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Successful Transplantation of Retinal Pigment Epithelial Cells from MHC Homozygote iPSCs in MHC-Matched Models

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SUMMARY

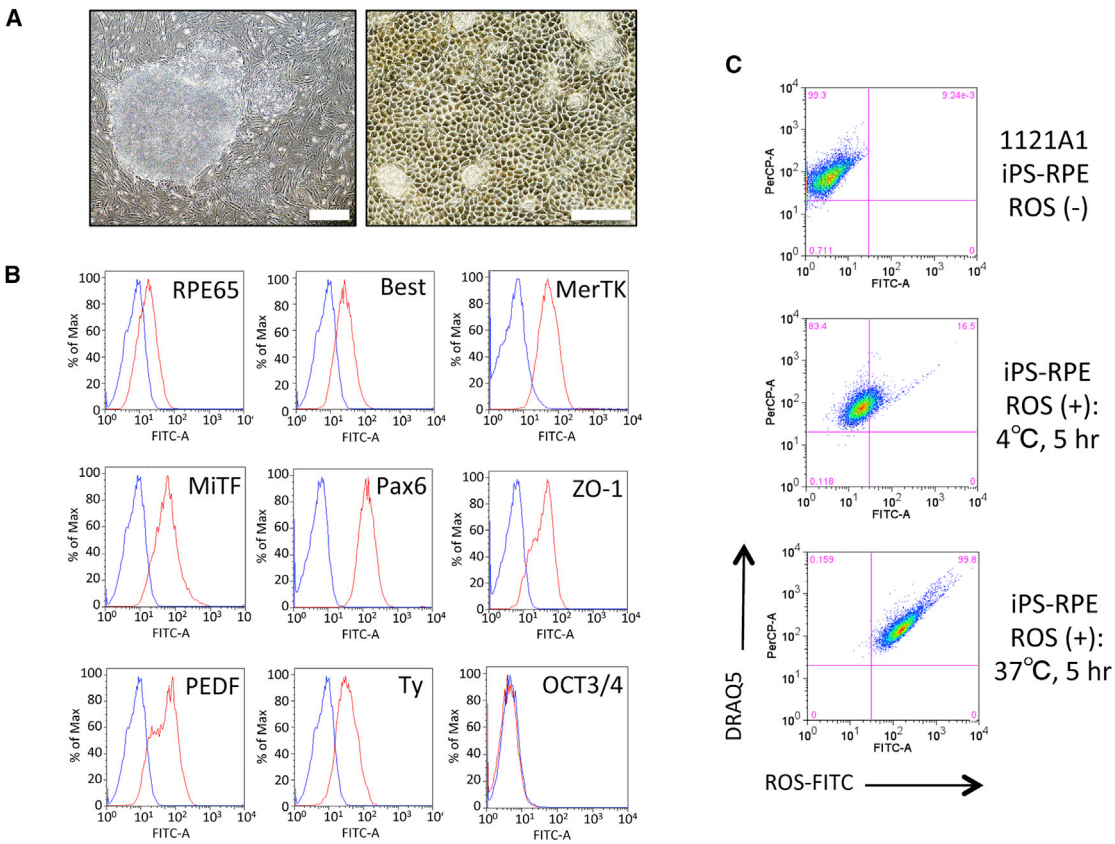
There is an ongoing controversy as to whether major histocompatibility complex (MHC) matching is a solution for allogeneic stem cell transplantation. In the present study, we established retinal pigment epithelial (RPE) cells from induced pluripotent stem cells (iPSCs) in MHC homozygote donors. We observed no rejection signs in iPSC-derived RPE allografts of MHC-matched animal models without immunosuppression, whereas there were immune attacks around the graft and retinal tissue damage in MHC-mismatched models. In an immunohistochemical examination of MHC-mismatched allografts, the transplanted RPE sheets/cells were located in the subretinal space, but the RPE exhibited inflammatory and hypertrophic changes, and many inflammatory cells, e.g., Iba1$^+$ cells, MHC class II$^+$ cells, and CD3$^+$ T cells, invaded the graft area. Conversely, these inflammatory cells poorly infiltrated the area around the transplanted retina if MHC-matched allografts were used. Thus, cells derived from MHC homozygous donors could be used to treat retinal diseases in histocompatible recipients.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) are generated from reprogrammed adult somatic cells by using Yamanaka pluripotent transcription factors (Park et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Recently, the potential for reprogrammed cells to be used as transplantation materials has been explored. The induced stem cells have the ability for self-renewal and the ability to generate several types of differentiated cells. Therefore, there might be a reduced risk for inflammatory immune rejection after transplantation because of the self-renewability. However, there have been problems with transplantation associated with immunogenicity in iPSCs, even after differentiation of cells/tissues. Even autologous mouse iPSCs induce an immune response, probably akin to an autoimmune reaction (Zhao et al., 2011). Although another group (Araki et al., 2013) reported that differentiated cells from iPSCs are eventually not recognized by the immune system, the immunogenicity of iPSCs and of iPSC-derived cells is still controversial.

The first clinical application of iPSCs has been initiated using autologous cells. Retinal pigment epithelium (RPE) cells are an especially safe cell type that will seldom form tumors; however, a major problem using autologous iPSCs for standard treatment is the high cost of cell production. To resolve these issues, we are studying allogeneic retinal cell lines derived from iPSCs. When we can prepare completely safe iPSC-derived retinal cells, and we use allogeneic retinal cells for the transplantation, we must consider the expression of major histocompatibility complex (MHC; also known as human leukocyte antigen [HLA]) antigens on the finally differentiated cells/tissues for the transplantation therapy as the next step. Although MHC expression is low in many types of stem cells, differentiated tissue expresses MHC, and this expression causes immune rejection.

Transplantation of RPE cells may be a treatment for retinal diseases, such as age-related macular degeneration (AMD). Many experimental clinical applications of allogeneic RPE cells for the treatment of AMD have been attempted (Algvere, 1997; Algvere et al., 1999; Kaplan et al., 1999; Peyman et al., 1991). The clinical application of iPSC-derived RPE (iPS-RPE) cells for AMD treatment was started in our associated hospital in 2014. Before transplantation studies of iPSCs are undertaken, questions concerning the survival of RPE cells in situ and the presence of immune attacks after retinal surgery must be addressed. It is assumed that MHC molecules on RPE cells, including cells derived from iPSCs, might be the main antigens in allogeneic inflammatory reactions. In previous reports (Mochizuki et al., 2013; Sugita, 2009; Sugita and Streilein, 2003;



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Sun et al., 2003), immune cells such as T cells were stimulated or inhibited by exposure to RPE cells. The dual effects of RPE cells are regulated by MHC and co-stimulatory molecules on RPE cells. Retinal antigen-specific T cells are stimulated by exposure to RPE cells that express MHC class II (MHC-II) on their surface (Sun et al., 2003). RPE cells maintain immune privilege in the eye (Mochizuki et al., 2013; Sugita, 2009), but allogeneic RPE grafts are immunogenic after ocular transplantation.

The purpose of the present study was to determine whether allogeneic RPE cells derived from iPSCs could survive after transplantation. We used an in vivo animal model with monkey iPS-RPE cells derived from MHC homozygote iPSC lines that were transplanted into the eyes of MHC-matched heterozygote donors.

RESULTS

Expression of MHC Classes I and II on iPSC-Derived RPE Cells

As a first step, we established RPE cells from monkey MHC homozygote iPSCs for transplantation materials. The monkey iPSCs 1121A1, which are MHC near-homozygous, were established from skin fibroblasts by using an episomal vector, as previously described (Kamao et al., 2014; Okamoto and Takahashi, 2011). The iPS-RPE cells showed polygonal morphology, mostly hexagonal, and contained melanin (Figure 1A). iPS-RPE cells expressed RPE65, MiTF, pigment epithelium-derived factor (PEDF), bestrophin, Pax6, tyrosinase, MerTK, and ZO-1, which are expressed by primary RPE cells but not the iPSC marker OCT3/4 (Figure 1B). The RPE cells had the ability to phagocytose shed photoreceptor rod outer segments (Figure 1C).

We also examined the expression of MHC molecules on 1121A1 iPS-RPE cells. MHC homozygote iPS-RPE cell line 1121A1, as well as control heterozygote iPS-RPE cell line 46a, constitutively expressed MHC class I (MHC-I), and recombinant interferon-γ (IFN-γ)-pretreated RPE cells expressed MHC-II (Figure 1D). In addition, these RPE cells did not express CD40, CD80 (B7-1), CD86 (B7-2), or CD275 (B7-H2), but constitutively expressed CD276 (B7-H3) co-stimulatory molecules (Figure 1E).

iPS-RPE Cells from Monkey MHC Homozygote iPSCs Have Poor Immunogenicity in an In Vivo Animal Model

Next, we transplanted the 1121A1 MHC homozygote iPS-RPE cells into monkey eyes in MHC-matched or -mismatched donors (cynomolgus monkeys without immunosuppression). The graft was monitored by color fundus pictures, fluorescein angiography (FA), and optical coherence tomography (OCT) after surgery at 1, 2, 4, 8, and 12 weeks, and at 6 months. MHC-typing profiles of iPS-RPE cells and transplanted animals are shown in Table S1.

We observed rejection signs in the allografts of the MHC-mismatched monkeys (1121A1 RPE cells into S2-4 monkey eye), e.g., disc redness in color fundus photographs, serous retinal detachment, and retinal edema including disappearance of the outer retinal layers around the graft in OCT findings (Figure 2A). On the other hand, there were no rejection signs in the allografts of the MHC-matched monkeys (1121A1 RPE cells into DrpZ1 monkey eye), and the grafts appeared to survive in the subretinal space of the eye (Figure 2B). Moreover, we observed severe rejection signs in the allografts of the MHC-mismatched monkeys (1121A1 RPE sheet into S3-2 monkey eye), e.g., leakage of fluorescein in the grafts and disc in FA, retinal cystic edema, and disappearance of the outer retinal layers around the graft in OCT (Figures 3A and S2). In the fellow eye (left eye), there were severe immune attacks in the retina (Figure S1). When 1121A1 RPE sheets were explanted into MHC-matched monkey eye (DrpZ17), the grafts survived until the final evaluation at 6 months (Figures 3B and S2). In addition, there were no immune attacks in the retina of the fellow eye (Figure S1).

In transplants with control MHC heterozygote iPS-RPE cells, there were inflammatory rejection signs around the grafts (46a iPS-RPE cells in K-177 and K-247 monkeys: Figure S3). For example, we observed rejection signs in the allografts (cell suspensions) of the MHC-mismatched

Figure 1. Establishment of iPS-RPE Cells from MHC Homozygote Monkey iPSCs

(A) RPE cells (right panel) induced from MHC homozygote monkey iPSCs (1121A1: left panel) clearly showed polygonal morphology and contained melanin. Scale bars, 500 μm.

(B) Expression of RPE-specific markers in monkey iPS-RPE cells (1121A1). iPS-RPE cells were stained with anti-RPE65, MiTF, PEDF, bestrophin (Best), Pax6, tyrosinase (Ty), MerTK, ZO-1, and OCT3/4 antibody (red). Isotype control is shown in blue.

(C) Measurement of phagocytosis by monkey iPS-RPE cells. 1121A1 iPS-RPE cells were cultured with FITC-shed photoreceptor rod outer segments (ROS) at 37°C for 5 hr (lower panel) and analyzed by flow cytometry. As a control, the RPE cells were cultured with FITC-ROS at 4°C (middle panel). iPS-RPE cells without ROS at 37°C were also analyzed (upper panel). DRAQ5 was bound within live/intact cells.

(D) Expression of MHC-I and MHC-II on monkey iPS-RPE cells. Monkey RPE cell lines 46a or 1121A1 were stained with anti-MHC-I or MHC-II antibody. The RPE cells in the presence of recombinant IFN-γ (100 ng/mL) were cultured for 48 hr.

(E) Detection of co-stimulatory molecules in monkey iPS-RPE cells, 46a, or 1121A1. iPS-RPE cells were stained with anti-CD40, CD80, CD86, B7-H2 or B7-H3 antibody (red). Isotype control is shown in blue.

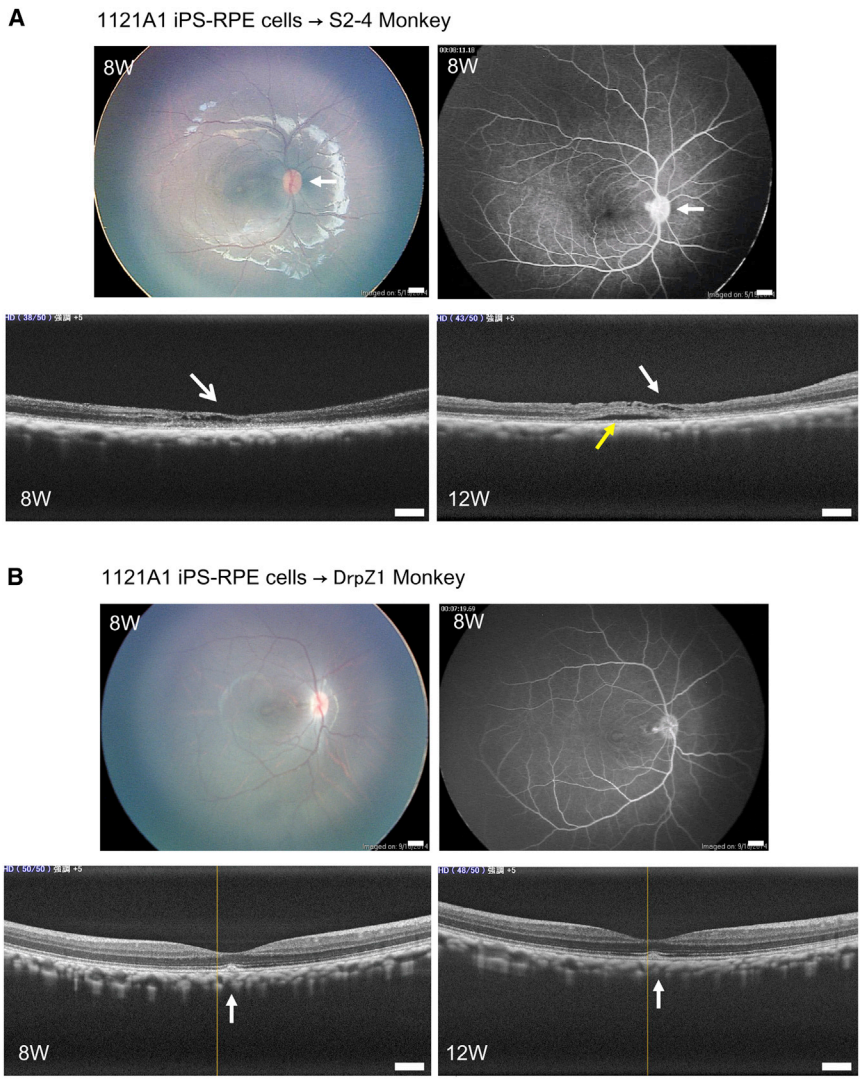


Figure 2. Allogeneic Transplantation of MHC Homozygote iPS-RPE Cells into Subretinal Space of MHC-Matched Monkey or Control Monkey

(A) Transplantation of monkey 1121A1 iPS-RPE cells (5×10^5, single-cell suspension) into the subretinal space in an S2-4 normal control monkey was performed without immunosuppression. Disc redness in a fundus photograph (upper left) and slight leakage from the disc in fluorescein angiography (FA, upper right) were observed at the 8-week (8W) evaluation. Optical coherence tomography (OCT, lower left) revealed the disappearance of the outer retinal layers around the transplanted RPE graft cells and the presence of retinal cystic edema (white arrow) and serous retinal detachment (yellow arrow) in the retina.

(B) Transplantation of monkey 1121A1 iPS-RPE cells (5×10^5, single-cell suspension) into the subretinal space in DrpZ1 MHC-controlled monkey (MHC-matched). At 8 weeks (8W) after surgery, the results of color fundus photographs (upper left) and FA (upper right) revealed no inflammation, and OCT indicated no signs of rejection. The transplanted iPS-RPE cells can be seen in the subretinal space (white arrow). Throughout the 6-month observation period, there were no rejection signs in the subretinal space or the retina.

Scale bars in color fundus and FA, 1.0 mm. Scale bars in OCT, 300 μm.

monkeys (46a RPE cells into K-177 monkey eye), e.g., leakage of fluorescein in the grafts in FA (Figure S3A). These rejection signs were seen 2 weeks after macular surgery. Moreover, we also observed rejection signs in the allografts of RPE sheets of the MHC-mismatched monkeys (46a RPE sheets into K-247 monkey eye), e.g., retinal infiltration around the graft in OCT (Figure S3B).

iPS-RPE Cells from MHC Homozygote iPSCs Have Poor Immunogenicity in Immunohistochemistry

Next, we examined whether the transplanted animal models with iPS-RPE sheets/cells have immunogenic inflammatory cells. We conducted H&E staining and inflammatory cell immune staining of retinal sections. The monkey retina was stained with anti-ionized calcium-binding adapter molecule 1 (Iba1), MHC-II, or CD3 antibodies using paraffin sections. In the eye of monkey K-247 (transplanted 46a iPS-RPE cell sheet: Figure 4), we found a piece of the RPE grafts in the center of the inflammatory nodules in the retina. $Iba1^+$ cells (ameboid-type microglia) invaded the inner nuclear layer (INL) and outer nuclear layer (ONL), and there were abundant $MHC\text{-}II^+$ cells and $CD3^+$ cells in the inflammatory nodules. In addition, we also observed rejection findings in the allografts of sheets/cells of the MHC-mismatched monkeys (S3-2, S2-4); the transplanted iPS-RPE cells/sheets disappeared in the subretinal space, and the retina was destroyed, with retinal cysts and serous retinal detachment in the operated eye (Figure S4).

On the other hand, the transplanted RPE cells survived in the subretinal space of the macula if we transplanted the 1121A1 iPS-RPE cell suspension into DrpZ1 monkey eye (MHC-matched: Figure 5). The retinal sections had poorly infiltrated $Iba1^+$ cells, $MHC\text{-}II^+$ cells, and $CD3^+$ cells around

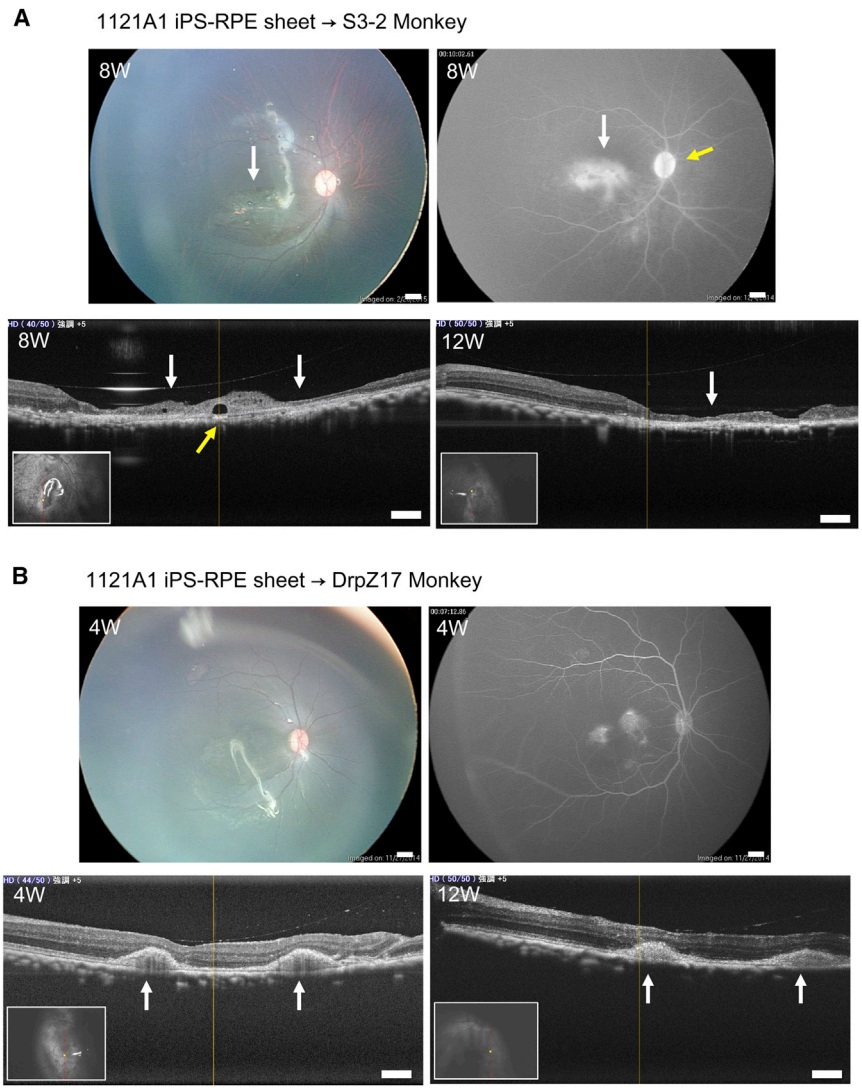


Figure 3. Allogeneic Transplantation of MHC Homozygote iPS-RPE Sheet into Subretinal Space of MHC-Controlled Monkey

(A) Transplantation of a monkey iPS-RPE cell sheet into the subretinal space of control normal S3-2 monkey was performed without immunosuppression. The RPE sheet graft is shown in the color photograph (white arrow in upper left panel). Results of late-stage FA (upper right) indicate severe FA leakage from the graft (white arrow) and the disc (yellow arrow) in the retina. OCT indicated characteristics of retinal destruction caused by graft rejection, such as the disappearance of the outer retinal layers (white arrows in lower panels) and cystic edema (yellow arrow).

(B) Transplantation of 1121A1 iPS-RPE cell sheets into the subretinal space of DrpZ17 MHC-controlled monkey (MHC-matched): two RPE sheet grafts survived in color photo, FA, and OCT (white arrows in lower left and right panels). Throughout the 6-month observation period, there were no rejection signs in the subretinal space or the retina. Small photos in the OCT indicate funduscopy images.

Scale bars in color fundus and FA, 1.0 mm. Scale bars in OCT, 300 μm.

the transplanted retina. We obtained similar results with DrpZ17 monkey eye (1121A1 iPS-RPE sheets of MHC-matched allografts: Figure 6). The transplanted RPE sheets survived in the subretinal space without any inflammatory signs, activated microglia, antigen-presenting cells (APCs), or T cells in the RPE sheets or retina. We also prepared control retinal sections that we injected with medium only (without iPS-RPE cells) (DrpZ10: Figure S5). We observed the Iba1$^+$ cells in the retina, but not MHC-II$^+$ cells or CD3$^+$ cells. These findings were similar to normal monkey sections (data not shown).

We summarized the results of allografts of iPS-RPE cells/sheets in monkey models (Table S2). Eventually there was no rejection between allogeneic RPE cells and the transplanted MHC-matched monkey eyes (n = 3), and there were no rejection signs between allogeneic RPE cells and the MHC-mismatched monkey eyes (n = 7) that had ocular inflammation after transplantation. Together, these results indicate that RPE cells including iPS-derived cells have immune attacks in the retina. Thus, if we transplant the iPS-RPE cells/sheets into the subretinal space of an MHC-matched monkey, the transplant remains intact, even if the monkey is not immunosuppressed.

iPS-RPE Cells from Monkey MHC Homozygote iPSCs Do Not Respond to MHC-Matched Allogeneic T Cells In Vitro

In the mixed lymphocyte reaction (MLR) assay with fresh peripheral blood mononuclear cells (PBMCs), monkey PBMCs proliferated when co-cultured with allogeneic monkey B cells (Figure S6A). In PBMCs, CD4$^+$ cells (helper T cells), CD8$^+$ cells (cytotoxic T cells), CD11b$^+$ cells (macrophages/monocytes), CD20$^+$ cells (B cells), and CD56$^+$ cells (natural killer [NK] cells/NKT cells) proliferated

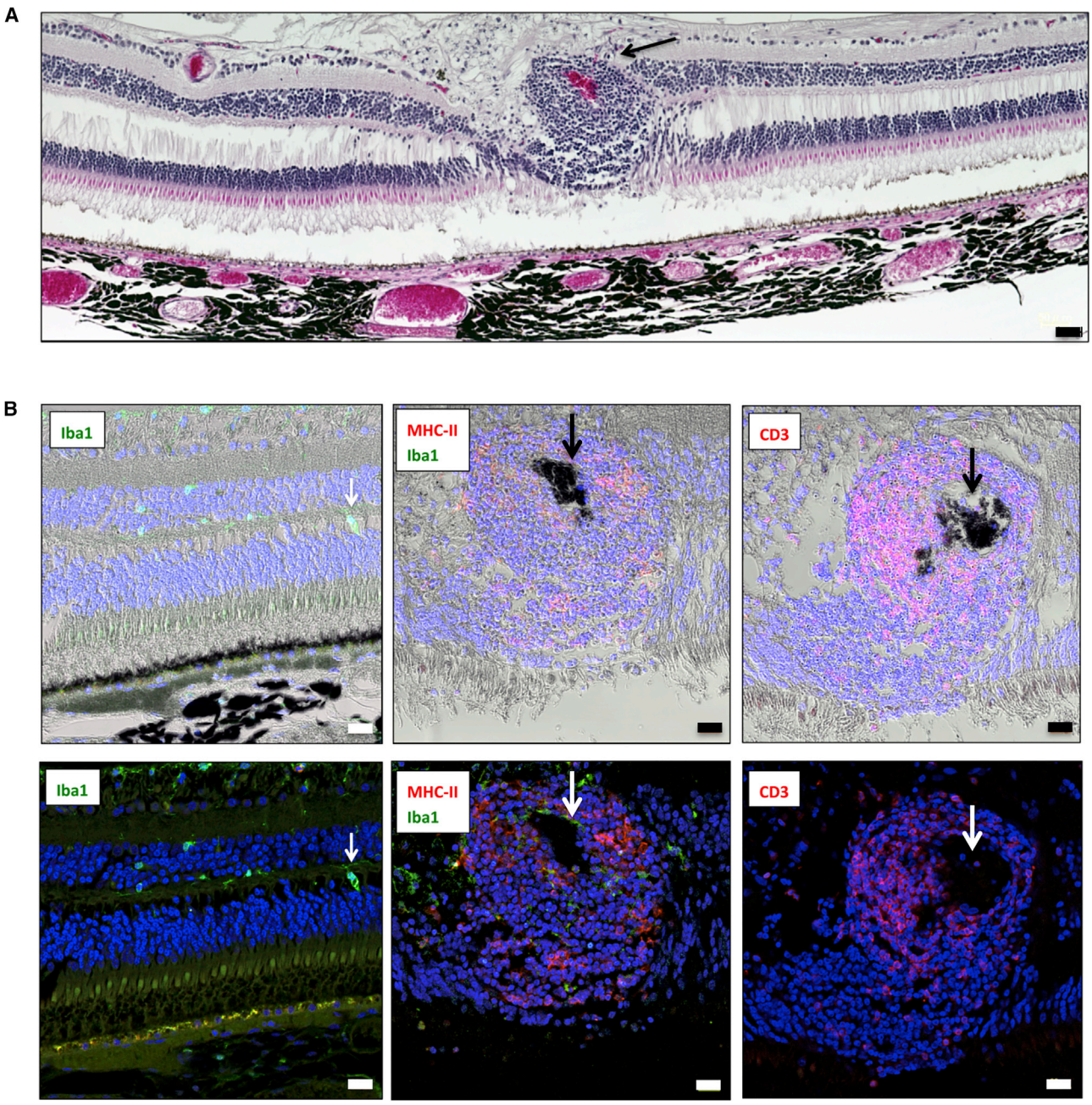


Figure 4. Immunohistochemistry of K-247 Monkey

(A) H&E staining for histological interpretation in K-247 monkey right eye (4M sections after transplantation: MHC-mismatched allograft). There was an inflammatory nodule (arrow) with many infiltrating cells in the retina. Scale bars, 50 μm.

(B) Photomicrographs showing labeling of K-247 monkey retina in right eye with anti-ionized calcium-binding adapter molecule 1 (Iba1), MHC class II (MHC-II), or CD3 antibodies using paraffin sections. Ameboid-type Iba1$^+$ cells (left panel, arrow) invaded the INL/ONL. There were many MHC-II$^+$ cells (middle panel) and CD3$^+$ cells (right panel) in the inflammatory nodules in the retina. We found a piece of the RPE grafts (arrows) in the center of the retinal nodules. Scale bars, 20 μm.

more than PBMCs only. The target B cells greatly expressed MHC-I, MHC-II, CD40, and CD86 molecules (Figure S6B).

In the PBMC-RPE MLR assay with allogeneic 1121A1 or 46a iPS-RPE cells, PBMCs proliferated when co-cultured with allogeneic iPS-RPE cells (Figure S6). However, the

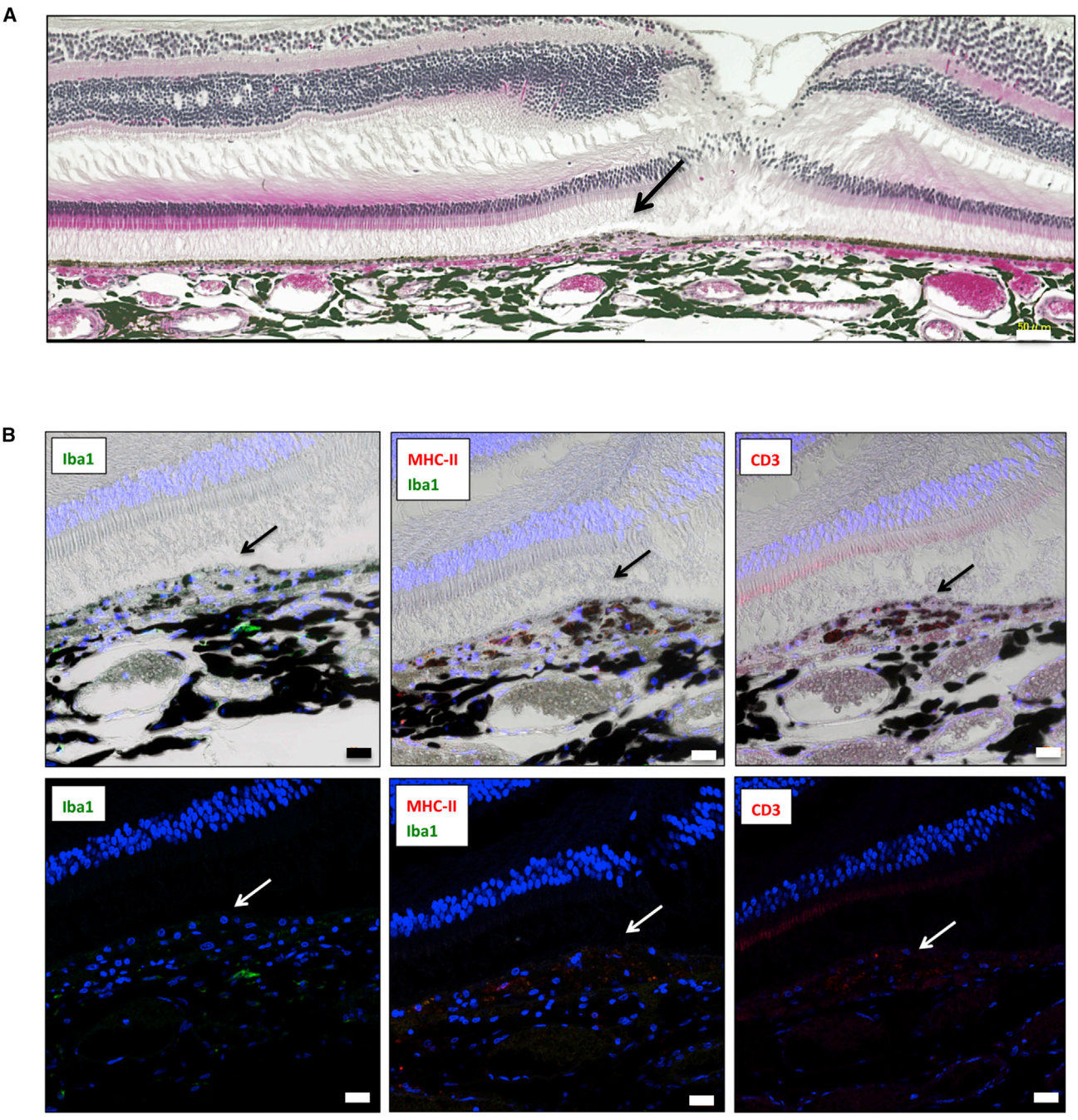


Figure 5. Immunohistochemistry of DrpZ1 Monkey

(A) H&E staining for histological interpretation in DrpZ1 monkey right eyes (MHC-matched allograft: 1121A1 iPS-RPE cell suspension [black arrow] → DrpZ1 MHC control monkey). The transplanted RPE cells survived in the subretinal space of the macula. Scale bars, 50 μm. (B) Photomicrographs showing labeling of DrpZ1 monkey retinal sections with anti-Iba1, MHC-II, or CD3 antibodies. $Iba1^+$ cells (left), $MHC\text{-}II^+$ cells (middle), and $CD3^+$ T cells (right) poorly infiltrated around the transplanted retina. Black and white arrows indicate iPS-RPE grafts. Scale bars, 20 μm.

PBMCs (DrpZ10 monkey) did not induce the proliferation of MHC-matched 1121A1 iPS-RPE cells, i.e., all cell types of inflammatory cells did not respond, whereas all cell types of inflammatory cells except $CD11b^+$ cells responded when co-cultured with MHC-mismatched 46a iPS-RPE cells (Figure 7).

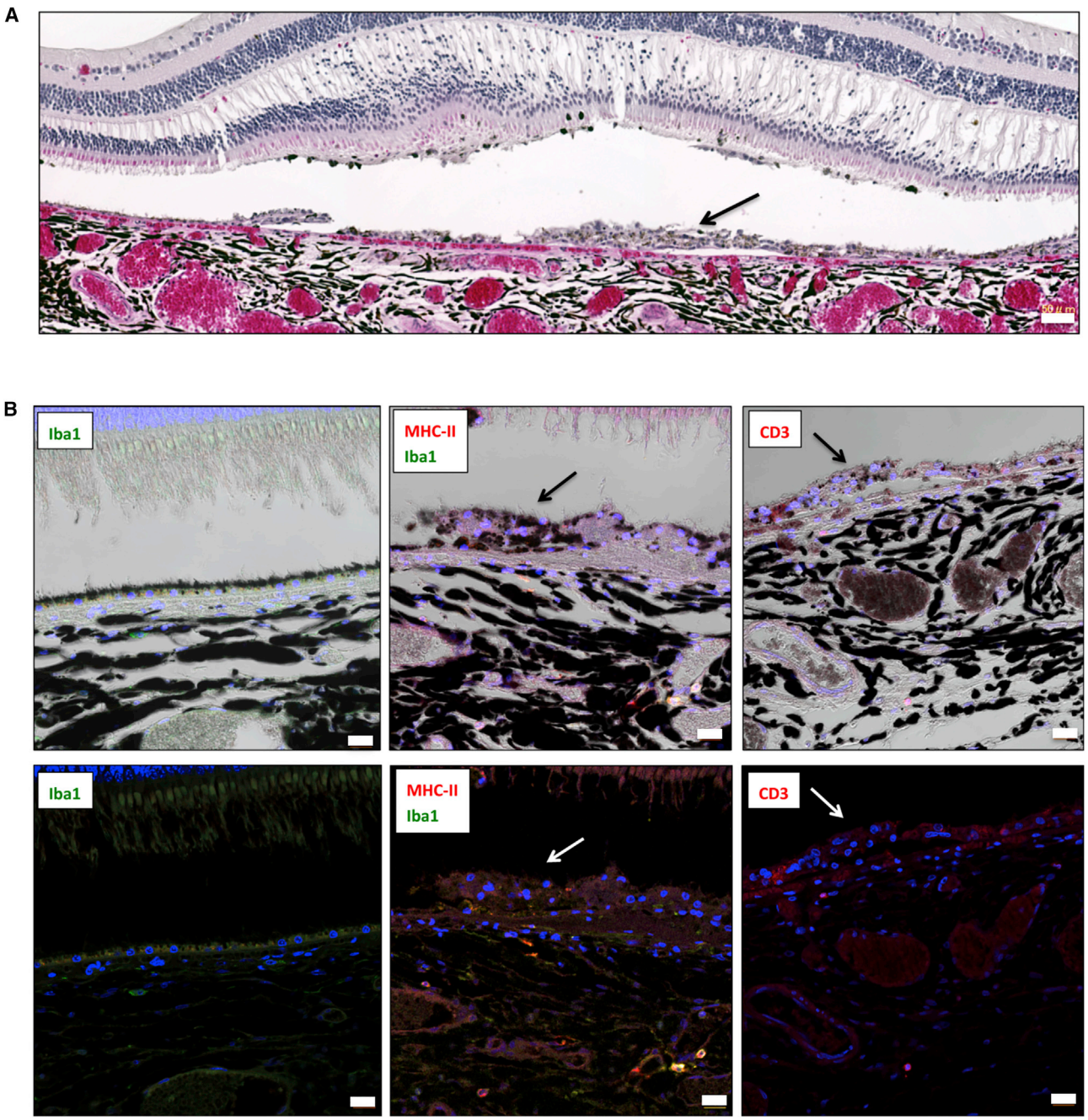


Figure 6. Immunohistochemistry of DrpZ17 Monkey

(A) H&E staining for histological interpretation in DrpZ17 monkey right eye (MHC-matched allograft: 1121A1 iPS-RPE sheets [black arrow] → DrpZ17 MHC control monkey). The transplanted RPE sheets survived in the subretinal space without any inflammatory signs. Scale bars, 50 μm.

(B) Photomicrographs showing labeling of DrpZ17 monkey retinal sections with anti-iba1, MHC-II, or CD3 antibodies. Compared with other allografts of iPS-RPE sheet transplantation, Iba1$^+$ cells (left), MHC-II$^+$ cells (middle), and CD3$^+$ T cells (right) poorly infiltrated the retina. The RPE cell sheet (black or white arrow) seems intact with no infiltrations of Iba1$^+$ cells, MHC-II$^+$ cells, or CD3$^+$ cells. Scale bars, 20 μm.

We also examined whether T cells can recognize allogeneic RPE cells in vitro (T cell RPE rejection assay). As expected, T cells produced significant amounts of IFN-γ with 46a iPS-RPE cells but not with autogenic iPS-RPE cells (Figure S7A). CD4$^+$ T cells produced high levels of IFN-γ with 1121A1 allogeneic MHC-mismatched iPS-RPE cells

(Figure S7B). However, $CD4^+$ T cells did not recognize MHC-matched iPS-RPE cells (Figure S7C). In addition, T cells failed to recognize MHC-mismatched allogeneic iPS-RPE cells if we added anti-B7-H3 blocking antibodies in the cultures (Figure S7D). Taken together, our results with in vivo and in vitro data demonstrate that MHC antigens play a prominent role in the inflammatory processes in iPSC-derived RPE allografts after transplantation.

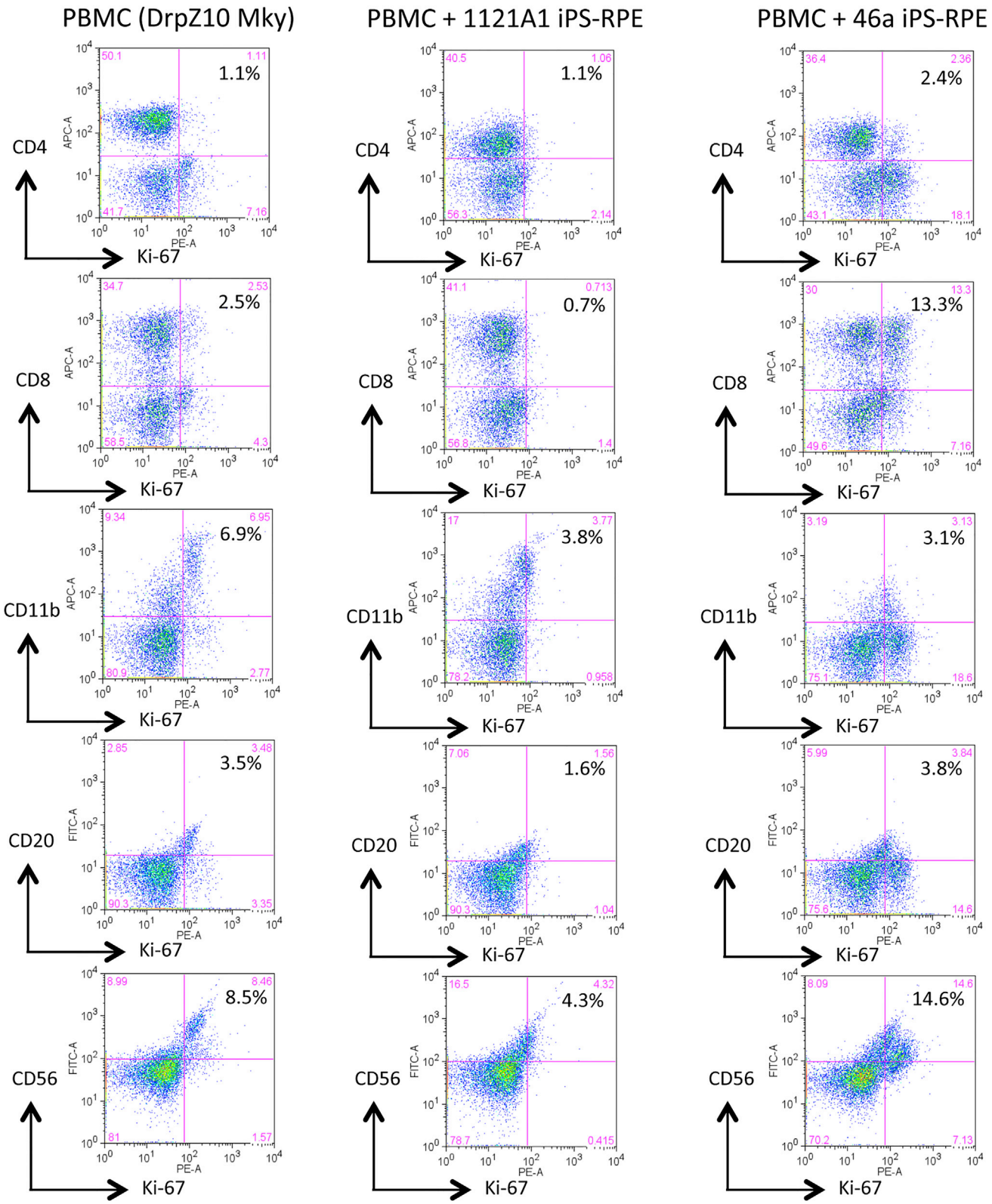
DISCUSSION

In the present study, we established RPE cells from iPSCs in MHC homozygote animals. We found no immune attacks in iPSC-derived RPE allografts of MHC-matched animal models without immunosuppression. By contrast, there were inflammatory immune rejections around the graft and retinal tissue damage together with inflammatory cell invasion in MHC-mismatched monkeys. In an immunohistochemical examination, the transplanted iPS-RPE cells of MHC-mismatched allografts did not survive in the subretinal space, and the retinal tissues exhibited hypertrophic changes, e.g., inflammatory nodules with many inflammatory cells, such as $Iba1^+$ microglia/macrophages, inflammatory $MHC\text{-}II^+$ APCs, and $CD3^+$ T cells that invaded the region around the RPE grafts. On the other hand, $Iba1^+$ cells, $MHC\text{-}II^+$ cells, and $CD3^+$ cells poorly invaded around the transplanted retina, and the allografts survived if we used MHC-matched allografts. In addition, T cells directly recognized allogeneic iPS-RPE cells in vitro as well as B cells (positive controls), whereas the T cells failed to recognize RPE cells from MHC homozygous donors when there was an MHC-matched combination between T cells and iPS-RPE cells.

The process of T cell activation after transplantation could be both direct and indirect. MHC-II-expressing APCs directly activate alloreactive T cells after transplantation. Alloreactive T cells of the direct type have T cell receptors (TCRs) that can directly recognize allogeneic MHC-I (mainly CD8) or MHC-II (mainly CD4), and these T cells are important mediators of allograft immune rejection. Most solid-tissue grafts contain $MHC\text{-}II^+$ APCs, i.e., dendritic cells and macrophages/microglia, which promptly migrate from the graft once it is in place. RPE cells do not contain $CD11b^+$, $CD11c^+$, $CD14^+$, or $CD45^+$ cells (Sugita and Streilein, 2003). In the case of solid-tissue grafts with $MHC\text{-}II^+$ APCs, by trafficking to draining lymph nodes these donor-derived cells activate alloreactive T cells with TCRs that directly bind donor MHC antigens. In the indirect pathway, T cells recognize graft MHC-II allogeneic antigens that have been processed and presented by host APCs. Ocular resident dendritic cells, macrophages, retinal microglia, and also RPE cells have the capacity to present retinal antigens to naive and sensitized T cells such as professional APCs (Butler and McMenamin, 1996; Percopo et al., 1990). Our results showed that the rejection process after allogeneic RPE transplantation in the subretinal space could be achieved by the direct and indirect activation of T cells.

As shown in this study, our iPS-RPE cells expressed MHC-I and MHC-II molecules (first signal). In addition, the cells expressed CD276 (B7-H3) molecules (second signal). Although there was no expression of CD40, CD80, CD86, or B7-H2 co-stimulatory molecules on iPS-RPE cells, the cells constitutively expressed MHC and co-stimulatory molecules B7-H3, and can activate T cells through the first and second signals. Although the function of B7-H3 molecules is still controversial, B7-H3 molecules that express APC can promote T cell-mediated immune responses and the development of acute and chronic immune rejection in allografts after transplantation (Chapoval et al., 2001). Our results indicated that the co-stimulatory signals from B7-H3 molecules on iPS-RPE cells could mediate allogeneic T cell activation. As the next step, we must consider other antigens (e.g., minor antigens and iPSC-specific antigens) and other immune cells (e.g., $CD8^+$ CTL, NK cells, B cells, and complement factors) to develop successful transplantation techniques for retinal diseases. The presentation of aberrant peptides (minor antigens) by MHC molecules could lead to significant T cell stimulation. There is substantial literature on major and minor antigen mismatch in bone marrow transplants (Dickinson et al., 2002; Goulmy et al., 1996; Tseng et al., 1999; Voogt et al., 1988). There are no previous reports of the correlation between minor histocompatibility antigens and T lymphocytes after retinal transplantation. The minor histocompatibility antigens, e.g., HA-1, are immunogenic allogeneic antigens responsible for graft-versus-host disease (GVHD) in HLA-matched bone marrow transplantation (Tseng et al., 1999; Voogt et al., 1988). Laurin et al. (2010) previously showed that minor histocompatibility antigen-associated alloreactivity plays a critical role in the development of GVHD and graft-versus-leukemia (GVL) after HLA-identical hematopoietic stem cell transplantation. They showed by IFN-γ assay that the alloreactivity from donor versus recipient or donor versus mismatched minor antigens was associated with acute GVHD and GVL. We currently have no evidence for the correlation between minor histocompatibility antigen and T cells after RPE transplantation, but we might be able to monitor the minor histocompatibility antigen-related immune rejection if we use similar methods using peripheral T cells in transplanted patients.

In in vivo animal models, we observed no rejection signs in the iPS-RPE allografts of MHC-matched monkeys



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(DrpZ1 and DrpZ17); for example, fundus photographs revealed no exudation, FA showed no vascular leakage of fluorescein, and OCT indicated there was no retinal edema around the transplanted RPE cells. In contrast, FA revealed hyperfluorescence staining, while OCT indicated retinal edema, serous retinal detachment, and disappearance of the outer retinal layers around the graft in MHC-mismatched animals. Thus, as T cells cannot recognize MHC near-homozygote iPS-RPE cells in MHC restriction, their derivatives from HLA homozygous donors may be useful in treating retinal diseases in histocompatible recipients. The iPSC bank (Nakatsuji et al., 2008; Okita et al., 2011) might be a useful source for allografts in retinal diseases, since T cell-mediated rejection may not occur if some of the main HLA antigens (at least HLA-A, B, and DRB1) are matched. This is new evidence that immune cells fail to attack iPS-RPE cells if we use human RPE cells from HLA homozygous donors.

We next examined whether the transplanted animal models with iPS-RPE cells have immunogenic inflammatory cells in the retina. First, we conducted H&E staining and then immune staining of inflammatory cells (Iba1$^+$, MHC-II$^+$, or CD3$^+$) by using retinal paraffin sections. Compared with OCT findings, the H&E staining of retinal sections was clearer. We found that the retina tissues in MHC-mismatched allografts exhibited hypertrophic changes in the subretinal space, inflammatory nodules and/or changes of cystic edema in the retina, retinal destruction, vitreous cells, and cell infiltration in the choroid. On the other hand, we observed that the grafts survived intact in the subretinal space if we transplanted MHC-matched RPE allografts. According to previous reports in monkey transplantation models (Valentino et al., 1995), the authors removed the RPE tissues from Bruch's membrane in the macula and outside the vascular arcades. In their results, the regrowth of RPE cells was associated with repaired, normal-appearing photoreceptor outer segments, suggesting that repopulation of RPE cells in the adult monkey could support the repair of damaged photoreceptors following retinal surgery.

On immune staining we found many inflammatory cells such as Iba1$^+$, MHC-II$^+$, and/or CD3$^+$ cells in the retina. For instance, K-247 monkey, ameboid-type Iba1$^+$ cells (activated microglia) invaded the INL/ONL after transplantation. Many MHC-II$^+$ cells (activated APCs) invaded the inflammatory nodules in the retina. Moreover, we were able to see the Iba1 and MHC-II double-positive cells in the retina, features that are not visible in normal sections and control sections (DrpZ10 left eye). More importantly, there were many CD3$^+$ cells (T cells) in the inflammatory nodules in the retina, and a piece of the RPE grafts in the center of the retinal nodules. In a previous report, IFN-γ-pretreated RPE cells that inducibly express MHC-II molecules were able to stimulate retinal antigen-specific T cells (Sun et al., 2003). The grafted RPE cells and the host RPE layer also expressed MHC-II molecules, and the infiltrated CD3$^+$ T cells in the retina greatly expressed IFN-γ in the inflamed eye (data not shown).

However, as shown in immunohistochemical analysis of DrpZ1 and DrpZ17 monkeys, the transplanted iPS-RPE cells (1121A1) survived in the subretinal space, and these monkey retinal sections with anti-Iba1, MHC-II, or CD3 antibodies had slight cell infiltration around the transplanted retina. In the present study we did not use any immunosuppressive medication for the transplantation, but we eventually found no immune attacks even if MHC-matched allografts were explanted. Tezel et al. (2007) previously reported that allogeneic RPE cell sheet transplants for exudative AMD patients showed no signs of immune rejection in patients who were able to continue the immunosuppressive medication for 6 months. Thus, systemic immune suppression prevented the rejection of the transplanted allogeneic RPE cells. Our results primarily show that MHC-matching transplantation between donor and recipient appears to prevent the immune attacks of allografts without immunosuppressants. We are now conducting experiments as to whether iPS-RPE MHC-mismatched allografts have immune rejection under local immunosuppressive medication (e.g., intraocular steroids), compared with systemic immunosuppressants (e.g., cyclosporine A).

In conclusion, T lymphocytes cannot recognize MHC antigens on allogeneic iPS-RPE cells established from MHC homozygote donors, as well as autogenic iPS-RPE cells in vitro. Importantly, in animal models without immunosuppressive medication, iPS-RPE cells from MHC homozygous donors did not have immune responses in the retina of MHC-matched donors after transplantation. This study is the first to show the possibility that the main HLA loci-matched allogeneic iPSC-RPE cell transplantation will need little or no immunosuppression.

Figure 7. Mixed Lymphocyte Reaction Assay with Fresh PBMCs Plus iPS-RPE Cells

In the PBMC-RPE MLR assay with allogeneic 1121A1 or 46a iPS-RPE cells, PBMCs (2×10^6 cells/well in DrpZ10 monkey) were cultured with allogeneic iPS-RPE cells for 5 days. Before the assay, RPE cells were irradiated (20 Gy), and 1×10^4 cells were cultured in a 24-well plate. Cells were stained with anti-CD4, anti-CD8, anti-CD11b, anti-CD20, anti-CD56, anti-Ki-67, and each isotype control antibody at 4°C for 30 min. The samples were analyzed on a FACS flow cytometer. Numbers (%) in the scatterplots indicate double-positive cells (e.g., CD4/Ki-67).

EXPERIMENTAL PROCEDURES

Preparation of Monkey iPS-RPE Cells

We prepared two iPSCs from normal cynomolgus monkey (HT-1 or Cyn46), 1121A1 iPSCs from HT-1 MHC homozygote monkey, and 46a iPSCs from Cyn46 MHC heterozygote monkey as a control. The monkey iPSCs were established from cynomolgus monkeys as previously described using *OCT3/4*, *SOX2*, *c-MYC*, and *KLF4* (Okamoto and Takahashi, 2011). To differentiate into RPE cells, we cultured monkey iPSCs on gelatin-coated dishes using Glasgow's minimal essential medium (GMEM) supplemented as previously described (Kamao et al., 2014; Sugita et al., 2015). Signal inhibitors Y-27632 (10 μM, Wako), SB431542 (5 μM, Sigma), and CKI-7 (3 μM, Sigma) were added to the GMEM (Kamao et al., 2014; Sugita et al., 2015). After the appearance of pigment epithelium-like colonies, the medium was switched to DMEM/F12 medium with B27 supplement (Invitrogen). In qRT-PCR analysis, these monkey iPS-RPE cells expressed mRNA for specific makers for primary RPE cells such as RPE65, Pax6, tyrosinase, transforming growth factor β1, β2, and β3, PEDF, and vascular endothelial growth factor, but not Nanog and Lin28 (data not shown).

The care and maintenance of the monkeys conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Use of Laboratory Animals, as well as to the Guidelines of the RIKEN CDB Animal Experiment Committee.

Transplantation of iPS-RPE Cells into the Subretinal Space of Monkeys

MHC-controlled monkeys (DrpZ1, 10, 17: adult cynomolgus monkeys/*Macaca fascicularis*) were purchased from Ina Research. Normal control cynomolgus monkeys (K-151, K-177, K-247, S2-4, S3-2) were purchased from Shin Nippon Biomedical Laboratories. The MHC-controlled monkeys or normal control monkeys were anesthetized with a mixture of ketamine and xylazine, and their pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride. For transplantation of iPS-RPE cells/sheets (MHC near-homozygote 1121A1 iPS-RPE cells), after induction of posterior vitreous detachment by active suction, a complete vitrectomy was performed (Accurus, Alcon) (Kamao et al., 2014). The transplantation site was enlarged with a subretinal injection cannula (Synergetics) and vitreoretinal scissors to enable implantation of iPS-RPE cells, and another retinotomy site for drainage was created by the cannula and scissors. The iPS-RPE cells/sheets were transplanted into the subretinal space, as previously described in detail (Kamao et al., 2014). The graft cells were monitored by color fundus photographs, FA (both RetCamII and Clarity) and OCT (Nidek) at 1, 2, 4, 8, and 12 weeks after surgery and at 6 months after surgery.

MHC Typing

The results of MHC-allele typing are described in Table S1. Genotyping of MHC-1 and MHC-II genes in *M. fascicularis* (cynomolgus monkeys) was performed by pyrosequencing as described in our recent report (Shiina et al., 2015). The MHC information for the MHC near-homozygote monkeys was described in the previous report (Shiina et al., 2015).

Mixed Lymphocyte Reactions with iPS-RPE Cells

PBMCs were isolated from healthy adult monkey donors, and allogeneic immune responses were assessed for proliferation by Ki-67 incorporation in the PBMCs. PBMCs were cultured with iPS-RPE cells (1121A1 or 46a) and monkey B cells. As a positive control, Epstein-Barr virus-transformed B cells (B95-8 B cells) that are MHC-I$^+$, MHC-II$^+$, CD40$^+$, and CD86$^+$ were also prepared. The culture medium used was RPMI-1640 medium containing 10% fetal bovine serum (BioWhittaker), human recombinant interleukin-2 (Becton Dickinson), 10 mM HEPES (Sigma), 0.1 mM nonessential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), penicillin-streptomycin (Gibco), and 1×10^{-5} M 2-mercaptoethanol (Sigma). Before the assay, the target RPE cells or B cells were irradiated (20 Gy). After 96–120 hr, PBMCs were washed and analyzed by flow cytometry (Ki-67 proliferation assay by fluorescence-activated cell sorting [FACS]). The PBMC-RPE MLR assay was also performed with similar methods (Kamao et al., 2014).

In Vitro T Cell RPE Rejection Assay

T cells were established from normal monkey PBMCs. Monkey CD4$^+$ T cells were prepared separately by using separation beads (MACS cell isolation kit, Miltenyi Biotec). These cells were more than 95% CD4-positive. Purified CD4$^+$ T cells ($5–8 \times 10^5$ cells/well in 96-well plates) from the PBMCs of healthy donors were co-cultured with target iPS-RPE cells ($5–8 \times 10^3$ cells/well: effector/target ratio = 100:1) for 48 hr. In some in vitro assays, T cells from the PBMCs of a healthy donor (K251) were co-cultured with iPS-RPE cells in the presence of anti-B7-H3 abs (CD276, BioLegend) or isotype-control mouse immunoglobulin G (IgG) (purified mouse IgG1, BioLegend). T cell activation was evaluated by measurement of monkey IFN-γ production by using ELISA (Mabtech).

Flow Cytometry

Expression of MHC-I and MHC-II on monkey iPS-RPE cells (1121A1 or 46a) was examined by FACS analysis. Before staining, these cells were incubated with an Fc block (Miltenyi Biotec) at 4°C for 15 min. The cells were stained with anti-MHC-I antibody, anti-MHC-II antibody, or isotype control (mouse IgG) at 4°C for 30 min. iPS-RPE cells co-cultured with recombinant IFN-γ (100 ng/mL) for 48 hr were also prepared. Expression of co-stimulatory molecules on these iPS-RPE cells was also examined by FACS analysis of CD40, CD80 (B7-1), CD86 (B7-2), CD275 (ICOS-L/B7-H2), and CD276 (B7-H3). The cells were stained with the above antibody or isotype control at 4°C for 30 min.

Expression of RPE65, bestrophin, MerTk, MiTF, Pax6, ZO-1, PEDF, tyrosinase, and OCT3/4 by monkey iPS-RPE cells (1121A1 or 46a) was evaluated by FACS analysis. After Fc block staining, these RPE cells were stained with anti-human antibodies (Table S3) at 4°C for 30 min. RPE cells were also stained with anti-rabbit IgG or anti-mouse IgG (isotype controls) at 4°C for 30 min.

To confirm phagocytic function, we cultured 1121A1 iPS-RPE cells with RPE medium in the presence of fluorescein isothiocyanate (FITC)-labeled porcine shed photoreceptor rod outer segments for 5 hr at 37°C, and phagocytosis was evaluated by FACS (Sugita et al., 2015).

In the Ki-67 proliferation assay by FACS analysis, the following antibodies were prepared: APC-labeled anti-CD4 (helper T cells),

APC-labeled anti-CD8 (cytotoxic T cells), APC-labeled anti-CD11b (macrophages/monocytes), FITC-labeled anti-CD20 (B cells), FITC-labeled anti-CD56 (NK cells/NKT cells), and phycoerythrin-labeled anti-Ki-67. The harvested PBMCs and PBMCs co-cultured with iPS-RPE cells (or B cells) were stained with these antibodies at 4°C for 30 min. For intracellular staining for Ki-67, the staining was performed after cell fixation and permeabilization (BioLegend). All antibody information including isotype controls is described in Table S3. All samples were analyzed on a FACSCanto flow cytometer (BD). Data were analyzed using FlowJo software (version 9.3.1).

Immunohistochemistry

Monkey eyes collected at 8 weeks, 12 weeks, or 6 months were fixed and embedded in paraffin (Sigma-Aldrich). Paraffin sections were sliced into 10-μm-thick sections in a series of five sequential slides by using an autoslide preparation system (Kurabo; http://www.kurabo.co.jp). The same immunochemical techniques and photographing methods as mentioned below were applied to all sections. Sections were then blocked with 5% goat serum in PBS for 1 hr at room temperature. Additional primary antibodies against the following proteins were used: ionized calcium-binding adapter molecule 1 (Iba1) (host: rabbit), CD3 (host: rabbit), and MHC-II (host: mouse) (Table S3), and the antibodies were incubated at 4°C with overnight cultures. After rinsing with Tween 20 in PBS three times, sections were incubated with appropriate secondary antibodies (Table S3) for 1 hr at room temperature and counterstained with DAPI (×1,000; Life Technologies). Images were acquired with a confocal microscope (LSM700, Zeiss; http://www.zeiss.com). Each retinal layer (anatomy) in the section is shown in Figure S5 (control section).

Statistical Evaluation

At least three independent experiments were performed for in vitro data. All statistical analyses were performed with the paired Student's t test. Values were considered statistically significant if p was less than 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.08.010.

AUTHOR CONTRIBUTIONS

S.S. was the principal investigator who designed and performed experiments, and wrote the manuscript. Y.I. and K.M. cultured and prepared iPSCs and iPS-RPE cells. H.K., Y.H., M.M., and Y.K. performed the transplantation. T.S. and K.O. performed monkey MHC analysis. M.T. designed and conceptualized the study, and drafted and edited the manuscript.

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