Natural Killer Cell Inhibition by HLA-E Molecules on Induced Pluripotent Stem Cell–Derived Retinal Pigment Epithelial Cells

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PURPOSE. To determine whether human induced pluripotent stem (iPS) cell–derived retinal pigment epithelial (RPE) cells (iPS-RPE) can suppress natural killer (NK) cell activation.

METHODS. iPS-RPE cells were cocultured with peripheral blood mononuclear cells (PBMCs) or purified NK cells from healthy donors after stimulation with cytokines. To confirm expression of NK cell–specific markers, flow cytometry and quantitative RT-PCR (qRTPCR) were performed. NK cells (or PBMCs) cocultured with iPS-RPE cells were assessed for proliferation by Ki67 expression with flow cytometry, and NK suppression by RPE cells was assessed for granzyme B production with ELISA. Human leukocyte antigen (HLA) expression including HLA-E on iPS-RPE cells was evaluated with flow cytometry and qRT-PCR. The effect of HLA-E downregulation was also investigated using small interfering RNA (siRNA) systems. Following iPS-RPE cell transplantation in vivo, we evaluated NK cell invasion in the retina with immunohistochemistry.

RESULTS. Activated NK cells expressed NK-related markers such as CD16, CD56, and CD11b, and NK cells produced cytotoxic agents such as granzyme B, perforin, and TNF-α. Human iPS-RPE cells inhibited cell proliferation and production of these cytotoxic agents by activated NK cells in vitro. iPS-RPE cells constitutively expressed HLA-E and suppressed NK cell activation through an interaction between HLA-E and CD94/NKG2A. Moreover, immunohistochemical evaluation of monkey RPE transplantation into in vivo immune rejection models showed no NK cell invasion in the retina in allografts or xenografts except for one xenografted eye.

CONCLUSIONS. Cultured iPS cell–derived RPE cells greatly suppress NK cell activation. Thus, NK cells might be inactivated when exposed to this type of retinal cell.

Keywords: iPS cells, natural killer cells, RPE cells, suppression

The subretinal space is a classic example of an immune-privileged site in which tissue/cell allografts escape immunologic rejection.1–3 The retinal pigment epithelium (RPE), which is located in the subretinal space, contributes to the immune-privileged site and creates a blood-retina barrier. Although the subretinal space is afforded an immune advantage in healthy eyes with an intact blood-retinal barrier, this is not the case in eyes with an inherited retinal degenerative disorder such as age-related macular degeneration in which the choriocapillaris, RPE, or photoreceptor layers are actually damaged.4–6 In clinical studies,3–9 patients treated with RPE allografts show immune rejection, and the grafts do not survive, despite the fact that RPE cells are immune-privileged tissues. One possible explanation is that immune-privileged tissues are able to attack explanted RPE cells due to the expression of major histocompatibility complex (MHC)/human leukocyte antigen (HLA) molecules.10,11 Retinal cells constitutively express MHC class I (MHC-I) molecules under normal conditions without inflammation.12 However, some ocular tissues such as corneal endothelial cells and lens epithelial cells also express little MHC-I, and mature adult neurons including retinal neurons poorly express these molecules.13 As a result, these cell types in the eye are highly vulnerable to lysis by natural killer (NK) cells because of low MHC-I expression.13 Several reports have demonstrated infiltrating NK cells within inflamed eyes.14–18 Intraocular infiltrating NK cells are observed in patients with noninfectious uveitis such as Behçet’s disease11–14 and infectious uveitis such as herpes virus infection.15 In addition, NK cells and natural killer T (NKT) cells are involved in ocular lymphoma by invading intraocular and ocular adnexal tissues.19–22 These activated NK cells can affect the retina, choroid, vitreous, and anterior chamber. These results suggest that intraocular NK cells, as well as T/B lymphocytes and antigen-presenting cells, have an important role in the generation of inflammatory responses during uveitis and intraocular lymphoma.

Organ transplantation with allografts is associated with early NK cell activation, which can directly kill allogeneic target tissues/cells. NK cells are a central component of the innate immune system, because NK cells are developmentally programmed to destroy targets either directly or via antibody-dependent cellular cytotoxicity in the absence of antigen priming.22–25 NK cells can discriminate between self and non-self (i.e., foreign tissues/cells) and play a critical role in the regulation of adaptive immune responses including effector T and B cells.
after transplantation. Based on studies assessing NK cell depletion, researchers have concluded that NK cells fail to contribute to acute rejection of solid organ allografts. Notably, early NK cell activation and graft invasion are only observed in recipients of allogeneic, but not syngeneic, transplants. These results suggest that NK cells become activated following direct recognition of allogeneic antigens on tissues. Manipulating the NK cell response or inducing inactivated tolerogenic NK cells may be useful for influencing the overall allogeneic immune response and the fate of an organ allograft.

NK cells traffic into the eye under inflammatory conditions, yet NK-mediated injury of RPE cells is unclear. In the present study, we showed that cultured human induced pluripotent stem (iPS) cell–derived RPE cells constitutively expressed classical MHC-I and nonclassical MHC-I (HLA-E) molecules. Furthermore, these HLA-E molecules on iPS-RPE cells inhibited NK cell activation in vitro.

### Materials and Methods

#### Establishment of Human iPS Cell–Derived RPE Cells

Human iPS cells were established from skin fibroblasts, dental pulp cells, or peripheral blood mononuclear cells (PBMCs) of healthy donors with an episcopal vector as previously reported. We then prepared primary cultures of iPS cell–derived RPE cells (454E2, 453F2, or TLH1D1 lines) with RPE-specific medium and signal indicators such as Y27632, SB431542, and/or CKI-7. After the appearance of RPE-like colonies, the medium was switched to Dulbecco’s modified Eagle’s medium (DMEM) supplemented with B27 supplement, and RPE colonies were transferred to CELLstart-coated dishes. Establishment of Human iPS Cell–Derived RPE Cells

#### Preparation of NK Cells

Target NK cells were established from PBMCs. NK cells were prepared separately by using separation beads and columns (negative selection, MACS cell isolation kits; Miltenyi Biotec, Auburn, CA, USA). These cells were more than 86% CD56 positive. Antibody information is described in Supplementary Table S1. NK cells were cultured with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and human recombinant proteins, interferon (IFN)–γ (50 ng/mL; R&D Systems), or phytohemagglutinin-P (Difco, Detroit, MI, USA), and cells were incubated for 1 hour. After washing with PBS, purified NK cells (5 × 10^5 cells/well in 96-well plates or 2 × 10^6 cells/well in 24-well plates) were stimulated as above with recombinant proteins, and then incubated with target B cells (5 × 10^4 cells/well; effector:target ratio = 10:1) for 5 hours. As negative control cells, we also prepared B cells from the same donor (autogenic B cells). After incubation, cells were harvested and analyzed on a FACSCanto II flow cytometer for Ki-67 expression and data were analyzed by the Institutional Ethics Committee of the Center for Developmental Biology, RIKEN.

#### NK-RPE Cell Assay for Cell Proliferation and Cytokine Production

Purified NK cells (5 × 10^5 cells/well in 96-well plates or 2 × 10^6 cells/well in 24-well plates) from healthy donor PBMCs were stimulated as above, and then incubated with iPS-RPE cells for 48 to 72 hours. Human iPS-RPE cells were cultured separately in 96-well plates (1 × 10^3 cells/well) or 24-well plates (5 × 10^3 cells/well), and NK cell activation in the presence of iPS-RPE cells was assessed with flow cytometry for Ki-67 expression (BioLegend, San Diego, CA, USA) and enzyme-linked immunosorbent assay (ELISA) for granzyme B production (Abcam, Cambridge, UK) or tumor necrosis factor (TNF)-α production (R&D Systems). We also used recombinant proteins, recombinant human HLA-E (0.01, 0.1, 1 µg/mL; Proteintech, Rosemont, IL, USA) and recombinant human macrophage migration inhibitory factor (MIF) (0.01, 0.1, 1 µg/mL; R&D Systems), anti-human HLA-E neutralizing antibody (1 µg/mL; eBioscience, San Diego, CA, USA), and an isotype control (1 µg/mL; eBioscience).

#### 51Cr Release Assay

Isolated NK cells from healthy donor PBMCs were incubated with target B cells in the 51Cr release assay. Before the assay, we labeled target B cells (human Epstein-Barr virus [EBV]-transformed B cells or monkey EBV-transformed B cells [B95-8 cell line]) with 51Cr radiolucide (0.37 MBq; PerkinElmer, Waltham, MA, USA), and cells were incubated for 1 hour. After washing with PBS, purified NK cells (5 × 10^5 cells/well in 96-well plates or 2 × 10^6 cells/well in 24-well plates) were stimulated as above with recombinant proteins, and then incubated with target B cells (5 × 10^4 cells/well; effector:target ratio = 10:1) for 5 hours. As negative control cells, we also prepared B cells from the same donor (autogenic B cells). After incubation, cells were harvested and analyzed with a liquid scintillation counter (LS6500; Beckman Coulter, Brea, CA, USA). We calculated % target cell killing with negative controls (i.e., target cells only) and positive controls (i.e., target cells plus 0.5% Triton X-100).

#### Flow Cytometry

NK cell activation markers, such as granzyme B and perforin, in NK cells exposed to iPS-RPE cells were assessed with flow cytometry. Intracellular staining was performed to assess granzyme B and perforin expression. After human Fc block staining and permeabilization, activated NK cells were stained with anti-human granzyme B or perforin antibodies plus CD56 antibody at 4°C for 30 minutes.

CD2, CD3, CD11b, CD16, CD19, CD56, CD94, NKG2A, and killer cell immunoglobulin-like receptor (KIR; CD158) expression on PBMCs or isolated NK cells was evaluated with fluorescence-activated cell sorting (FACS) analysis. Purified NK cells or PBMCs were also stained with FITC–, allophycocyanin (APC), or phycoerythrin (PE)-labeled isotype control antibodies (mouse IgG) at 4°C for 30 minutes.

HLA-E expression on iPS-RPE cells was evaluated with FACS analysis. IFN–γ–pretreated RPE cells (rIFN–γ 0.1, 1, 10, 100 ng/mL) were also prepared. After human Fc block staining, RPE cells were stained with anti-human HLA-E antibodies at 4°C for 30 minutes. RPE cells were also stained with anti-human HLA class I (A, B, C), HLA class II (DR, DP, DQ), HLA-G, and β2–microglobulin antibodies at 4°C for 30 minutes. For isotype control staining, RPE cells were stained with FITC– or PE–labeled anti-mouse IgG at 4°C for 30 minutes. All samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (version 9.3.1; Tree Star, Ashland, OR, USA). All antibody information is described in Supplementary Table S1. To avoid experimental bias in FACS analysis, our technical staff masked the identity of the samples, specifically with respect to gating approaches during data capture.
Transfection of Small Interfering RNAs (siRNAs) Targeting HLA-E

siRNAs targeting human HLA-E and β2-microglobulin (both Santa Cruz, Dallas, TX, USA) were transfected into iPS cell-derived RPE cells. On day 0, cells were cultured in DMEM supplemented with 5% FBS (antibiotic free). After overnight culture, RPE cells were transfected with HLA-E or β2-microglobulin siRNA reagent or control siRNA (Santa Cruz) at 37°C for 6 to 7 hours and then cultured in DMEM supplemented with 10% FBS at 37°C for 24 hours. After incubation, cells were harvested and examined for HLA-E and β2-microglobulin expression with quantitative PCR (qRT-PCR), flow cytometry, or Western blots.

Quantitative RT-PCR

Total RNA was isolated from iPS-RPE cells (836B1, 454E2, and TLHD1), HLA-E, or β2-microglobulin siRNA-transfected iPS-RPE cells, and control cells such as human iPS cells, human ES cells, and human fibroblasts. After cDNA synthesis, HLA-E, β2-microglobulin, and β-actin expression in triplicate samples was analyzed with qRT-PCR with a LightCycler 480 instrument by using qPCR Mastermix and Universal ProbeLibrary assays (all Roche Diagnostics, Mannheim, Germany). Primers and the universal probe used in this study are described in Supplementary Table S2. qRT-PCR was performed by denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 second. Relative mRNA expression was calculated with Relative Quantification Software (Roche Diagnostics) by using an efficiency-corrected algorithm with standard curves and reference gene normalization to β-actin (ΔΔCt).

Western Blots

We prepared iPS-RPE cells, including control siRNA-transfected, HLA-E siRNA-transfected, and β2-microglobulin siRNA-transfected cells. The cells were pretreated and then heat shocked at 95°C for 5 minutes. We used 10% polyacrylamide gel (SarSepAce; Waco, Osaka, Japan) with samples (1.5 × 10⁴ RPE cells/lane). The samples were electrophoresed at 80 V for 30 minutes, and then 200 V for 50 minutes. Following equilibration of the gel in transfer buffer, separated samples were blotted at 25 V for 10 minutes with the Trans-Blot Semi-dry Transfer cell (Bio-Rad, Hercules, CA, USA). Following washes with TBS-T, blots were incubated overnight at 4°C with primary antibodies diluted 1:500 for HLA-E (Abcam), 1:5000 for β2-microglobulin (Abcam), or 1:5000 for GAPDH (Cell Signaling Technology, Danvers, MA, USA). Following washes with TBS-T (Tris-buffered saline, 0.1% Tween 20), blots were incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:20,000 (Dako, Santa Clara, CA, USA) for 1 hour at room temperature. After washes with TBS-T, the target proteins were detected with Chemi-Lumi One Super kit (Nacalai Tesque, Kyoto, Japan).

Immunohistochemistry

In our previous reports, we transplanted the monkeys with allografts (monkey iPS-RPE cells → monkey eyes, n = 8) and xenografts (human iPS-RPE cells → monkey eyes, n = 3). After monkeys were killed, monkey eyes that were collected at 16 weeks or at 6 months were fixed and embedded in paraffin (Sigma-Aldrich Corp., St. Louis, MO, USA). We performed immunohistochemistry (IHC) as in previous reports. All antibody information for IHC is described in Supplementary Table S1. All animal experiments were approved by the Institutional Animal Research Committee of RIKEN CDB, and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Statistical Evaluation

All statistical analyses were conducted with the paired Student’s t-test. Differences were considered statistically significant at P < 0.05.

RESULTS

Inhibition of NK Cell Activation by iPS Cell–Derived RPE Cells

First, we isolated NK cells from PBMCs obtained from healthy donors. To confirm NK cells, we evaluated CD2, CD11b, CD16, and CD56 expression. We also evaluated CD3 (T-cell marker and non-NK marker) and CD19 (B-cell marker and non-NK marker) expression. PBMCs included 13.2% CD3+CD56+ cells, 80.0% CD19+CD2+ cells, and 11.6% CD16+CD11b+ cells, indicating that PBMCs contained approximately 10% NK cells (Fig. 1A). After isolation of NK cells, purified NK cells included 87.9% CD3+CD56+ cells, 80.6% CD19+CD2+ cells, and 84.5% CD16+CD11b+ cells (Fig. 1B). In addition, isolated NK cells included 2.6% CD3+CD56+ cells, indicating that isolated NK cells include 2% to 3% NKT cells.

We next tried to establish activated NK cells from healthy donor cells. We used purified NK cells and stimulated them with human recombinant proteins such as IFN-γ, IL-12, and IL-2 in vitro. We evaluated production of granzyme B, which is secreted by NK cells, along with perforin (a pore-forming protein), which mediates apoptosis of target cells. Compared with IL-2-supplemented medium, NK cells highly produced granzyme B in the presence of recombinant interleukin (rIL)-12 only, but not IFN-γ only (Fig. 1C). Moreover, compared with IL-2-supplemented medium, NK cells significantly secreted cytotoxic agents when all recombinant proteins (IFN-γ, IL-12, and IL-2) were added to the cultures (Fig. 1C). FACS analysis demonstrated that activated CD56+ NK cells expressed granzyme B and perforin (Fig. 1D). Moreover, activated NK cells greatly killed EBV-transformed B cells in the 51Cr release assay (Fig. 1E). Therefore, we used activated NK cells in the following experiments.

We next assessed whether human iPS cell–derived RPE cells could suppress NK cell activation. As shown in Figure 2A, PBMCs that included CD2+ (mainly T cells and NK cells), CD16+ (mainly NK cells and monocytes), or CD56+ cells (mainly NK and NKT cells) expressed Ki-67. However, CD2+, CD16+, or CD56+ cells among PBMCs exposed to iPS-RPE cells poorly expressed Ki-67, suggesting that iPS-RPE cells are able to suppress NK cell proliferation in vitro. To confirm this finding, we measured concentrations of cytokines generally produced by activated NK cells. ELISA results demonstrated that the production of inflammatory cytokines, such as TNF-α and granzyme B, by NK cells was significantly suppressed when cocultured with iPS-RPE cells, whereas NK cells without iPS-RPE cells produced high levels of these cytokines (Supplementary Fig. S1A). We obtained similar results for granzyme B with FACS analysis (Supplementary Fig. S1B). Quantitative RT-PCR analysis demonstrated that iPS-RPE cells significantly suppressed CD16a, TNF-α, granzyme B, and IFN-γ mRNA levels in activated NK cells (Fig. 2B). Next, we prepared different numbers of RPE cells in the in vitro assay. NK cell activation was not suppressed in the presence of 5 × 10⁴ to 5 × 10⁵ iPS cell-derived RPE cells (rather, NK cells were activated),
whereas NK cell activation was significantly suppressed in the presence of $5 \times 10^4$ to more than $5 \times 10^5$ RPE cells (Supplementary Fig. S1C). When the same number of NK cells and RPE cells was used in the assay (both $5 \times 10^5$ cells/well: ratio = 1:1), NK cell activation was greatly suppressed.

**Capacity of iPS-RPE Cells to Suppress NK Cell Activation via Cell-to-Cell Contact**

We then investigated whether other RPE cells (derived from human ES cells and human primary RPE cells) and iPS cells could suppress NK cell activation. As expected, all types of RPE cells significantly suppressed granzyme B (Fig. 3A) and TNF-$\alpha$ (data not shown) production, similar to iPS cell-derived RPE cells. Cultured iPS cell-derived RPE cells also greatly suppressed NK activation, whereas iPS cells did not (Fig. 3B).

To clarify the role of cell contact in the suppression of NK cell activation by iPS cell-derived RPE cells, these cell types were cultured separately in culture plates. CD56$^+$ NK cells highly expressed cytotoxic agents such as granzyme B and perforin, but the expression was reduced if NK cells were cocultured with iPS-RPE cells in the absence of transwell cell inserts (Fig. 3C). However, iPS-RPE cells failed to suppress NK cell activation in the presence of the transwell membrane. These results suggest that cell-to-cell contact is essential for NK cell suppression by iPS-RPE cells. Thus, RPE cells express immunosuppressive factor(s) on their surface to suppress NK cells.

**HLA-E Expression by iPS Cell–Derived RPE Cells**

Cultured human RPE cells including iPS cells constitutively express MHC-I molecules, but not MHC-II molecules such as...
Among MHC and MHC-related molecules, HLA class I and nonclassical HLA class I (HLA-E and HLA-G) suppress NK cells. We therefore examined whether iPS-RPE cells expressed HLA class I (A, B, C), HLA-E, HLA-G, β2-microglobulin, and HLA class II (DR, DP, DQ) molecules. Flow cytometric analysis demonstrated that iPS-RPE cells expressed HLA class I and β2-microglobulin, but not HLA class II (Fig. 4A). For nonclassical HLA class I molecules, iPS-RPE cells constitutively expressed HLA-E, whereas HLA-G expression was poor. Human fibroblasts exhibited a similar phenotype excluding HLA-G expression (Fig. 4A). For nonclassical HLA class I molecules, iPS-RPE cells constitutively expressed HLA-E, whereas HLA-G expression was poor. Human fibroblasts exhibited a similar phenotype excluding HLA-G expression (Fig. 4A). Conversely, human iPS cells expressed HLA molecules on their surfaces, but this expression was poor compared with iPS-RPE cells. Because HLA class I and nonclassical HLA class I molecules, such as HLA-E, inhibit NK cells, we focused on HLA-E expression on iPS-RPE cells as a candidate molecule for NK cell suppression.

Compared to human iPS cells, iPS-RPE cells expressed high HLA-E mRNA levels (Fig. 4B). In addition, compared to human ES cells, ES-RPE cells expressed high levels of HLA-E mRNA (Fig. 4B). FACS analysis demonstrated that iPS-RPE cells pretreated with recombinant IFN-γ expressed significant amounts of HLA-E compared with nontreated cells (Fig. 4C). In addition, high HLA-E and β2-microglobulin mRNA levels on IFN-γ–treated RPE cells were observed compared with nontreated cells (Fig. 4D). When iPS-RPE cells were treated with 100 ng/mL recombinant IFN-γ, we observed significantly higher levels of both HLA-E and β2-microglobulin mRNA (Supplementary Fig. S2).

Detection of CD94, NKG2A, and KIR on NK Cells

The CD94/NKG2A complex serves as an inhibitory receptor that is specific for HLA-E, and KIR (CD158) also has an inhibitory function on NK cells. We therefore confirmed the expression of CD94/NKG2A and KIR on target NK cells. CD16+ or CD56+ cells from fresh PBMCs expressed CD94 and KIR (Fig. 5). Moreover, NK cells isolated from PBMCs greatly expressed these receptors (Fig. 5). In addition, cultured NK cells among PBMCs that were pretreated with recombinant cytokines (IL-2, IL-12, and IFN-γ) highly expressed CD94/NKG2A and KIR compared with nontreated cultured cells (data not shown). These results imply that iPS-RPE cells are able to suppress NK cells through the interaction between HLA-E and the CD94/NKG2A complex.
Capacity of Recombinant HLA-E to Suppress NK Cell Activation In Vitro

We next tested the ability of HLA-E to suppress NK cells. Because RPE cells also express other molecules, for example, macrophage migration MIF, which inhibits NK activation, we used these human proteins. Recombinant human HLA-E proteins suppressed proliferation of NK cells from PBMCs, that is, suppression of NKG2A/Ki-67 molecules on PBMCs (Fig. 6A). Recombinant HLA-E (10, 100, and 1000 ng/mL) also suppressed NKG2A/Ki-67 molecules (Fig. 6A, right). In addition, HLA-E proteins suppressed NK cell killing of virus-infected B-cell lines (Fig. 6B). Conversely, recombinant MIF proteins did not suppress NK cell activation (Figs. 6A, 6B), suggesting that HLA-E is critical for RPE cell-mediated suppression. Although human iPS-RPE cells can produce MIF, this cytokine might be unnecessary for NK cell suppression.

FIGURE 3. Capacity of iPS-RPE cells to suppress NK cell activation via cell contact. (A) NK cells were cocultured with other RPE cells, human ES cell–derived RPE cells, and human primary RPE cells in the presence of rIFN-γ, rIL-12, and rIL-2 for 72 hours. The supernatants were evaluated by ELISA for granzyme B. Data are shown as mean ± SEM of three ELISA determinations. *P < 0.05, **P < 0.005, as compared to the positive control (open bar). (B) Ability of iPS-RPE cells and iPS cells (both TLHD1 lines) to suppress NK activation (granzyme B ELISA). Data are representative of three independent experiments with similar results. (C) NK cells and iPS-derived RPE cells were separately cultured in culture plates. Transwell cell inserts were placed in wells, and each cell insert contained NK cells (iPS-RPE cells were outside of cell inserts). FACS histograms indicate the percentage of CD56/granzyme B or perforin double-positive cells. Data are representative of three independent experiments with similar results.
To determine whether HLA-E expressed by iPS-RPE cells was necessary to suppress NK cells, we next examined the effect of siRNA-mediated downregulation of HLA-E. HLA-E siRNA-transfected iPS-RPE cells poorly expressed HLA-E compared to control siRNA-transfected iPS-RPE cells (Fig. 7A). Similarly, compared with control siRNA-transfected cells, β2-microglobulin siRNA-transfected iPS-RPE cells expressed lower levels of HLA-E and β2-microglobulin (Fig. 7A). HLA-E mRNA levels were downregulated in HLA-E siRNA-transfected iPS-RPE cells (Supplementary Fig. S3A). Similar results were obtained in β2-microglobulin siRNA-transfected iPS-RPE cells (Supplemen-
tary Fig. S3A). We obtained similar results with Western blotting. We did not observe a specific band in HLA-E- or β2-microglobulin siRNA-transfected RPE cells (Supplementary Fig. S3B).

Subsequently, we examined whether HLA-E siRNA-transfected iPS-RPE cells could suppress NK cell activation. HLA-E siRNA-transfected iPS-RPE cells failed to suppress NK cell activation (i.e., granzyme B production) (Fig. 7B). Moreover, β2-microglobulin siRNA-transfected iPS-RPE cells, which poorly express HLA-E, failed to suppress NK cells in vitro when anti-human HLA-E blocking antibodies were added, whereas iPS-RPE cells significantly suppressed NK cells in the presence of isotype control antibodies (data not shown). Taken together, our results indicate that HLA-E is important for RPE cell-mediated NK suppression.

Detection of NK Cells in the Retina After Transplantation of iPS Cell–Derived RPE Cells in a Monkey In Vivo Immune Attack Model

Recently, we reported the presence of inflammatory T/B lymphocytes and antigen-presenting cells in the retina and around grafts when allografts were transplanted (monkey iPS-RPE cells → monkey eyes). In addition, we found that these inflammatory cells mediate immune attacks in xenografts (human iPS-RPE cells → monkey eyes). Therefore, to evaluate NK cell–associated immune attacks, we conducted IHC using these retinal sections from the monkeys. We found no NK cells throughout the retinal sections, even when we transplanted RPE cell allografts (n = 8). However, we found obvious NK cell invasion in the retina in one of the xenografts (1/3 eyes) (Fig. 8). Importantly, donor iPS-derived RPE cells clearly expressed HLA-E molecules in the retina after RPE cell transplantation (Fig. 8D).

DISCUSSION

In the present study, we assessed whether human iPS cell–derived RPE cells could suppress NK cell activation in vitro. Our established iPS-RPE cells suppressed NK cell proliferation and suppressed NK cell activation, for example, granzyme production. To suppress NK cells, iPS-RPE cells expressed immunosuppressive factor(s) on their surfaces, indicating that cell-to-cell contact is essential for NK cell suppression. Because HLA class I and nonclassical HLA class I (e.g., HLA-E) molecules can inhibit NK cells, we selected HLA-E on iPS-RPE cells as a candidate molecule for NK cell suppression. iPS-RPE cells expressed high levels of HLA-E. Moreover, we demonstrated that iPS-RPE cells suppressed NK cell activation via HLA-E (perhaps through the CD94/NKG2A complex on NK cells), because RPE cells in which HLA-E was downregulated failed to inhibit NK cell activation, and recombinant HLA-E proteins suppressed NK cell activation in vitro. Thus, one possibility for the in vivo observation (Fig. 8) is that explanted RPE cells suppress NK cells after RPE cell transplantation.

The eye is one of the immune-privileged sites in the body, which includes areas such as the subretinal space and anterior chamber of the eye, the testis, and the central nervous system behind the blood–brain barrier. Immune-privileged tissues must suppress a direct cytotoxic immune attack on target cells, that is, NK cells and cytotoxic T cells (CD8 T cells). This is achieved by a range of mechanisms, among which downregulation of MHC-I molecules and local expression of potent immunosuppressive factors (e.g., TGF-β and s-MSH) are main strategies. In addition, ocular parenchymal cells express the CD95 ligand (CD95L/Fas ligand) that triggers apoptosis of inflammatory cells. Absent or low MHC-I expression in the immune-privileged eye constitutes a basic problem in self- or non-self-discrimination and self-tolerance, because NK cells...
are primed to recognize and eliminate such cells. Because many retinal disorders result from a disrupted blood-retina barrier and absent or low MHC-I expression in the eye, intraocular NK cells are able to infiltrate and attack the tissues/cells of the eye.

Activated NK cells directly kill allogeneic target tissues/cells after organ transplantation. An ocular study showed little evidence of NK-related allogeneic immune rejection. Similar to T cells, NK cells play a crucial role in corneal allografts in rat transplantation models. Compared with draining lymph node cells, NK cells are present in the anterior chamber at a 10- to 15-fold higher percentage after transplantation, suggesting that NK cells can expand in the local space and become activated after antigen priming. However, to our knowledge, NK-associated immune attacks in retinal diseases have not been demonstrated. We recently established allogeneic transplantation using iPS cell–derived RPE cells in animal immune rejection models. Compared with draining lymph node cells, NK cells are present in the anterior chamber at a 10- to 15-fold higher percentage after transplantation, suggesting that NK cells can expand in the local space and become activated after antigen priming. However, to our knowledge, NK-associated immune attacks in retinal diseases have not been demonstrated. We recently established allogeneic transplantation using iPS cell–derived RPE cells in animal immune rejection models. Inflammatory immune attacks were observed around iPS-RPE grafts and retinal tissue damage together with invasion of inflammatory cells such as T cells, microglia, and antigen-presenting cells in the retina. As shown in this study, we investigated whether NK-specific marker-positive cells (e.g., NKG2A) are present in retinal sections from experimental animals in an immune attack model.

HLA-E is a nonclassical MHC-I (MHC-Ib) molecule, and HLA-E, similar to other MHC-I molecules, heterodimerizes with β2-microglobulin. HLA-E is characterized by limited polymorphism and ubiquitous expression. HLA-E interacts with CD94/NKG2A to regulate NK cell cytolytic activities. iPS-RPE cells pretreated with recombinant IFN-γ expressed significant amounts of HLA-E compared with nontreated cells (Figs. 4C, 4D; Supplementary Fig. S2). In inflammatory conditions such as an IFN-γ–rich environment, in vivo RPE cells likely express high levels of HLA-E. In fact, activated NK cells in patients with ocular inflammation secrete IFN-γ, and this and other cytokines, which are also produced by Th1 cells, can stimulate NK cells. Inflammatory cytokines secreted by NK cells, T cells, and other immune cells may mediate HLA-E expression on RPE cells. In a recent report, the investigators proposed HLA-ABC null cells as candidate cells for transplantation, but HLA-E–expressing pluripotent stem cells, which are termed universal donor cells, escape allogeneic lysis by NK cells without T-cell stimulation.

NK cells constitutively express the inhibitory receptors KIR and CD94/NKG2A. If target cells express MHC-I molecules, then NK cell activation can be prevented through interaction with KIR by phosphorylation of the immunoreceptor tyrosine inhibitory motif followed by binding to phosphatases. However, target cells lacking MHC-I expression (e.g., virus-infected and tumor cells) do not inhibit NK cells that are triggered by a signal from an activating receptor such as NKG2D. As shown in Figure 5, isolated NK cells constitutively expressed CD94/NKG2A on their surfaces. Therefore, we speculate that HLA-E on iPS-RPE cells can bind and inhibit NK cell activation through these interactions via cell-to-cell contact. In fact, iPS-RPE cells failed to suppress NK cell activation in the presence of transwell cell inserts (Fig. 3C), suggesting that cell contact is essential for NK cell suppression by iPS-RPE cells.

In addition to NK cell receptors and ligands, other factors should be considered. In the anterior chamber of the eye, MIF suppresses NK cell activity. MIF is a pleiotropic cytokine that is present in the aqueous humor and inhibits NK cell–mediated cytolysis. Moreover, human RPE cells, including iPS cell–derived cells, secrete this cytokine. However, as shown in this study, recombinant MIF failed to inhibit NK cell activation (Fig. 6). Although human RPE cells can produce MIF, we believe that HLA-E molecules expressed by RPE cells play a critical role in suppressing intraocular NK cells.

We also evaluated whether NK cell attacks are relevant in transplantation of iPS cell–derived RPE cells. For this, we used retinal sections from immune rejection eyes in allografts (monkey RPE cells → monkey eyes) and xenografts (human...
FIGURE 7. RPE cell–related suppression of NK cell activation via HLA-E. (A) Expression of HLA-E and β2-microglobulin (β2-MG) on HLA-E siRNA-transfected iPS-RPE cells by FACS analysis. β2-MG siRNA-transfected iPS-RPE cells or control siRNA-transfected iPS-RPE cells were also prepared. Upper: iPS-RPE cells. Lower: IFN-γ–pretreated iPS-RPE cells. Left: HLA-E expression on HLA-E siRNA-transfected iPS-RPE cells. Middle: HLA-E expression on β2-MG siRNA-transfected iPS-RPE cells. Right: β2-MG expression on β2-MG siRNA-transfected iPS-RPE cells. Blue lines in histograms indicate control siRNA-transfected iPS-RPE cells; red lines indicate target siRNA (HLA-E or β2-MG)–transfected iPS-RPE cells; and green lines indicate isotype control antibodies. Numbers in histograms indicate mean fluorescence intensity. Data are representative of three independent experiments.
In the eye, many CD3+ observed in the choroid. (Left: CD3+ T cells (allograft/xenograft) showing immunohistochemistry for HLA-E in the retina at 6 months were labeled with the antibody and costained with 4′,6-diamidino-2-phenylindole (DAPI) to label nuclei. (Right: graft RPE cells — monkey eyes). The RPE allografts demonstrated immune attacks mediated by T/B lymphocytes and antigen-presenting cells,11,27 but no evidence of NK cell attacks. These results imply that the suppressive effects of RPE cells on NK cells occur in vivo. Grafted RPE cells that express HLA-E (MHC-E) may suppress infiltrating NK cells. However, in one of the xenografts, we did find NK cell invasion together with infiltration of T cells, B cells, and antigen-presenting cells in the inflamed retina (Fig. 8), suggesting that HLA-E-expressing human RPE cells do not suppress NK cells because monkey NK cells do not have human HLA-E receptors. Another possibility is that monkey NK cells can recognize ligands for NK cell activation (e.g., NKG2D) on iPS-RPE cells. If so, we will need to consider the possibility of NK cell activation in allogeneic iPS-RPE cell transplantation for retinal diseases. KIR ligand matching may be necessary for patients. Interestingly, a recent report by Ichise et al.42 clearly demonstrated that NK cell lysis is prevented when iPSC-derived graft cells overexpress the missing KIR ligand. Thus, KIR ligand matching, as well as HLA matching (HLA haplotype identity), may be necessary for RPE cell transplantation.

In conclusion, cultured RPE cells including iPSC-derived RPE cells are able to suppress activated NK cells in vitro. Although T cell–mediated rejection of iPSC-derived cells has been proposed to be irrelevant when cells are transplanted back into the donor of the somatic cells, their lack of MHC antigens could make them targets for NK cells.40 NK cells favor and recognize graft cells with little or no MHC-I expression. RPE cells can inhibit NK cells (i.e., inactivated tolerogenic NK cells) via HLA-E expression; however, NK cells can activate and attack RPE cells if HLA-E expression is poor. Therefore, we recommend establishment of human iPSC-RPE cells that constitutively express MHC-I and HLA-E molecules before transplantation.

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Supported by a Scientific Research Grant (B, 25293357) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by the Research Center Network for Realization of RPE cells — monkey eyes). The RPE allografts demonstrated immune attacks mediated by T/B lymphocytes and antigen-presenting cells,11,27 but no evidence of NK cell attacks. These results imply that the suppressive effects of RPE cells on NK cells occur in vivo. Grafted RPE cells that express HLA-E (MHC-E) may suppress infiltrating NK cells. However, in one of the xenografts, we did find NK cell invasion together with infiltration of T cells, B cells, and antigen-presenting cells in the inflamed retina (Fig. 8), suggesting that HLA-E-expressing human RPE cells do not suppress NK cells because monkey NK cells do not have human HLA-E receptors. Another possibility is that monkey NK cells can recognize ligands for NK cell activation (e.g., NKG2D) on iPS-RPE cells. If so, we will need to consider the possibility of NK cell activation in allogeneic iPS-RPE cell transplantation for retinal diseases. KIR ligand matching may be necessary for patients. Interestingly, a recent report by Ichise et al.42 clearly demonstrated that NK cell lysis is prevented when iPSC-derived graft cells overexpress the missing KIR ligand. Thus, KIR ligand matching, as well as HLA matching (HLA haplotype identity), may be necessary for RPE cell transplantation.

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References


