) were used as positive controls for this experiment. The cells were dissociated into single cells. mkiPSC-retinas, with and without co-culture with recombinant IFN- γ (R&D systems:100 ng/mL) for 48 h, were dissociated using the Neuron Dissociation solution (Wako Pure Chemical Industries) for 10 min at 37°C and incubated with an Fc block (Miltenyi Biotec) at 4°C for 15 min. The cells were stained with anti-MHC-I antibody (host: mouse, Sigma-Aldrich), anti-MHC-II antibody (host: mouse, AbD Serotec), or isotype control (mouse IgG) at 4°C for 30 min. All samples were analyzed using a FACS Canto flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (version 9.3.1).

Transplantation of hiPSC-retinas into subretinal space of monkey eye

Transplantation was performed at least one month after focal photoreceptor ablation using laser treatment. After a regular vitrectomy, the post-vitreous membrane was detached from the retinal surface. Focal retinal detachment was created at the laser-induced degeneration area using a MicroDose™ injection device connected to an extension tube and 38G PolyTip cannula (MedOne®). The graft sheet was transplanted subretinally using 20-24-G cannula tips, as described previously. After regular fluid-gas exchange, the vitreous cavity was filled with silicone oil. This procedure was also reported by Shirai et al. (4)

In vivo imaging of monkey eyes

The transplanted eyes were monitored using optical coherence tomography (RS3000, Nidek) and a fundus color camera (CX-1, Canon and Retcam II, Clarity). Fluorescein angiography was performed using Retcam II or CX-1 with an intravenous injection of 2.5 ml of 10% fluorescein sodium. We intermittently captured images of both eyes until approximately 11 minutes after injection.

LGIR test for monkeys

The LGIR test was performed as previously described (5). PBMCs were isolated from the blood and stored at -80°C in CELLBANKER1 plus (Nippon Zenyaku Kogyo) freezing medium until use. Two different developmental stages of the iPSC-retinas at DD41-55, a premature stage for transplantation, and >DD120 of the matured stage were used as immunogens. These retinas were dissociated into single cells by incubating in neuron dissociation solutions (Wako) for 10 min, and 1×10^4 cells were co-cultured with 3×10^6 recipient PBMCs. After 96 h of incubation, we evaluated the ratio of Ki67⁺ lymphocytes to other immune cell markers such as CD4⁺ helper T-cells, CD8⁺ cytotoxic T cells, CD20⁺ B cells, CD11b⁺ monocytes/macrophages, and NKG2A⁺ natural killer cells using flow cytometry. The procedure for Ki-67 staining and antibody information has been previously described (6). All FACS samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences, CA, USA), and data were analyzed using FlowJo software version 9.3.1 (BD Biosciences). We evaluated the proliferating population of five types of immune cells (CD4⁺, CD8⁺, CD11b⁺, CD20⁺, and NKG2A⁺ cells) among PBMCs co-cultured with mkiPSC-retinas.

Quantification of Iba1⁺ cells in transplanted eyes by immunohistology

Images of the left eye (3 months after transplantation) were used for quantification. For Iba1 counting, 7-12 sections containing the transplants were used for the quantitative analysis of each eye. Composite images were obtained by averaging the intensity using ImageJ, the graft area was marked as a region of interest (ROI), and the ROI area was converted to a binary image using the threshold Otsu algorithm program to identify the Iba1 positive area. The Iba1 area in the graft area was calculated as the Iba1% area.

Maintenance and induction of retinal organoid from mouse embryonic stem cells (ESCs)

The ROSA::Nrl-CtBP2-tdTomato mESC line (C57BL/6J) was used for the transplantation experiments in the mice (7, 8). We used differentiation protocols based on previous studies (7, 9). The mESCs were seeded at 5000 cells/well in a V-bottom-shaped 96-well plate (Falcon) containing a differentiation medium (DMEM/F12 with GlutaMAX, 1% N2 supplement), and 2% Matrigel was added on day 2. The aggregates were transferred to 6-cm Petri dishes (Falcon). Retinoic acid (0.5 μM) (Sigma) and L-taurine (1 mM) (Sigma) were added on day 10, while the mESC-retinas were cut into approximately 0.5 x 1 mm grafts using micro scissors on day 13 for transplantation.

Surgical procedures of the retinal transplantation into the subretinal space of rd1 mice

The mice were anesthetized by the inhalation of 3–5% isoflurane, and the pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine. Topical 0.4% oxybuprocaine hydrochloride was additionally used when needed. A glass tip for transplantation was prepared from a disposable micropipette (1-000-0500, Drummon, Alabama, USA) using a micropipette puller (P-97/IVF Puller, Sutter Instruments, California, USA). The tip was cut and sharpened using a microgrinder (EG-400, Narishige, Tokyo, Japan). The glass tip was adapted to a microelectrode holder (MPH310, World Precision Instruments, FL, USA) with an electrode handle (2505, World Precision Instruments) and connected to a 10 µl micro-syringe (1701LT, Hamilton, MA, USA) with an extension tube. The graft was loaded onto the glass tip by gentle aspiration with HBSS and gently pushed into the subretinal space. The mice undergoing transplantation were administered 10 mg/L indomethacin (Wako) in their drinking water.

Immunostaining

Monkey eyes were fixed in SUPERFIX (Kurabo) for one week at 4°C, processed using automatic fixation (Excelsior; Thermo Fisher Scientific), and embedded in paraffin (P3683; Sigma). A 10-µm thick section was prepared using an automatic slide preparation system (AS-200; Kurabo). For cryosections, the eyes were fixed in 4% paraformaldehyde for 1 h for mice and rat eyes and up to 4 h for monkey eyes at room temperature and placed in 30% sucrose in PBS for at least three nights at 4°C. Eye cups were subsequently embedded in the O.C.T compound (Sakura Finetek), and the frozen blocks were sectioned at 12 µm thickness using a cryostat (CM3050S; Leica).

For immunostaining, paraffin-embedded sections were deparaffinized using an auto-slide staining system (ST4020; Leica) in xylene and alcohol, followed by antigen retrieval using citrate buffer in a microwave at 100°C for 20 min. After rinsing with phosphate buffered saline (PBS,) deparaffinized sections and cryosections were blocked with 0.3% TritonX-100 (Sigma)/PBS in 5% goat or BSAe serum albumin BSA (Sigma) for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C, and secondary antibodies were incubated for 1 h at room temperature after three washes with 0.05% Tween-20 (Sigma-Aldrich)/PBS. The antibodies used are listed in Supplemental Table 1. For the immunostaining of synapse markers, the primary and secondary antibodies were

incubated for three and two nights, respectively. Images were obtained using a confocal microscope (SP8; Leica).

Recordings using Multiple electrode arrays (MEA)

The animals were dark-adapted overnight, and the retinas were carefully isolated from the eye and mounted on electrodes (60MEA200/30iR-Ti-gr) with the host RGCs beneath and placed in constantly oxygenated warm Ames' medium (A1420, Sigma-Aldrich) in the dark (34-35°C). Opsinamide (10 µm; AA92593, Sigma-Aldrich) was added to the perfusion medium to suppress the melanopsin-expressing ganglion cells. Spontaneous RGC activity, a characteristic feature of degenerating retinas, was monitored during the 30-min recovery before starting the recordings. We used three stimulus intensities of full-field light stimuli (10.56 (weak), 12.16 (medium) and 12.84 (strong] log photons/cm²/s) with a white LED (NSPW500C) to cover the low to high mesopic range, with durations of 10 ms (filtered at 1 Hz) and 1000 ms (filtered at 100 Hz) for mERG (field potential recording) and ON/OFF types of RGC recordings, respectively. After the primary recordings, we added the mGluR6 receptor agonist L-AP4 (10 µm; 016–22083, Wako) to block the photoreceptor to ON-bipolar signals, followed by a 10-min washout for ON signal recovery. The recordings were obtained using three sequential stimuli of the same intensity and the duration of light under each condition (pre, L-AP4, washout). After the recordings, the retina on the electrodes was imaged with IR and 546-nm light for tdTomato under a microscope to map the graft location on the electrodes.

Spike sorting and RGC light response analysis

The light responses of the host RGCs were analyzed as described by Matsuyama et al. (8). The RGC spikes were sorted using Spike2 (CED.version 7.2). Recordings from before, during, and after L-AP4 treatment with the same intensity stimulus were merged and processed to trace the RGC responses under different conditions. The detected RGCs were categorized using an automated program as spontaneous firing, ON/OFF/ON-OFF/Delayed-ON light response type, or hypersensitive type, according to their temporal spiking pattern corresponding to the light stimulus onset and offset. RGCs with light-responsive patterns were considered to compute the RGC response probability for each retina.

We mapped the graft area referring to CtBP2::tdTomato fluorescence, as shown in the upper panel

of Fig.6E, and categorized each electrode as "on" if completely covered, "edge" if the graft boundary was partially on the electrode, and "off" if not covered by the graft. "Control" is the result of the other eye that has been not transplanted.

Statistical analysis

We used full hierarchical Bayesian statistical inference with Markov chain MCMC sampling for statistical modeling using Rstan (10). We show the posteriori distributions of the parameters of interest, which indicate the probability of the value of the parameter given the data, with 95% confidence intervals (CIs) and the mean indicated above.

Rate of Iba1 area

The area of Iba1 positive regions within grafts was analyzed by employing linear regression with varying intercepts and slopes and using the gamma distribution as the likelihood that the Iba1 area is always positive. For a graft with an area x_i , the Iba1 area y_i is

$$y_i \sim \text{Gamma}\left(\frac{\mu_i^2}{\sigma^2}, \frac{\mu_i}{\sigma^2}\right)$$

$$\mu_i = a_{aml} + b_{aml}x_i$$

where a_{aml} and b_{aml} denote intercept and slope, respectively. Note that the gamma distribution was parameterized using the mean (μ) and standard deviation (σ). The model was run with the following priors, which were vague regarding the scale of the data.

$$\sigma \sim \text{Normal}(0, sd(y))$$

$$a_{aml} \sim \text{Normal}(0,1)$$

$$b_{aml} \sim \text{Normal}(0,1)$$

RGC response probability

The response probability of RGCs was analyzed from the MEA recordings (10 ms stimuli). Following spike sorting and response assignment, we modeled the probability of each spike train to be light-responsive, that is, the probability of each RGC responding to light.

We obtained 51070 observations for 21257 cells collected from 58 retina samples (retinas). The

probability of each cell responding to a light (θ_i) stimulus was modeled using logistic regression. Thus, for a particular outcome y_i (no response or response),

$$y_i \sim \text{Bernoulli}(\theta_i)$$

$$\theta_i = \frac{1}{2} \text{guess} + (1 - \text{guess}) * \text{inv.logit}(\beta_0 + \beta_{tpl} + \beta_{hst} + \beta_{stm} + \beta_{cnd} + \beta_{smp} + \beta_{hst \times tpl} + f_i * \beta_{spt})$$

β_0 represents the overall mean, and the effect of different predictors was calculated as a deviation from this mean with a sum-to-zero constraint on each predictor ($\sum \beta_{[predictor]} = 0$). β_{tpl} is the effect of graft coverage (*on, edge, off, or control*), β_{hst} is the effect of the host (*Rd1-2J or C3H*), β_{stm} is the effect of stimulus strength (*weak, medium, strong*), β_{cnd} is the effect of L-AP4 treatment (*before, L-AP4, after*), β_{smp} represents the per-sample (retina sample) bias, $\beta_{hst \times tpl}$ is the interaction term between host and graft coverage, β_{spt} is a coefficient for the effect of the frequency of spontaneous activity, and f_i is the spontaneous spiking frequency (normalized and standardized) of cell i . The prior for the overall mean (β_0) was estimated from the data (in log odds, probability of response: $p_{\neq 0}$), with an SD of 1.

$$\beta_0 \sim \text{Normal}(\text{logit}(p_{\neq 0}), 1)$$

For the mouse effect, we estimated the hyperparameter (σ_{mus}) from the data, using a Gamma distribution with mode 2 and SD 4 ($\text{Gamma}(1.64, 0.32)$) following the recommendation of Kruschke (11).

$$\beta_{smp} \sim \text{Normal}(0, \sigma_{smp})$$

$$\sigma_{smp} \sim \text{Gamma}(1.64, 0.32)$$

For the rest of the regression coefficients (β_{spt} , β_{hst} , β_{stm} , β_{cnd} , β_{tpl} , $\beta_{hst \times tpl}$), we used generic weakly informative priors for logistic regression ($\text{Student}_t(3, 0, 1)$). For the guess parameter, which represents outliers, we followed the recommendation of Kruschke (Kruschke, 2014) for robust logistic regression (Chapter 21.3). The use of $\text{Beta}(1, 9)$, which emphasizes small values, reflects our expectation that the proportion of outliers is small. In particular, $\text{Beta}(1, 9)$ provides values greater than 0.5, which is a very small but nonzero probability.

$$\text{guess} \sim \text{Beta}(1, 9)$$

Supplemental references

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