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Title	Macrophage-like iPS-derived Suppressor Cells Reduce Th1-mediated Immune Response to a Retinal Antigen
Author(s)	Hase, Keitaro; Namba, Kenichi; Wada, Haruka et al.
Citation	Current eye research, 46(12), 1908-1916 https://doi.org/10.1080/02713683.2021.1952605
Issue Date	2021-12-02
Doc URL	https://hdl.handle.net/2115/87439
Rights	This is an Accepted Manuscript of an article published by Taylor & Francis in Current eye research on 2021/12/02, available online: http://www.tandfonline.com/10.1080/02713683.2021.1952605.
Type	journal article
File Information	iPS-SCs Manuscript revised 2 clean copy.pdf



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Macrophage-like iPS-derived suppressor cells reduce Th1-mediated immune response to a retinal antigen

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Macrophage-like iPS-derived suppressor cells reduce Th1-mediated immune response to a retinal antigen

Abstract

Purpose: To investigate the immunotherapeutic effects of macrophage-like induced pluripotent stem (iPS) cell-derived suppressor cells (SCs) in ocular immune response and experimental autoimmune uveoretinitis (EAU).

Methods: The genes of *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* were transferred to B cells enriched from the spleen cells of C57BL/6 mice by using retrovirus vectors. Transferred B cells were cultured for 17 days to obtain colonies of iPS cells. Through additional steps, iPS-SCs were induced.

An antigen-specific T cell proliferation assay was performed with $CD4^+$ T cells collected from draining lymph nodes of the mice immunized with human interphotoreceptor retinoid-binding protein (hIRBP) peptide and co-cultured with iPS-SCs. Cytokine concentrations in the culture supernatant were examined. Mice were immunized with hIRBP peptide to induce EAU. The iPS-SCs were administered into the mice one day before the induction of EAU.

Results: The iPS-SCs decreased hIRBP-specific T cell proliferation depending on the number of cells. Productions of tumor necrosis factor-α and interferon-γ were significantly decreased; however, transforming growth factor-β1, nitric oxide, interleukin (IL)-13, IL-17A, and IL-17F levels were elevated in the supernatant when the collected T cells were co-cultured with iPS-SCs. The iPS-SCs had immunosuppressant effects even without cell-to-cell contact, and their effects were non-specific to the antigen preloaded on iPS-SCs. EAU was significantly milder in the mice administered iPS-SCs prior to immunization.

Conclusions: Macrophage-like iPS-SCs reduced Th1 immune response to a retinal antigen and Th1-mediated EAU in mice. These results showed the possibility of the application of iPS technology to the treatment of noninfectious ocular inflammation, endogenous uveitis, in the future.

Keywords: retinal antigen, type 1 helper T cells, cellular immunity, immunosuppression, iPS cells

Introduction

Uveitis is the inflammation that occurs in the uvea composed of the iris, ciliary body, and choroid, and is the cause of approximately 10% of blindness over the age of 40. The average age of onset of 1,616 uveitis patients who visited Hokkaido University Hospital in 2004–2014 was 38.8 years in men and 50.0 years in women [1]. This shows that uveitis often occurs in working age, which is an important issue not only for patients themselves but also for society.

Corticosteroids, non-steroid anti-inflammatory drugs, and immunosuppressants are mainly used in the treatment of uveitis. They have a certain degree of risk of side effects with long-term administration, and patients often show a recurrence of uveitis while reducing the dosage of the medicines. Recently, biological agents targeting inflammatory cytokines have been developed. For instance, Tumor necrosis factor (TNF) inhibitors, one group of biologics, show high efficacy against refractory non-infectious uveitis [2-5]. However, these therapies including biologics, are palliative treatments and cannot be a radical treatment for every case; therefore, they need to be continued for a long time.

Other studies offered a different therapeutic approach for uveitis, “immunotherapy”. The purpose of the therapy is to control excessive autoimmune reactions considered to be the cause of uveitis by transferring immunosuppressive cells, such as regulatory T cells and

regulatory dendritic cells. Previously, we reported that experimental autoimmune uveoretinitis (EAU) in mice was ameliorated by transferring the regulatory T-cells induced by α-melanocyte stimulating hormone (MSH) and transforming growth factor (TGF) -β2 [6]. It was also reported that regulatory dendritic cells induced by IL-10 suppressed EAU in mice [7].

Recently, induced pluripotent stem (iPS) cells have been gathering attention in the fields of regenerative medicine as a way to generate grafts for transplantation. These cells can proliferate semi-permanently and differentiate into various tissues or organs in the human body. The benefit of treatment with iPS cells is that they can be administered to patients with various diseases in a large amount repeatedly without the fear of allogenic rejection. It is expected that iPS cell-derived immunosuppressive cells will be applied to inflammatory diseases [8]. We succeeded in inducing macrophage-like iPS cell-derived immunosuppressor cells (iPS-SCs) and reported that those cells contributed to the prolonged survival of allografts [9]. In this study, we focused on the immunosuppressive potency of iPS-SCs in antigen-stimulated T cell proliferation and then investigated the therapeutic effects in EAU, an animal model of human autoimmune uveitis.

Materials and methods

Animals

C57BL/6J (CD45.2) mice (6 week-old males) were obtained from CLEA Japan (Tokyo, Japan). C57BL/6J (CD45.1) mice (8–12 week-old females) were obtained from Japan SLC (Shizuoka, Japan). All studies were conducted in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Review Committee for Animal Experimentation of Hokkaido University.

Reagents

Human interphotoreceptor retinoid-binding protein (hIRBP) peptide sequence 1–20 (GPTHLFQPSLVLDMAKVLLD, hIRBPp1-20), pigeon cytochrome c (PCC) peptide sequence 43-58 (AEGFSYTEANKAKGIT, PCCp43-58), and purified *Bordetella pertussis* toxin (PTX) were synthesized by Sigma-Aldrich (St. Louis, MO, USA). Complete Freund's adjuvant (CFA) and *Mycobacterium tuberculosis* strain H37Ra were purchased from Difco (Detroit, MI, USA).

Preparation of iPS cells and induction of iPS-SCs

The iPS cells were prepared according to the protocol of the Kyoto University iPS Cell Research Institute [10]. By using retrovirus vectors, four genes of *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* were transferred to B cells enriched from the spleen cells of C57BL/6J (CD45.1) mice. Then, transferred B cells were cultured for 17 days to obtain colonies of iPS cells [11,12]. These cells expressed embryonic stem (ES) cell marker genes including *Nanog*, *Ecat* and *Gdf*. To induce iPS-SCs, iPS cells were cultured in αMEM (Thermo Fisher Scientific, Waltham, MA, USA) medium including 20% fetal bovine serum (GE healthcare, Chicago, IL, USA), 0.1 mM non-essential amino acids (NACALAI TESQUE, Kyoto, Japan), 1mM sodium pyruvate (NACALAI TESQUE), 1% penicillin/streptomycin (NACALAI TESQUE), and 55 μM 2-mercaptoethanol (NACALAI TESQUE) using untreated 100-mm dishes. On day 3 of culture, 5 μg/ml basic fibroblast growth factor (bFGF) (Peprotech, Cranbury, NJ, USA) and 10 μg/ml vascular endothelial growth factor (VEGF) (Peprotech) were added to the medium for inducing embryoid bodies (EBs). On day 6 of culture, EBs were collected and disseminated on OP9 cells (bone marrow stroma cell line derived from op/op mice lacking macrophage colony-stimulating factor (M-CSF) gene) to support differentiation into hematopoietic cells previously seeded in 100-mm dishes [13]. To the culture medium, 10 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) (Peprotech) and 10 ng/ml M-CSF (Peprotech) were added. On

day 10, hematopoietic cells were collected and seeded on untreated 100-mm dishes to induce differentiation into immunosuppressive cells. Then, 10 ng/ml GM-CSF was added to the culture medium. From day 10 of culture, RPMI (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), including 10% fetal bovine serum (GE healthcare), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1% penicillin/streptomycin, 55 μM 2-mercaptoethanol, was used as the culture medium.

On day 15, the cultured cells were collected and disseminated on untreated 100-mm dishes. Then, 5 ng/ml GM-CSF and 10 ng/ml IL-4 were added to the medium. On day 24, the cells were collected and disseminated again on untreated 100-mm dishes filled with the medium including 10 ng/ml IL-4 and 1 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich). On day 25, hIRBPp1-20 (4 μM) was added to the culture medium to bind the peptide to the major histocompatibility complex (MHC) class II of iPS-SCs, that is, to preload the peptide on iPS-SCs. On day 26, iPS-SCs were obtained by detaching cells from dishes using cell scrapers after incubating at 37 °C for 20 minutes with 10 mM ethylenediaminetetraacetic acid (EDTA). In the end, iPS-SCs were washed three times with PBS to remove the peptide adequately. Then, we checked the expression of CD11b and F4/80 by fluorescence-activated cell sorting analysis

and the production of nitric oxide (NO) by using an NO assay kit described below to confirm the differentiation of iPS-SCs cells before the experiment.

Immunization to induce EAU

EAU was induced by immunization of C57BL/6J (CD45.2) mice. Mice were subcutaneously injected with hIRBPp1-20 (200 μg/mouse) emulsified in CFA containing 2.5 mg/ml *M. tuberculosis* strain H37Ra into the upper and lower back. Concurrent with immunization, 0.1μg PTX in 100 μl phosphate buffered saline (PBS) was injected intraperitoneally as an additional adjuvant.

Extraction of $CD4^+$ T cells from immunized mice

Extraction of $CD4^+$ T cells from mice immunized with hIRBPp1-20 was performed as follows. C57BL/6J (CD45.2) mice were immunized with hIRBPp1-20 (200 μg/mouse). Draining lymph nodes were obtained from the mice at day 9 after immunization. The extracted lymph nodes were homogenized in a petri dish containing PBS. The single cell suspension flowed through a cell strainer (Greiner Bio-One, Kremsmünster, Austria) with a pore diameter of 100 μm to remove extraneous tissues. The obtained suspension and MACS® magnetic microbeads

(Miltenyi Biotec, Bergisch Gladbach, Germany) combined with $CD4^+$ T cell antibodies were reacted at 4 °C for 15 minutes. Then, the reacted suspension was passed through an LS column (Miltenyi Biotec) attached to the magnetic field of the separator. The magnetically labeled $CD4^+$ T cells were obtained according to the manufacturer's protocol.

T cell proliferation assay

CellTrace Violet^{TM} (5μM) (Thermo Fisher Scientific) was added to hIRBP-primed $CD4^+$ T cells and reacted at 37 °C for 20 minutes in a water bath.

Spleen cells isolated from naive C57BL/6J (CD45.1) mice were irradiated with 30 Gy to stop cell cycles and used as antigen-presenting cells (APCs). The hIRBP-primed $CD4^+$ T cells, APCs, and hIRBPp1-20 were co-cultured with or without iPS-SCs in a 96-well plate at 37 °C for 4 days. On day 4 of culture, the cells were collected and reacted with 1.3 μg/ml allophycocyanin anti-mouse CD45.2 antibody (Biolegend, San Diego, CA, USA) on ice for 10 minutes under light shielding to label $CD4^+$ T cells. Then, the cells were reacted with 1 mg/ml propidium iodide (PI) (Sigma-Aldrich) for 15 minutes on ice in the dark to stain dead cells. On the same day, proliferation of labeled $CD4^+$ T cells was assessed by the following flow cytometry analysis using a BD FACSCanto^{TM} II flow cytometer (BD Biosciences, San Jose,

CA, USA). At first, debris was excluded using forward scatter-area (FSC-A) and side scatter-area (SSC-A). Then, doublets were excluded using forward scatter-width (FSC-W) and forward scatter-height (FSC-H). The PI-negative living cells were gated on $CD45.2^+$ that were mainly primed $CD4^+$ T cells to exclude CD45.1 mice-derived cells used as APCs. Generation of T cell division was determined by generational dilution of the CellTrace Violet dye and gates were drawn to determine the percentage of proliferated T cells that were in cellular division (Fig 1-A). The fluorescence intensity of CellTrace Violet™ was measured for assessment of $CD4^+$ T cell proliferative response, and T cell proliferation was analyzed using Flow Jo (Tree Star, Ashland, OR, USA).

Cytokine concentration measurements

Cytokine concentrations of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, and IL-17F in the culture supernatant produced during T cell proliferation assay were quantified by LEGENDplex™ (Biolegend), which is a bead-based immunoassay kit that can quantify multiple soluble proteins simultaneously in a sample using flow cytometry. Transforming growth factor (TGF)-β1 was measured using a Mouse TGF-β1 ELISA kit (R & D Systems, Minneapolis, MN, USA). The concentrations of NO were measured

with an NO assay kit (Promega) using a Griess Reagent System for measuring nitrite (NO^{2-}), which was a decomposition product of NO^{-}.

Assay without cell-to-cell interaction

Using Millicell-96 Cell Culture Insert Plates (Merck Millipore, Burlington, MA, USA), while hIRBP-primed $CD4^{+}$ T cells and APCs were cultured in the lower well, iPS-SCs were cultured in the upper well to prevent contact with the other cells. The culture medium containing hIRBPp1-20 was filled in the reservoir plate. The cells were co-cultured at 37 ℃ for 4 days. Then, $CD4^{+}$ T cells were labeled and T cell proliferation was determined by using flow cytometry as previously described.

Clinical evaluation of EAU

Clinical severity of retinal inflammation was assessed by funduscopic examination every 3 or 4 days from 6 days to 20 days after induction of EAU. The clinical scoring was based on the number or extent of vasculitis and soft exudate in the retina, and graded on a five-point scale, as previously reported [14]. The clinical evaluations were performed by two ophthalmologists in a blind fashion.

Statistical analysis

All results are shown as mean ± standard deviation (SD). Analysis between the two groups was performed using the Mann-Whitney U test. In addition, Tukey's test was performed for comparing three or more groups. When the *p*-value was less than 0.05, it was considered statistically significant.

Results

Inhibitory effect of iPS-SCs on uveitogenic T cell proliferation

Firstly, we examined the ability of iPS-SCs to suppress hIRBP-specific T cell proliferation associated with the pathogenesis of uveitis *in vitro*. $CD4^+$ T cells extracted from draining lymph nodes of immunized mice, hIRBPp1-20 and spleen cells isolated from naïve mice were co-cultured for 4 days. After co-culture, proliferation of $CD4^+$ T cells was analyzed by using flow cytometer. As a result, proliferation of $CD4^+$ T cells was suppressed by adding iPS-SCs in the culture in a number-dependent manner ($p < 0.01$, Fig. 1-B).

Examination of inflammatory cytokines in the culture supernatant

The culture supernatant produced in the T cell proliferation assay was collected after four days of coculture. The concentrations of inflammatory cytokines in the culture supernatant were examined. Among pro-inflammatory cytokines, Th1 cytokines (IL-2, TNF-α, IFN-γ), Th2 cytokines (IL-4, IL-5, IL-10, IL-13) and Th17 cytokines (IL-17A, IL-17F), which are respectively associated with protection against intracellular or extracellular pathogens, allergy and autoimmunity, were examined. Among anti-inflammatory cytokines, TGF-β1 and NO involved in immunosuppression were examined. TNF-α and IFN-γ concentrations were significantly decreased in the presence of iPS-SCs ($p < 0.05$, Fig. 2-A, B). Concentrations of IL-13, IL-17A, IL-17F, and TGF-β1 were significantly increased in the presence of iPS-SCs ($p < 0.05$, Fig. 2-C, D, E, F). NO production was significantly enhanced depending on the number of iPS-SCs ($p < 0.01$, Fig. 2-G). IL-2, IL-4, IL-5, and IL-10 were below the detection limits. The exact value and the *p*-value between each group are shown in Supplemental Table.

The effect of iPS-SCs on the suppression of hIRBP-specific T cell proliferation with or without cell-to-cell contact

To reveal the mechanism of iPS-SCs in the suppression of T cell proliferation, we examined whether iPS-SCs need cell-to-cell contact in the suppression of T cell proliferation *in vitro*.

Using a cell culture insert plate, hIRBP-primed $CD4^+$ T cells and APCs were cultured separated from iPS-SCs. iPS-SCs could suppress T cell proliferation in the culture without cell-to-cell contact as the same level as in the culture with cell-to-cell contact (Fig. 3).

Verification of antigen specificity of iPS-SCs

Next, the antigen specificity in the immunosuppression of iPS-SCs was examined. Twenty-four hours before the collection of iPS-SCs, hIRBPp1-20 or PCC p43-58, which also binds to MHC class Ⅱ molecules, $H\text{-}2A^{b}$ [15] was preloaded into iPS-SCs by adding these peptides to the culture medium. Then, hIRBP-primed $CD4^+$ T cells, APCs, hIRBPp1-20, and peptide-preloaded iPS-SCs were co-cultured. Four days later, T cell proliferation was measured using flow cytometry. T cell proliferation was also suppressed with PCC-preloaded iPC-SCs at the same level as with hIRBP-preloaded iPC-SCs ($p < 0.01$). There was no significant difference between them (Fig. 4).

Amelioration of EAU with injection of iPS-SCs *in vivo*

On one day prior to the induction of EAU, iPS-SCs (5×10^5 cells/mouse) suspended in PBS or PBS alone were injected intraperitoneally into the mice (non-treated group: 5 mice, 10 eyes; iPS-SCs-injected group: 6 mice, 12 eyes).

The clinical severity of the iPS-SCs-injected group was significantly milder 13, 17, and 20 days after immunization than that of untreated mice [score: 13 days = 1.3 ± 0.5 (iPS-SCs-injected mice), 1.9 ± 0.3 (non-treated mice), $p < 0.01$; 17 days = 1.5 ± 0.5 (iPS-SCs-injected mice), 2.2 ± 0.4 (non-treated mice), $p < 0.05$; 20 days = 2.2 ± 0.4 (iPS-SCs-injected mice), 2.8 ± 0.4 (non-treated mice), $p < 0.01$)] (Fig. 5-A). Fundus of representative cases are presented in Fig. 5-B, C. Vascular sheathings and retinal exudates were observed in untreated mice, while those were not observed in iPS-SCs-injected subjects.

Discussion

We previously reported that iPS-SCs expressed CD11b, F4/80, CD206, and CD14 as macrophage markers, as well as mRNA of immunosuppressive molecules (Arginase-1 and Nos2) [9]. They suppressed the allogeneic T cell proliferation when T cells were co-cultured with allogeneic dendritic cells *in vitro*, and the survival of the allograft in iPS-SCs-injected mice was prolonged compared with non-treated mice *in vivo* [9]. In this study, we found that macrophage-

like immunosuppressive iPS-SCs have an ability to suppress antigen stimulated Th1 immune response and consequently to ameliorate Th1-mediated EAU.

It is known that pathogenic Th1 cells are induced and activated by immunization of hIRBPp1-20, a sequence of retinal antigens, and the cells induce EAU in C57BL/6 mice [16]. In the present study, we examined the suppressibility of iPS-SCs to hIRBP-stimulated T cells. The iPS-SCs significantly suppressed hIRBP-specific T cell proliferation in a number-dependent manner and decreased the production of Th1 cytokines, such as TNF-α and IFN-γ in the culture supernatant *in vitro* (Fig. 1, Fig. 2-A, B). Th1 cytokine is known to be involved in the pathogenesis of EAU [17]. Th17 cytokines such as IL-17A and IL-17F in the culture supernatant were conversely elevated (Fig. 2-D, E). According to previous reports, Th1 and Th17 are shown to antagonize each other [18,19]. Th17 cytokines are considered to increase in response to the decrease of Th1 cytokines in this study. The levels of IL17-A and IL17-F detected in the culture supernatant were almost 100 times smaller than those of IFN-γ. Therefore, we consider that although IL17-A and IL17-F may also be increased reactively *in vivo*, they would not cause the exacerbation of the uveitis. Similarly, Th1 and Th2 are known to keep the balance, which are called Th1/Th2 paradigm [20]. Elevated concentrations of Th2 cytokine IL-13 were possibly associated with a decrease in Th1 responses (Fig. 2-C). It has been reported that TGF-β1

inhibited $CD4^+$ T cell activation on inflammatory sites [21,22]. Consistent with these previous reports, TGF-β1 concentrations were elevated in the supernatant with iPS-SCs (Fig. 2-F). Also, it has been reported that NO has a suppressive function against activated T cells and is associated with infection-preventing immunity by reducing the T cell response of the host [23,24]. NO concentrations were predictably increased in the supernatant in the presence of iPS-SCs (Fig. 2-G). Considering these results, TGF-β1 or NO produced in the presence of iPS-SCs may suppress T cell activity.

Next, it was investigated whether iPS-SCs need cell-to-cell contact to establish the T cell suppressibility; however, we found that they did not need cell-to-cell contact to suppress T cell activation. These results suggest that immunosuppressive effects of iPS-SCs on the activated T cells were dependent on humoral factors such as TGF-β1 and NO regardless of cell contact.

Additionally, the antigen specificity of iPS-SCs was examined using PCC p43-58 known to bind to the same $H\text{-}2A^b$ as hIRBPp1-20 binds to [15]. PCC-preloaded iPS-SCs showed almost the same level of suppression effect on the T cell activation as hIRBP-preloaded ones (Fig. 4), indicating that the immunosuppressive effect of iPS-SCs is antigen non-specific. The fact that iPS-SCs express macrophage markers suggests that they have the ability of antigen

presentation, indicating that they may suppress T cell activation in an antigen-specific manner; however, the result obtained in this study does not indicate it. To act in an antigen-specific manner, the cells usually need cell-to-cell contact. The result that iPS-SCs does not require cell-cell adhesion when they suppress T cell activation is consistent with the result that they do not act in an antigen-specific manner. In summary, iPS-SCs suppress T cell activation by secreting humoral factors, but not by cell-to-cell contact associated with antigen specificity.

In the present study, immunosuppressive ability of iPS-SCs on hIRBP-stimulated Th1 cells was confirmed. EAU, a Th1 cell-mediated autoimmune disease model for human endogenous uveitis, was consistently ameliorated (Fig. 5). We also investigated the effect of iPS-SCs given after the onset of EAU by injection of iPS-SCs on day 10 and day 17 after immunization (data not shown). However, EAU was suppressed in iPS-SCs-treated mice on days 14 and 17 but not on day 21, indicating that the administration of iPS-SCs after the onset of the disease cannot suppress ocular inflammation adequately. We consider that the administration of iPS-SCs would rather be the preventive treatment to be performed during the remission phase to suppress the recurrence of uveitis. The expected benefit of iPS-SCs is to obtain a sufficient number of immunosuppressive cells from the patient's own soma. Another benefit is good cell viability in the induction of iPS-SCs. Almost 100 % cells are alive and

healthy after being cultured. Administration of iPS-SCs is possibly a promising immunotherapy for autoimmune diseases, including uveitis.

In conclusion, this study suggests that iPS-SCs suppress Th1-mediated immune response to a retinal antigen by secreting humoral factors that are non-relevant to cell contact and non-specific to a particular antigen, leading to amelioration of EAU in mice. To elucidate the more detailed mechanism of immunotherapeutic effects brought by iPS-SCs, however, further studies are still needed.

Acknowledgements

We thank Miyuki Murata, Ikuyo Hirose and Shiho Yoshida (Department of Ophthalmology, Hokkaido University) for their technical assistance.

Declaration of interest

The authors report no conflicts of interest

Funding

This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture

of Japan (K.N. Grant #16K11310).

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Figure legends

Fig. 1. Suppression of antigen-specific T cell proliferation by iPS-SCs administration

Lymphocytes were obtained from the draining lymph nodes of immunized mice 9 days after immunization with hIRBPp1-20. $CD4^+$ T cells (4×10^5 cells/well) extracted from the obtained lymphocytes were labeled by CellTrace Violet™ and they were incubated for 4 days with APCs (1×10^5 cells/well) and hIRBPp1-20 (0.1 μM) in the presence or absence of iPS-SCs (1×10^4 or 1×10^5 cells/well). And proliferation of labeled $CD4^+$ T cells was assessed by flow cytometer. (A) The representative flow plots, gating strategy and histogram to determine T cell proliferation are shown. At first, debris was excluded using forward scatter-area (FSC-A) and side scatter-area (SSC-A). Then, doublets were excluded using forward scatter-width (FSC-W) and forward scatter-height (FSC-H). Dead cells were excluded by gating propidium iodide (PI) and APCs derived from $CD45.1^+$ mouse were excluded by gating CD45.2. Therefore, PI^- $CD45.2^+$ cells show alive $CD4^+$ T cells derived from immunized mice. A histogram indicates generation of alive $CD4^+$ T cell division by dilution of the CellTrace Violet label and a gate indicates percentage of proliferated $CD4^+$ T cells that were in division. (B) The representative

results of T cell proliferation assay are shown. The iPS-SCs significantly suppressed hIRBP-specific T cell proliferation depending on the cell number (n = 3 in each group, $^{**}p < 0.01$, $^{*}p < 0.05$). Data are shown as representative of 3 independent experiments.

Fig. 2. Cytokine concentrations in the supernatant under the influence of T cell proliferation with iPS-SCs

The culture supernatant obtained on day 4 of the T cell proliferation assay (Fig. 1) was assessed. Cytokine concentrations of (A) TNF-α, (B) IFN-γ, (C) IL-13, (D) IL-17A, (E) IL-17F, (F) TGF-β1 and (G) NO in the culture supernatant were measured. TNF-α and IFN-γ concentrations were significantly decreased in the supernatant cultured with iPS-SCs (1×10^5/well) (TNF-α: $p < 0.05$, IFN-γ: $p < 0.01$). IL-13 concentrations were significantly increased in the supernatant cultured with iPS-SCs (1×10^5/well) ($p < 0.05$). IL-17A and IL-17F concentrations were significantly increased in the supernatant cultured with iPS-SCs (1×10^5/well) ($p < 0.01$). TGF-β1 concentrations were significantly increased in the supernatant cultured with iPS-SCs (1×10^5/well) ($p < 0.01$). NO concentrations were significantly increased depending on the number of iPS-SCs ($p < 0.01$) (n = 3 in each group, $^{**}p < 0.01$, $^{*}p < 0.05$). Data are shown as representative of 3 independent experiments.

Fig. 3. Suppression of antigen-specific T cell proliferation by iPS-SCs with or without cell-to-cell contact

After separating iPS-SCs from hIRBP-primed $CD4^+$ T cells and APCs, hIRBP-specific T cell proliferation was determined (n = 4 in each group). The hIRBP-specific $CD4^+$ T cell proliferation was suppressed by iPS-SCs regardless of cell-to-cell contact ($^{**}p < 0.01$). Data are shown as representative of 3 independent experiments.

Fig. 4. Verification of the antigen specificity of iPS-SCs

T cell proliferation assay was performed after the culture with iPS-SCs preloaded with hIRBPp1-20 or PCCp43-58. The iPS-SCs preloaded with PCC suppressed T cell proliferation as much as those preloaded with hIRBP (n = 4 in each group, $^{**}p < 0.01$). Data are shown as representative of 3 independent experiments.

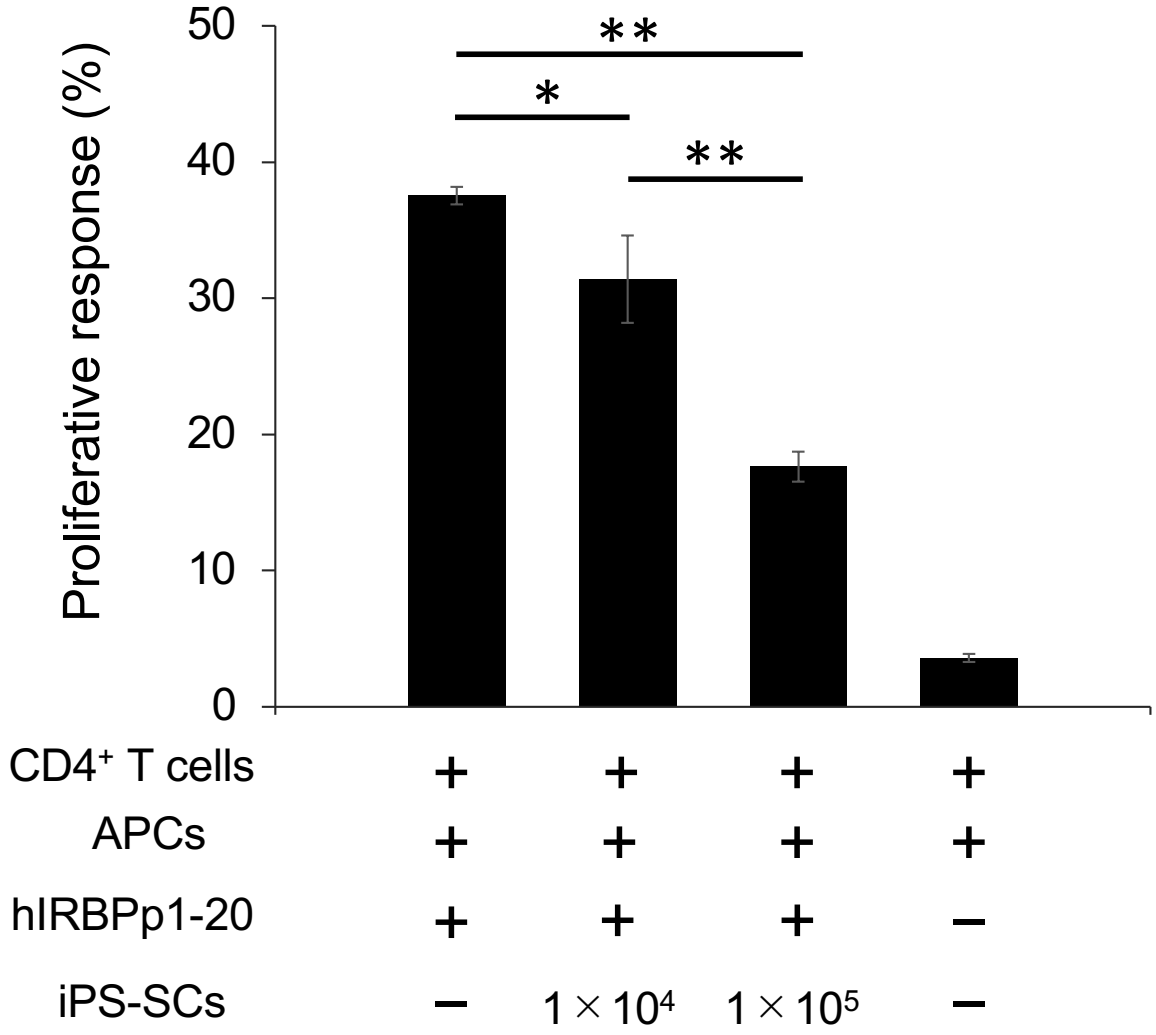
Fig. 5. Clinical evaluation of EAU in mice pretreated with iPS-SCs

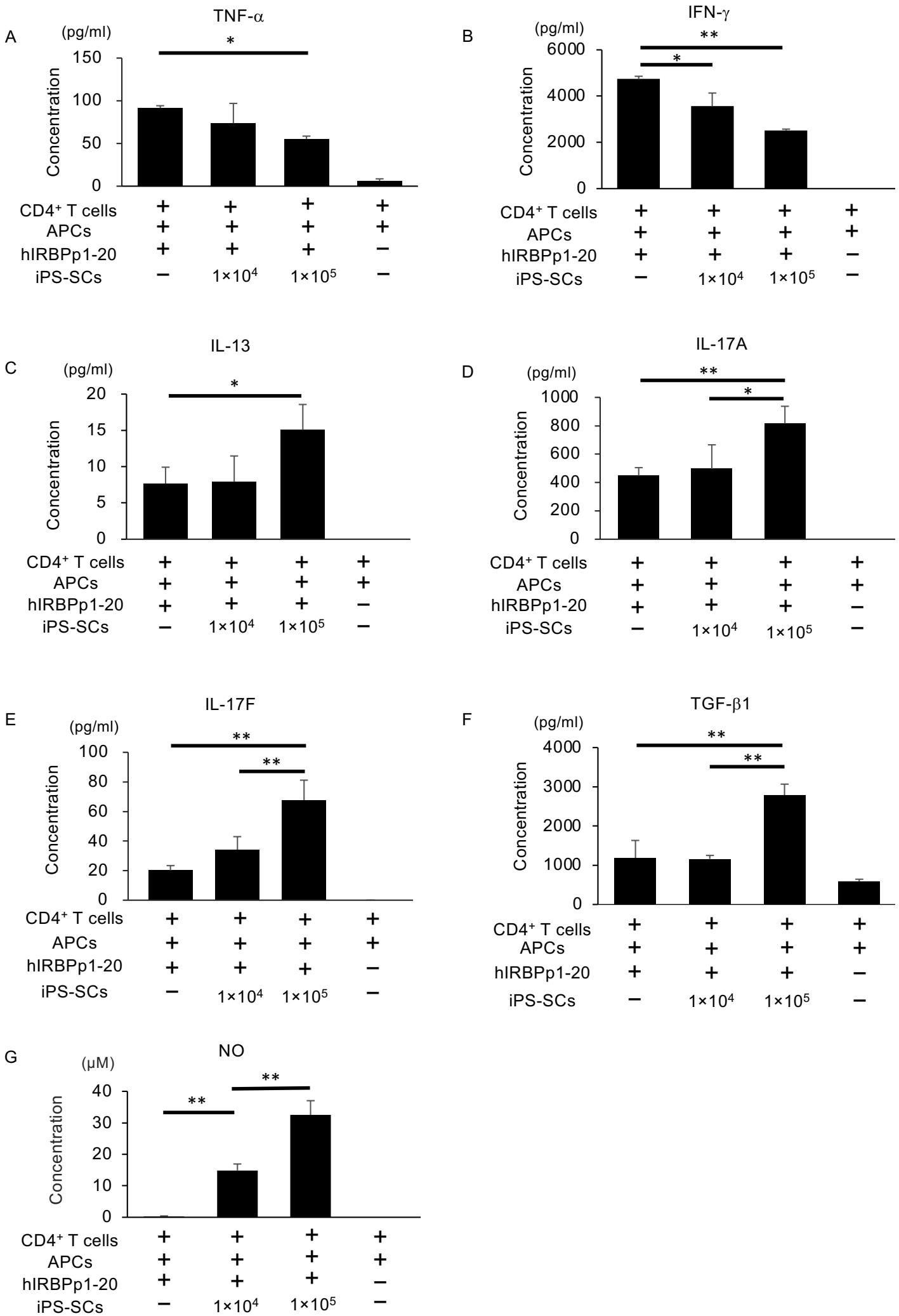
(A) The mean of clinical EAU score in non-treated mice (closed circle; n = 10) or iPS-SCs-injected mice (closed triangle; n = 12). The clinical severity in iPS-SCs-injected mice was significantly milder than that in non-treated mice on day 13 ($^{**}p < 0.01$), day 17 ($^{*}p < 0.05$) and day 20 ($^{**}p < 0.01$). Data are shown as representative of 3 independent experiments.

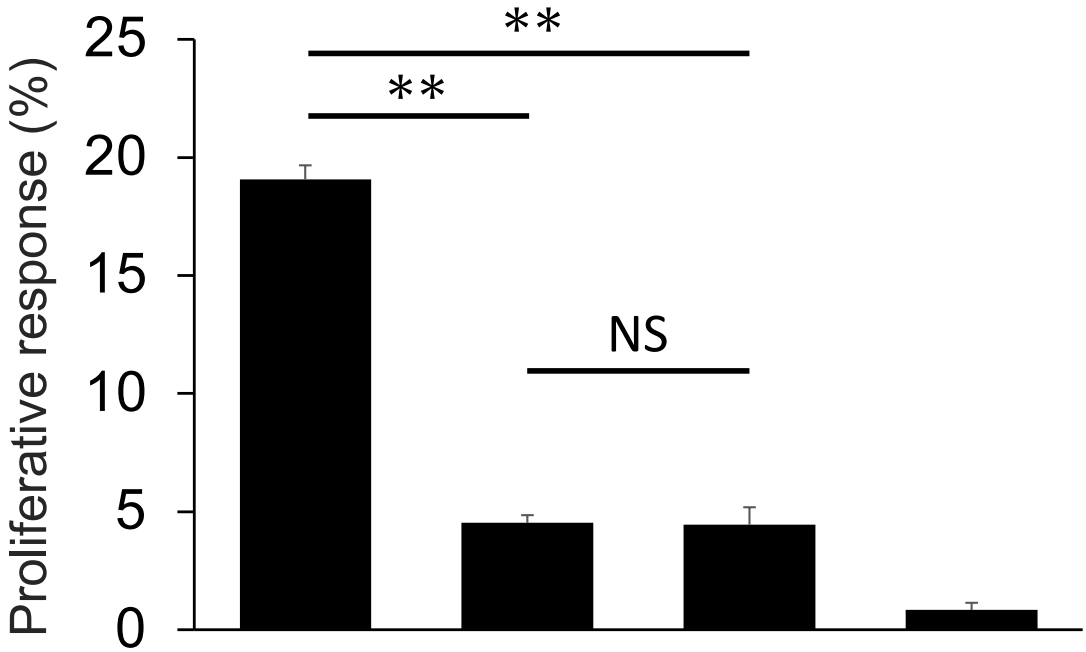
(B) The fundus of the representative case of an EAU mouse in which iPS-SCs were not injected. Vascular sheathings (yellow arrow) and a retinal exudate (yellow arrow head) were seen. (C) The fundus of the representative case of an EAU mouse in which iPS-SCs were injected. Vascular sheathings and retinal exudates were not seen.

Supporting information

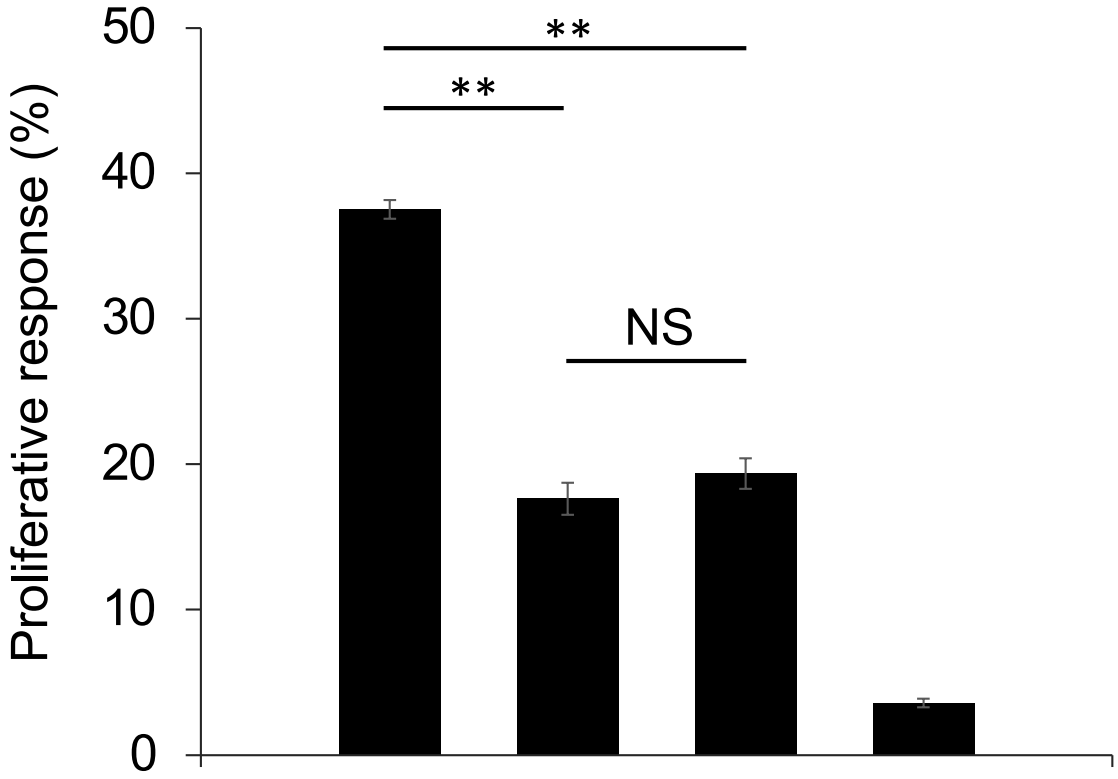
Supplemental Table. Inflammatory cytokine concentrations in the culture supernatant of T cell proliferation assay.


Proliferative response (%)
50
40
30
20
10
0
**
*
**
CD4+ T cells + + + +
APCs + + + +
hIRBPp1-20 + + + −
iPS-SCs − 1 × 10^4 1 × 10^5 −


A
TNF-α
(pg/ml)
Concentration
150
100
50
0
*
B
IFN-γ
(pg/ml)
Concentration
6000
4000
2000
0
**
*
C
IL-13
(pg/ml)
Concentration
20
15
10
5
0
*
D
IL-17A
(pg/ml)
Concentration
1000
800
600
400
200
0
**
*
E
IL-17F
(pg/ml)
Concentration
100
80
60
40
20
0
**
**
F
TGF-β1
(pg/ml)
Concentration
4000
3000
2000
1000
0
**
**
G
NO
(μM)
Concentration
40
30
20
10
0
**
**
CD4⁺ T cells + + + +
APCs + + + +
hIRBPp1-20 + + + −
iPS-SCs − 1×10⁴ 1×10⁵ −

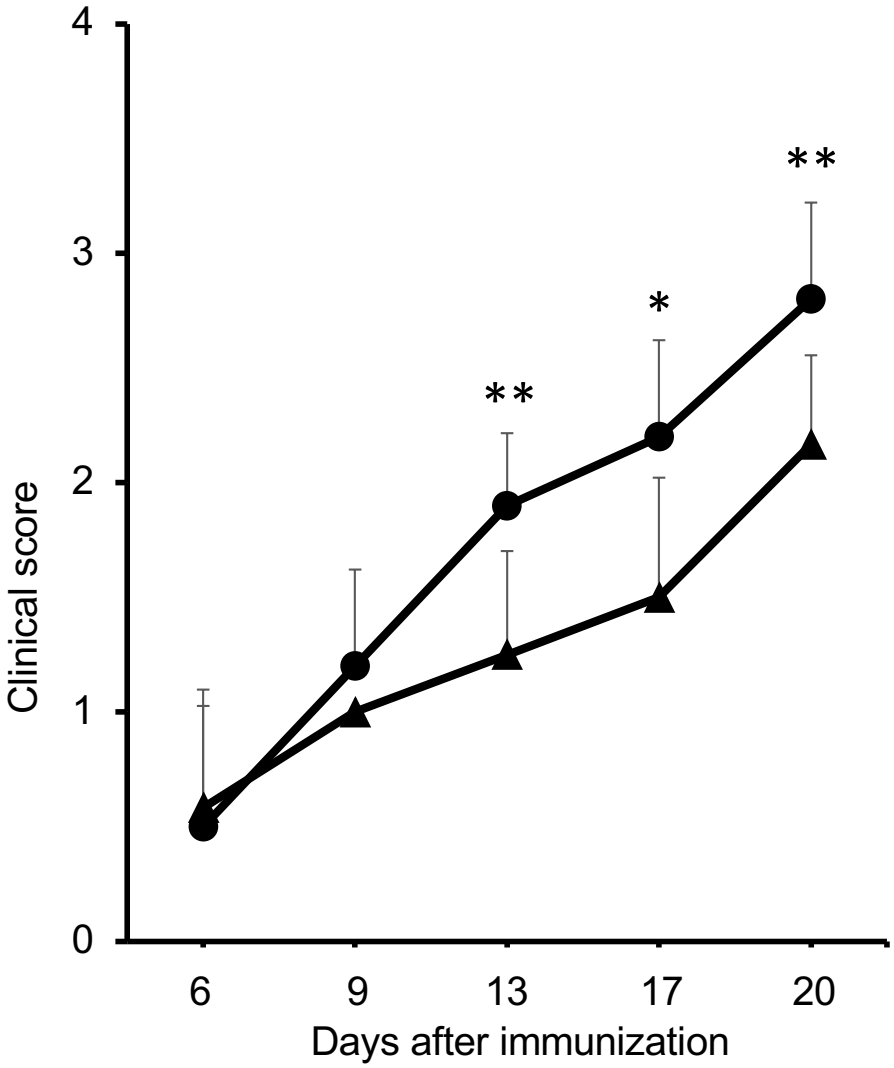


$CD4^+$ T cells	+	+	+	+
APCs	+	+	+	+
hIRBPp1-20	+	+	+	−
iPS-SCs	−	1×10^5	1×10^5	−
Cell-to-cell contact	+	+	−	+

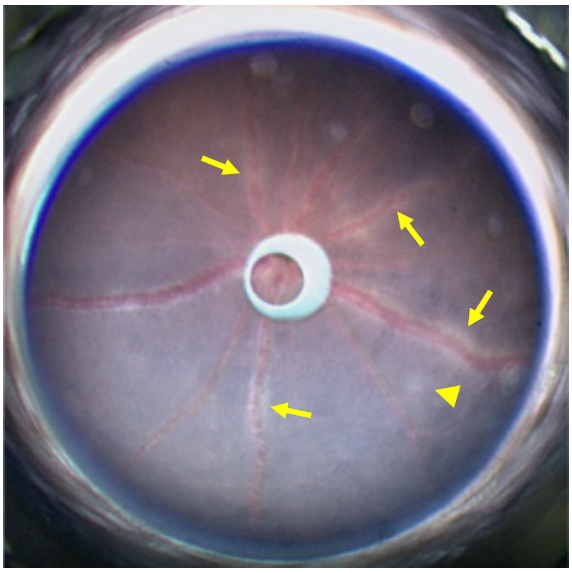


$CD4^+$ T cells	+	+	+	+
APCs	+	+	+	+
hIRBPp1-20	+	+	+	–
hIRBP-preloaded iPS-SCs	–	1×10^5	–	–
PCC-preloaded iPS-SCs	–	–	1×10^5	–

A



B



C

